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Combination approach of solid lipid nanoparticles and dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

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**Combination approach of solid lipid nanoparticles and dry floating in situ delivery systems
for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)**

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as its bioactive compounds possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as lipid matrix, SLNs were less than 200 nm in size with > 80% of compounds were encapsulated into SLNs. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment were significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system was able to control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, antioxidant, solid lipid nanoparticles, floating gel in situ, controlled release

1. Introduction

Recently, oxidative stress, caused by free radicals has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason of this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effect and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. Antioxidant properties of safflower is mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting its bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop delivery system to improve the solubility of these compounds.

Among several delivery system, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

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4 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
5 compounds [22–24]. However, the poor stability and high propensity to aggregate in acid
6 conditions limited their application through oral route [25]. Previously, hydroxypropyl beta-
7 cyclodextrin (HP β CD) has been found to improve the stability of SLNs administered orally [26].
8 The decoration of SLNs containing amphotericin B, paramomycin and paclitaxel using HP β CD
9 could improve their efficacies for their respective pharmacological effects [26,27]. Therefore, the
10 incorporation of bioactive compounds of safflower into SLNs decorated with HP β CD could be an
11 appropriate strategy for improved solubility.
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19 To facilitate the oral administration of the SLNs, it was important to develop a suitable
20 system. One of delivery systems to control and sustain the release of drugs through oral
21 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
22 drugs would stay in the stomach for long period [28–30]. As one of GRDDS, in situ forming gel
23 system has attracted the interest in drug delivery due to the transformation of the formulation from
24 solution into floating gel when administered orally and reaching the gastric environment [31–33].
25 Numerous studies have explored the effectiveness of this system to control the release some drugs
26 administered orally [29,34,35].
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33 In this study, we extracted the bioactive compounds from safflower and formulated the
34 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
35 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
36 incorporated into floating gel in situ system in the dry form which would be dispersed in water
37 prior to the oral administration. The floating gel in situ was evaluated for their properties and
38 release behavior of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-
39 glucopyranoside as the interest compounds. The effect of food intake on the dissolution profile
40 was further investigated. The main finding of this investigation showed the potential of using
41 natural product as antioxidant sources in sustain release formulation for potential treatment of
42 oxidative stress induced diseases.
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2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvent, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used in this study room temperature. The mixtures were then filtered and the filtrate was subjected to rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoids content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [36], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used a standard compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [36], with a slight modification. The extract was initially

solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to mL) in volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as standard compound. The concentration of TPC were reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as control. The extraction solvents were utilized were used as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of extract were assessed using thiocyanate, with slight modification. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g) and 20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL)

was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 μm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 μL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1).

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [26]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

2.8. Determination of Particle Size, PDI and Zeta Potential

Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK) was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations. The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the SLNs was diluted in water accordingly.

2.9. Encapsulation Efficiency Determination

Indirect method was applied to determine the encapsulation efficiency of the compounds, by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes. The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-dissolved compounds were quantified by determining the compounds from the filtrate from the purification step to concentrate the SLNs using HPLC. The encapsulation efficiency of both compounds was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

2.10. FTIR study

To evaluate the possible intervention between the compounds in extract and excipients used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used. In this study, all samples were scanned between 4000 and 600 cm⁻¹ wavenumbers.

2.11. DSC evaluation

The thermal properties of the compounds in extract, as well as in SLNs formulation were evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments)). The samples were weighed of around 5 mg and were closed in aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min under N₂ flow (40 mL/min)

2.12. XRD study

X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state of the compounds in extract, as well as in SLNs formulation. All formulations were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min.

2.13. SEM evaluation

In this study, the morphologies of SLNs were detected using a scanning electron microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan).

2.14. Solubility Analysis

In order to investigate the solubility of both compounds in extract and SLN preparations, two different solvents, namely water and n-octanol were used. Briefly, extract and SLNs were added in excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from lipid matrix was carried out using dialysis method was used [37–40]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium were withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [41,42]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBC was prepared in the concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C

and spun for 10 min at 7000 rpm. The supernatant was collected and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of the hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	F1	F2	F3	F4	F5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

2.19. In Vitro Gelation Study

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4 The gelation properties of the formulations were carried out by dispersing the dry powder
5 into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into
6 FaSSGF media (100 mL) under the stirring at 50 rpm at 37 ± 0.5 °C. The formation of gel was
7 observed and recorded as gelation time [43].
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11 12 13 **2.20. In Vitro Floating Study**

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15 The formulation was evaluated for the floating study using using USP Dissolution
16 Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish.
17 Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at $37 \pm$
18 0.5 °C. The floating lag time was recorded by observing time required by the gel formed to float
19 in the surface. Furthermore, the floating duration time was also recorded by observing the time
20 required by the gel to stop floating [43].
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27 28 **2.21. Measurement of Viscosity and Gel Strength**

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30 A brookfield Viscometer DV III (Brookfield Engineering Laboratories, MA) was used to
31 determine the viscosity of the formulation after being dispersed in water. The study was carried
32 using using spindle number 2 at 50 rpm. In an attempt to measure the gel strength, a Rheometer
33 (TA Instrument, USA) was utilized. The study was conducted at 25 °C.
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39 40 **2.22. Preparation of dissolution media**

41 Two different type of dissolution media were used, namely FaSSGF (Fasted-State
42 Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of
43 FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of
44 the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGFF was
45 carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. The pH of the
46 mixture was adjusted to pH 5 using 1 M HCl [44].
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53 54 **2.23. In Vitro Drug Dissolution Study**

55 The dissolution study of both compounds in safflower extract from the formulation was
56 carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled
57 with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the
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4 formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the
5 media was taken, replaced with the fresh media and the amount of both compounds was analyzed
6 using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of
7 both compounds in three different conditions was performed, as previously reported in our study,
8 namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [34]. The release profile
9 of both compounds from crude extract loaded dry floating gel in situ was also investigated.
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17 **2.24. Statistical analysis**

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19 The statistical analysis of all results was carried out using GraphPad Prism® version 6
20 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when p
21 < 0.05 .
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26 **3. Results and Discussion**

27 **3.1. Extraction Yield, TFC and TPC of Safflower Extract**

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29 Safflower has been found to show strong antioxidant properties against free radical. This
30 is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been
31 previously reported that both compounds and their glycoside forms can protect the damage caused
32 by oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower
33 using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC,
34 TPC and antioxidant properties of all extract are depicted in Figure 1. It was found that the high
35 concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest
36 values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC
37 of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin)
38 and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type
39 could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive
40 compounds, including flavonoid and phenolic contained in safflower possessed low solubility in
41 water which could further limit its application.
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55 **3.2. Antioxidant Activity using DPPH**

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57 Furthermore, as the main purpose of this study was related to antioxidant properties, the
58 antioxidant activities of all extract were assessed. Antioxidant could have various benefits in
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medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPD as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was statistical difference in IC₅₀ values of all extract ($p < 0.05$).

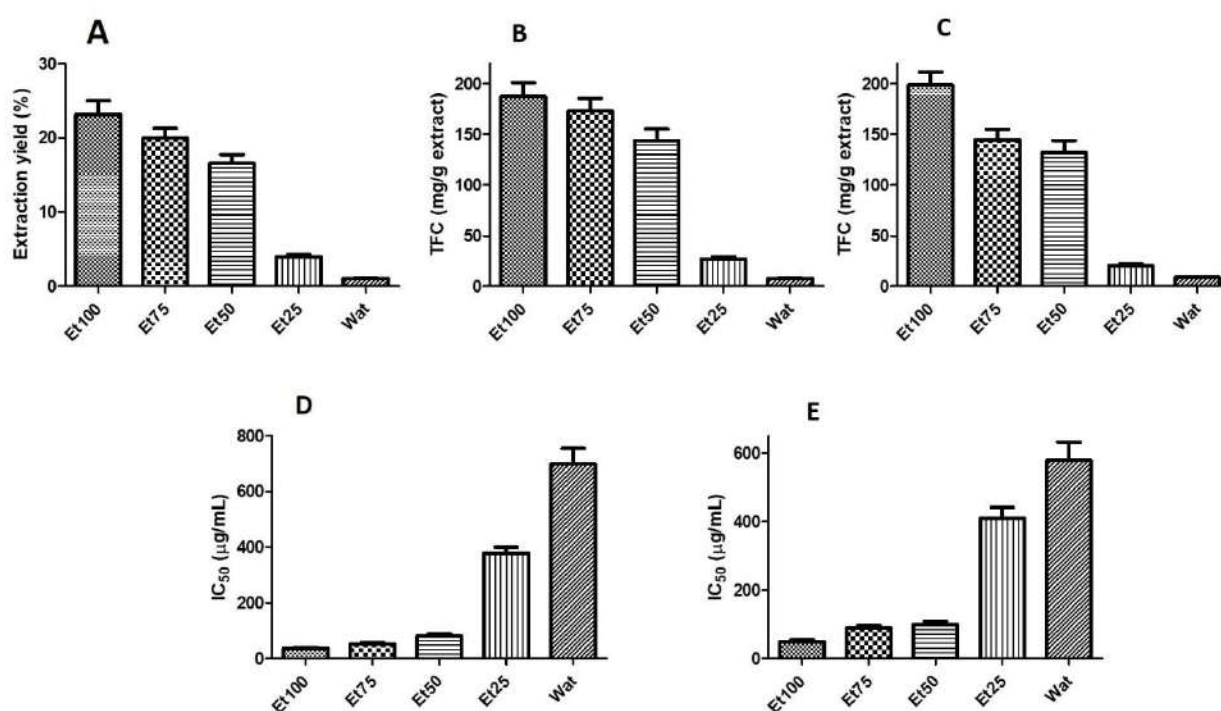


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPD (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, n=3)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extract were also investigated using lipid peroxidation method. As lipids are the major component of cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [36]. In this evaluation, it was found that the IC₅₀ against lipid peroxidation were of Et100, Et75,

Et50, Et25 and Wat are depicted in Figure X, showing the values of $48.75 \pm 3.42 \mu\text{g/mL}$, $89.04 \pm 6.56 \mu\text{g/mL}$, $98.97 \pm 8.91 \mu\text{g/mL}$, $409.51 \pm 31.67 \mu\text{g/mL}$ and $577.43 \pm 43.87 \mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC50 values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were $7.65 \pm 0.63 \text{ mg/g}$ for Et100, $4.32 \pm 0.32 \text{ mg/g}$ for Et75, $2.97 \pm 0.19 \text{ mg/g}$ for Et50, $0.74 \pm 0.05 \text{ mg/g}$ for Et25 and $0.02 \pm 0.001 \text{ mg/g}$ for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were $2.32 \pm 0.21 \text{ mg/g}$, $1.92 \pm 0.15 \text{ mg/g}$, $0.67 \pm 0.05 \text{ mg/g}$, $0.29 \pm 0.03 \text{ mg/g}$ and $0.01 \pm 0.001 \text{ mg/g}$, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

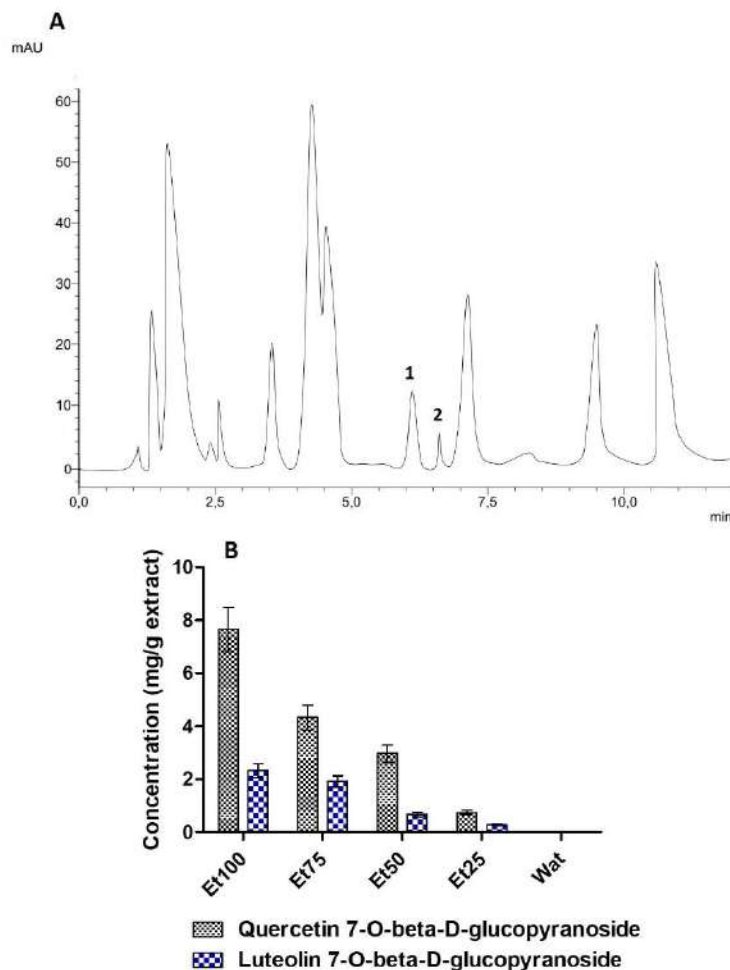


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [36]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [45]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [46]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency of both compounds in lipid matrix [36]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum

formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. Moreover, the encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [25,26]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

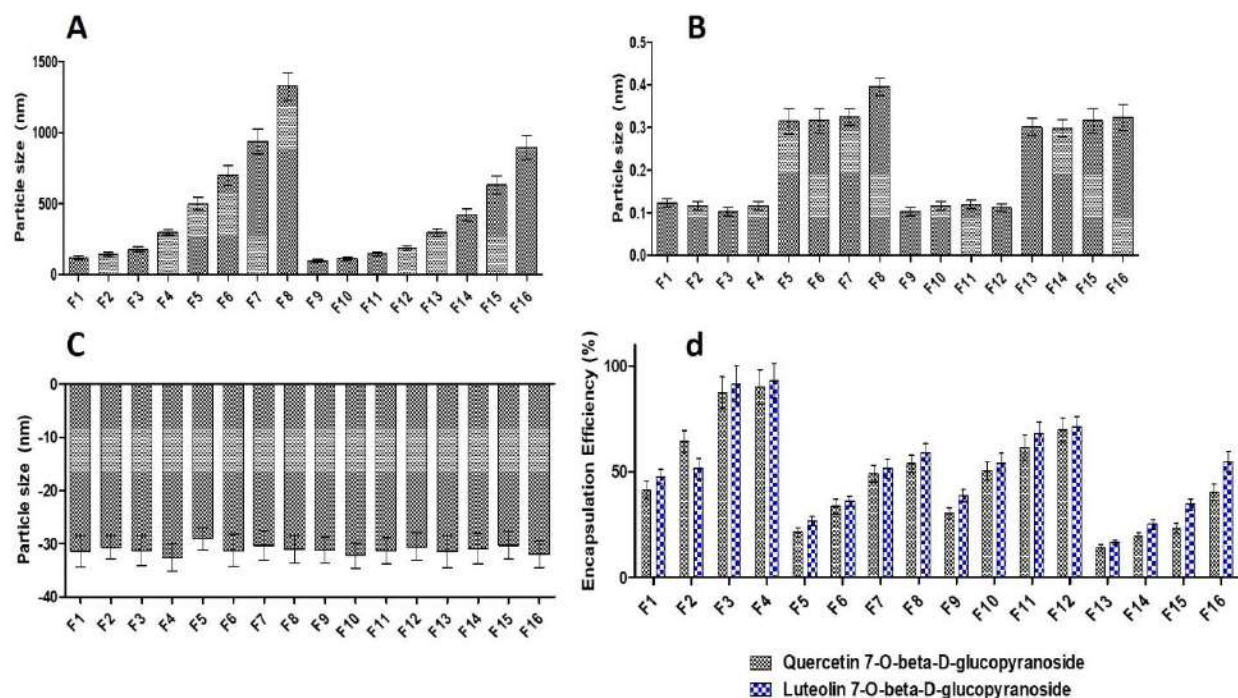


Figure 3. Particle size (A), PDI (B), zeta potential (C) and encapsulation efficiency results of SLNs prepared from safflower extract (mean $\pm$ SD, $n=3$)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both

compounds. Additionally, another peak was detected at 1684 cm⁻¹ which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm⁻¹ due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm⁻¹, 1363 cm⁻¹ and 1237 cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds.

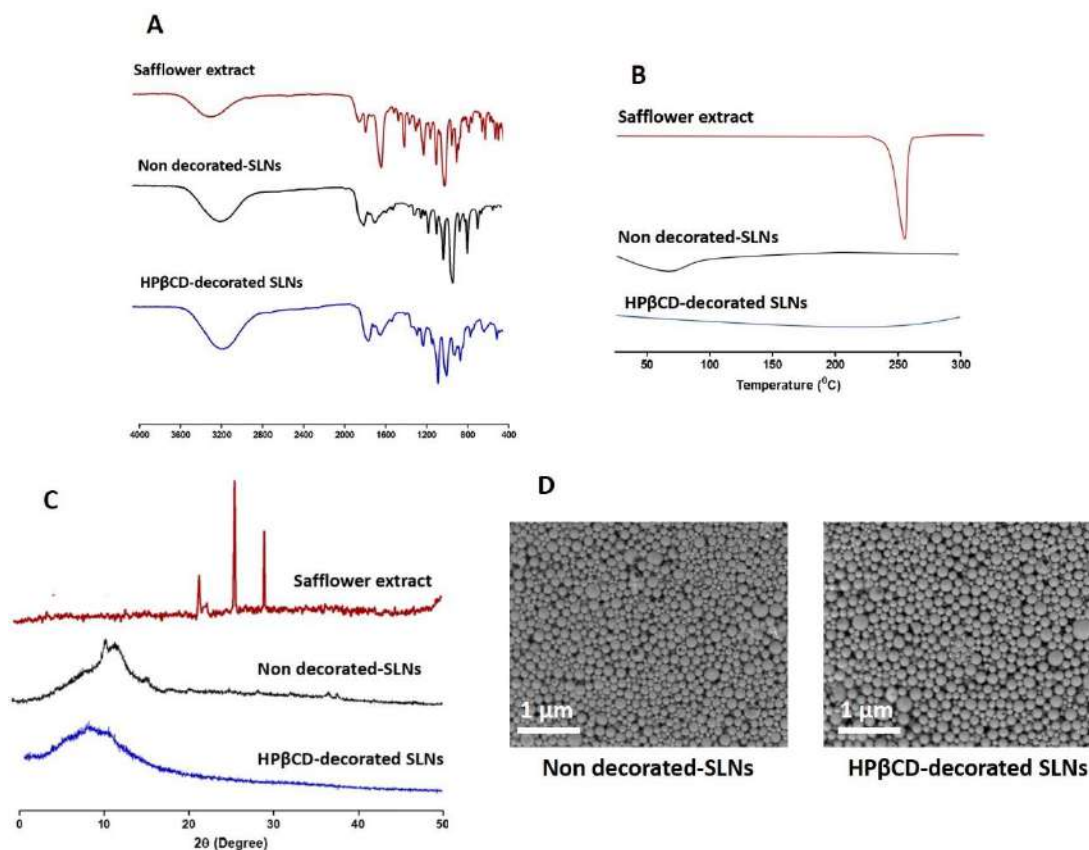


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4 shows the DSC thermograms of safflower extract and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the SLN formulations, the sharp peak was not

observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21].

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were 18.23 ± 1.32 µg/mL and 16.54 ± 1.36 µg/mL for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely 408.45 ± 29.34 µg/mL and 434.31 ± 39.31 µg/mL. Following the incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HPβCD-SLNs (mean ± SD, n=3)

Compound	Samples	Aqueous solubility (µg/mL)	n-octanol solubility (µg/mL)
Quercetin 7-O-beta-D-glucopyranoside	Extract	18.32 ± 1.32	408.45 ± 29.34
	SLNs	278.43 ± 20.31	411.23 ± 31.72
	HPβCD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside	Extract	16.54 ± 1.36	434.73 ± 39.42
	SLNs	201.23 ± 17.44	454.31 ± 40.38
	HPβCD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [47–51].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustain release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [52]. Following this, the slow-release patterns was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,41].

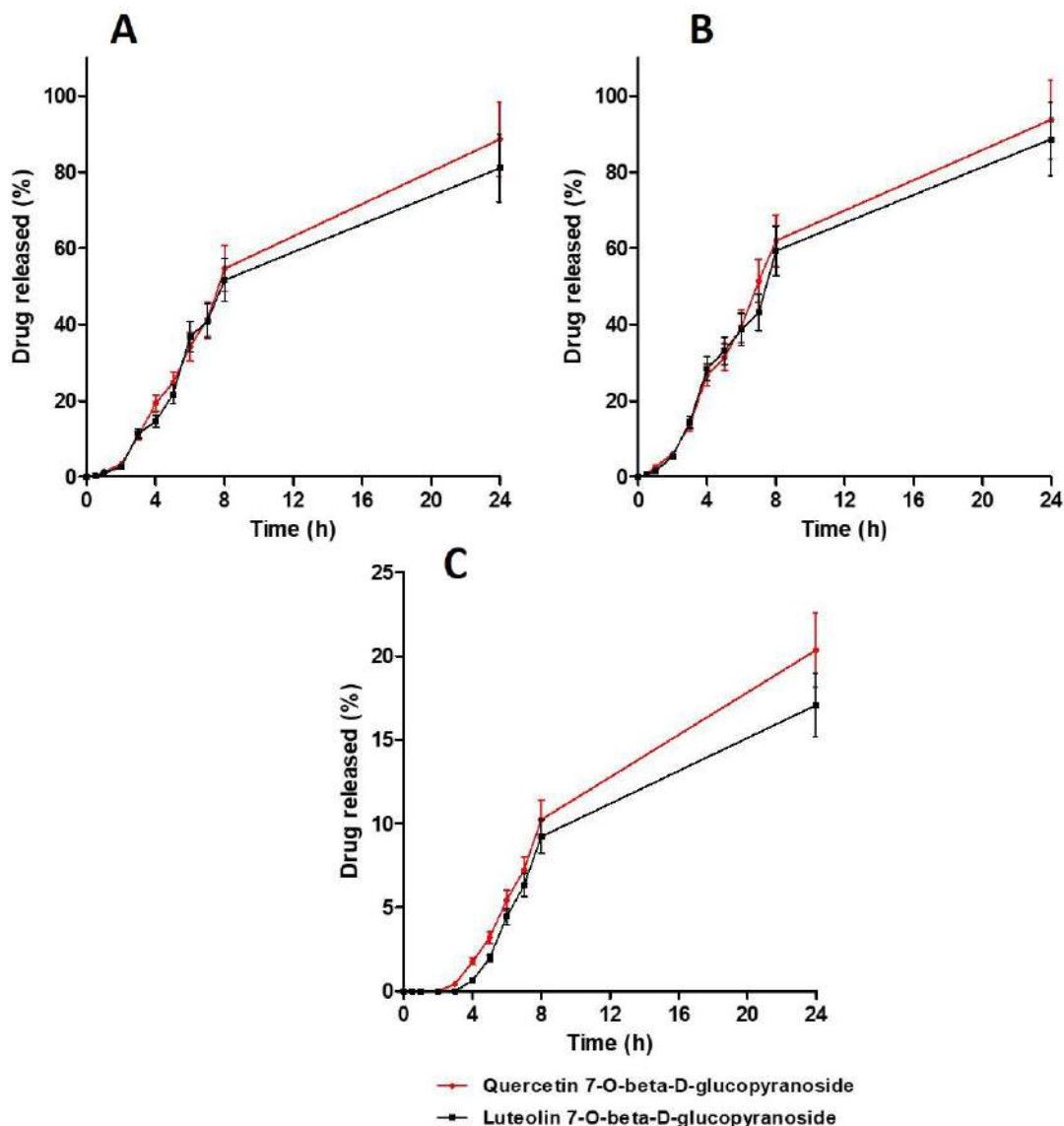


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD -SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [39,40].

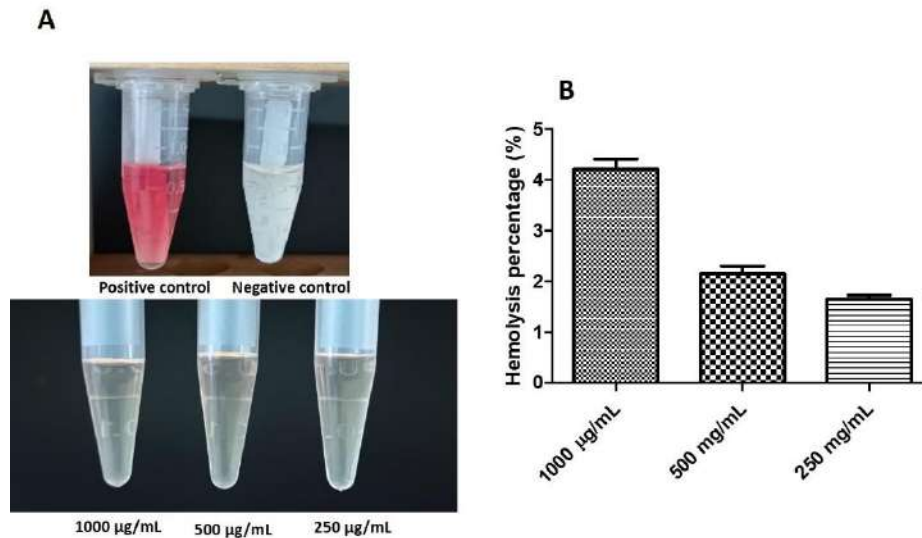


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporate HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [29]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [34]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [53]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [54,55]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for F1, F2, F3, F4 and F5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

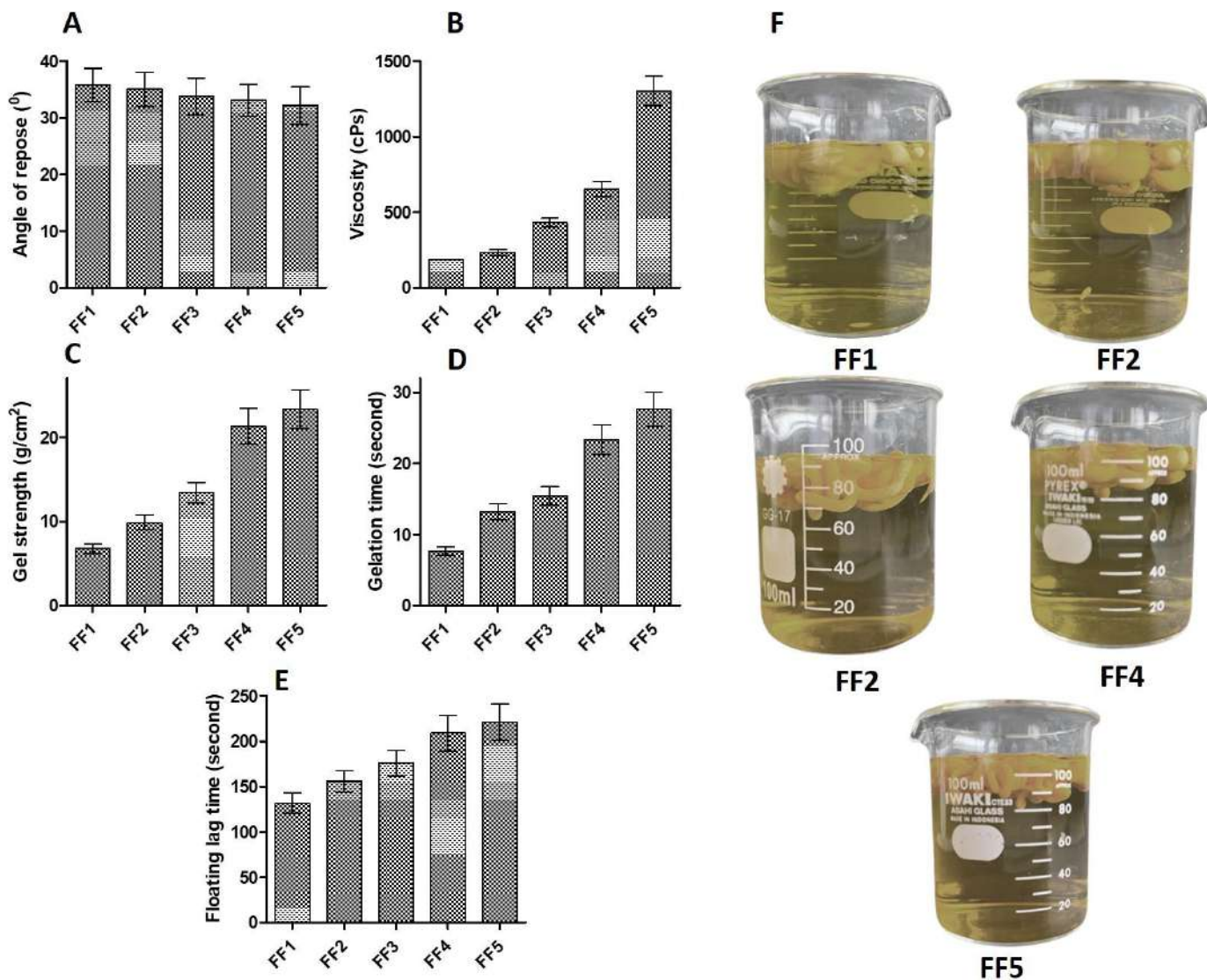


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, n=3). Representative images of floating gel in situ after the floating evaluation in FaSGGF, showing that all formulations were able to float (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The viscosity of the formulation was in the range of 187.98 ± 1.67 cps to 1302.62 ± 98.21 cps. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic

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4 F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling
5 agent, causing the increase in viscosity with the increase of the concentrations [54,55].
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8 The strength values of the gel formulation are shown in Figure 7C. It was found that the
9 increase of Pluronic F127 concentration from 2.5% to 10% could significantly improve ($p < 0.05$)
10 the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show
11 any significant improvement in the gel strength. This was in a good agreement with the viscosity
12 determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary
13 to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix
14 would release to gastric environment depending on the strength of the complex 3D structure of the
15 gel network [53]. Accordingly, the gel should be strong enough to maintain its integrity. The
16 strength values of the gel were similar to the values reported previously, showing the ability of the
17 formulation in maintaining the gel form in the gastric environment.
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28 **3.15. Gelation time**

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30 Gelation time is one of critical parameters in in situ gel formulation. In order to control the
31 release of drugs, the formulation should be able to form gel before the release of the drugs from
32 the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure,
33 showing that all formulation were able to form a gel in FaSGGF media at body temperature less
34 than 30 seconds. As explained in previous section, gellan gum could form gel following the
35 presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration
36 of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic
37 concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation.
38 Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely
39 polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel
40 conversion following the change of the temperature [41].
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51 **3.16. Floating lag time and duration of floating**

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53 Following the gel formation of the in situ formulation, to further sustain the release based
54 on its gastroretentive characteristics, the formulation should be able to float in the gastric
55 environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag
56 times of the formulations. Following the concentration of Pluronics, the time required of the gelled
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4 formulation to float also increased. This might be associated with the increase of the viscosity of
5 the formulation when the concentration of Pluronics increased. As a result, the penetration of the
6 gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which
7 affected the release of CO₂ [56]. A previous study has shown the same phenomenon showing that
8 the floating lag time increased following the increase of the polymeric material in the system [57].
9 It should be noted that all formulations remained floating after 24 h (Figure 7F).
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17 **3.17. In Vitro Drug Dissolution Study**

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19 The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-
20 glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three
21 different conditions. To ensure that the formulation could control the dissolution of both
22 compounds, the burst release should be avoided [58]. In this study, initially we evaluated the
23 dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-
24 glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are
25 depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ
26 could potentially control the dissolution profile of active compounds from safflower extract.
27 Without the SLNs formulation, the dissolution percentages were found to be significant lower (p
28 < 0.05) in all cases. Specifically, the difference in Pluronic concentrations resulted in various
29 control release patterns. As shown in Figure X, below 7.5% of Pluronics (F1 and F2), the sustained
30 release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (F3),
31 10% (F4) and 15% (F5) of Pluronics were capable of controlling the release of the compounds
32 over 24 h.
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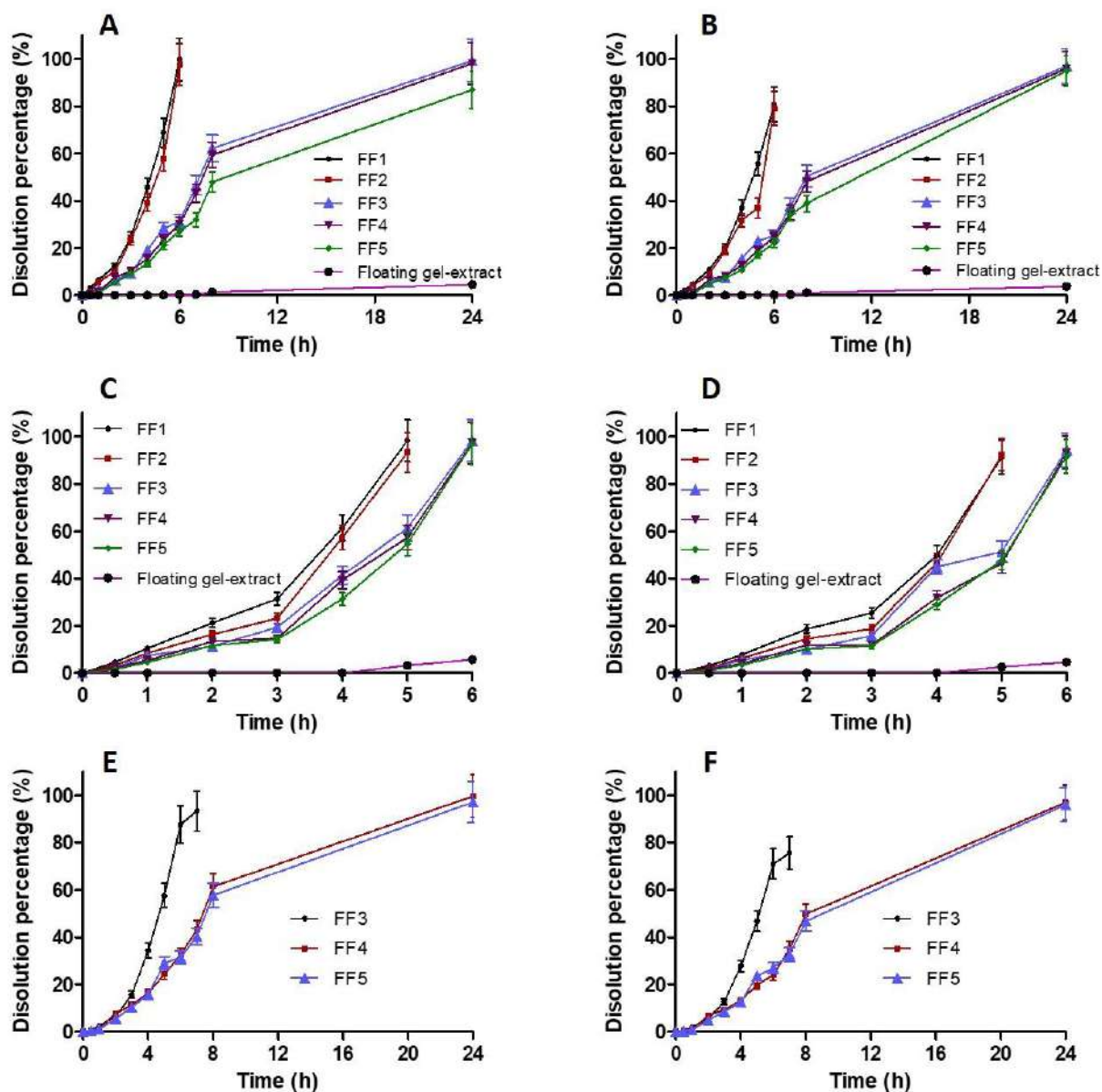


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (E) and luteolin 7-O-beta-D-glucopyranoside (F) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

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4 In FeSGGF, all formulations were not able to control the release of active compounds. As
5 shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of
6 gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should
7 be taken in fast state. However, it was important to evaluate the effect of the release of active
8 compounds from the developed formulations after food intake. In this investigation, only F3, F4
9 and F5 were selected due to their ability to control the dissolution behavior for 24 h. After 2 h in
10 FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution
11 behaviour of both compounds. On the other hand, F4 and F4 could potentially maintain and control
12 the release of the active compounds encapsulated into SLNs. This could be related the difference
13 in the gel strengths between the formulations. As the gel strength of F3 was significantly lower (p
14 < 0.05) than F4 and F5, the ability to maintain its rigid gel decreased when the formulation was
15 moved into FaSGGF. Thi study showed the possibility to improve the solubility of bioactive
16 compounds in safflower using SLNs and further control the release using floating gel in situ
17 system. To further evaluate its efficacy, in vivo studies should now be conducted using appropriate
18 animal model.
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34 **4. Conclusions**

35 In this study, safflower extract with antioxidant activity was obtained using ethanol as
36 extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of $36.87 \pm$
37 $2.81 \mu\text{g/mL}$ against DPPD and $48.75 \pm 4.52 \mu\text{g/mL}$ against lipid peroxidation. Specifically, the
38 extract contained $7.65 \pm 0.63 \text{ mg/g}$ extract of quercetin 7-O-beta-D-glucopyranoside and $2.32 \pm$
39 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract
40 was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-
41 cyclodextrin (HP β CD) with particle size of $178.97 \pm 16.57 \text{ nm}$, showing the improvement of 15-
42 fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement
43 after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan
44 gum as gelling agent. With desired gelation and floating properties, the formulation could control
45 the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in
46 biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of
47 this approach in a suitable animal model.
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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The Editor

International Journal of Biological Macromolecules

29th September 2022

Dear Sir/Madam,

I wish you to consider our manuscript entitled: “**Combination approach of solid lipid nanoparticles and dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)**” for publication in International Journal of Biological Macromolecules.

Safflower (*Carthamus tinctorius* L.) is a potent natural antioxidant because of active compounds such as quercetin and luteolin. These components prevent damage to the skin caused by free radicals from UV rays. However, due to the poor solubility and transdermal permeation, the effectiveness of the compounds in showing their activity was limited. In this study, we develop solid lipid nanoparticles (SLNs)-based hydrogel formulations to increase penetration of bioactive compounds of safflower extract. The hot emulsification-ultrasonication method was used to produce SLN, and to obtain high antioxidant activity, 100% v/v ethanol was used in the extraction procedure. The results showed that this approach could encapsulate > 80% of both quercetin and luteolin. Moreover, FTIR, DSC, and PXRD spectra indicated that most of the drug was trapped in a lipid matrix and dispersed homogeneously at the molecular level, increasing the solubility. The formulations showed rapid release due to the large surface area of the small particles. Additionally, hydrogel application in SPE-loaded SLNs demonstrated increased skin retention and penetrability of the two primary compounds up to 19-folds. The results of the SPF test show a value above 15, showing the excellent promising application to prevent symptoms associated with photo-induced skin aging.



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We believe that this work will be of great interest to the readers of International Journal of Biological Macromolecules. The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in International Journal of Biological Macromolecules and look forward to hearing from you in due course.

Yours sincerely,

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Combination approach of solid lipid nanoparticles and dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as its bioactive compounds possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as lipid matrix, SLNs were less than 200 nm in size with > 80% of compounds were encapsulated into SLNs. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment were significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system was able to control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, antioxidant, solid lipid nanoparticles, floating gel in situ, controlled release

BUKTI PROSES REVIEW



Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

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Kind regards,
Daniela Giacomazza
Editor

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Reviewer's Responses to Questions

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1. Are the objectives and the rationale of the study clearly stated?

Please provide suggestions to the author(s) on how to improve the clarity of the objectives and rationale of the study. Please number each suggestion so that author(s) can more easily respond.

Reviewer #1: The objectives and the rationale of the study are not clearly stated. The authors have written the article in very confusing way.

2. If applicable, is the method/study reported in sufficient detail to allow for its replicability and/or reproducibility?

Please provide suggestions to the author(s) on how to improve the replicability/reproducibility of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #1: Mark as appropriate with an X: NO

Yes ☐ No ☐ N/A ☐

Provide further comments here:

3. If applicable, are statistical analyses, controls, sampling mechanism, and statistical reporting (e.g., P-values, CIs, effect sizes) appropriate and well described?

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Yes ☐ No ☐ N/A ☐

Provide further comments here: Statistical data is extremely poor.

4. Could the manuscript benefit from additional tables or figures, or from improving or removing (some of the) existing ones?

Please provide specific suggestions for improvements, removals, or additions of figures or tables. Please number each suggestion so that author(s) can more easily respond.

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5. If applicable, are the interpretation of results and study conclusions supported by the data?

Please provide suggestions (if needed) to the author(s) on how to improve, tone down, or expand the study interpretations/conclusions. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #1: Mark as appropriate with an X: NO

Yes ☐ No ☐ N/A ☐

Provide further comments here:

6. Have the authors clearly emphasized the strengths of their study/methods?

Please provide suggestions to the author(s) on how to better emphasize the strengths of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #1: The authors failed to provide clear rational and interpretation of the data.

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8. Does the manuscript structure, flow or writing need improving (e.g., the addition of subheadings, shortening of text, reorganization of sections, or moving details from one section to another)?

Please provide suggestions to the author(s) on how to improve the manuscript structure and flow. Please number each suggestion so that author(s) can more easily respond.

Reviewer #1: The manuscript is poorly written.

9. Could the manuscript benefit from language editing?

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Reviewer #1: Comments
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1. The authors should performed in vivo pharmacokinetic analysis of the developed formulation to check the effect of the SLN-loaded gel in complex in vivo settings.
2. What was the yield of safflower flowers in the process of extraction?
3. The authors should add references to all the methods reported in the manuscript.
4. Please write the detailed methods of particle properties, FTIR, XRD, and SEM.
5. Why the authors used 2 methods (UV and HPLC) for quantification of drugs. Why not all the analysis were performed using HPLC?
6. Did the authors develop HPLC method? What was the linearity, inter day & intraday variations?
7. Beside the entrapment efficiency, the authors are advised to add drug content of the SLN.
8. Why the in vitro dissolution study was performed at 100 rpm. Does it mimic stomach conditions?
9. Why the gel strength was determined at 25 C, when the formulation has to become gel in situ at physiological temperature? Please explain.
10. Why the pH of the dissolution media was adjusted to pH 5? Please explain
11. Please add the details of statistical evaluation
12. What is the difference between the in vitro drug release and in vitro drug dissolution study reported in the manuscript?
13. What is the rational of incorporation of SLNs in HP β CD? If it is only stability, it can be achieved by simply lyophilizing the SLNs into dry powder? Please explain in the manuscript.
14. Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-Obeta-D-glucopyranoside (2) in safflower extract is very confusing. There are so many peaks with high intensities, indicating the presence of other metabolites. I wonder how the authors justify the safety or no pharmacological effect of these metabolites. Do the authors have any evidences to rule out it?
15. Did you check the viscosity of the SLNs with Geleol® as the main phenomenon of its selection is made on viscosity?
16. FTIR analysis. Why the peak of safflower extract at 1100-1200 is absent in the corresponding SLNs spectra's? Please explain
17. Why the authors didn't choose to perform the FTIR, DSC and XRD analysis of lipids, surfactants and their physical mixture?

18. Moreover, the results of DSC and XRD very poorly properly discussed.
19. Please explain with logical reasons the role of HP β CD-SLNs in enhancing the solubility of extract, even more than the SLNs?
20. Figure 5 shows no significant difference between the in vitro release profiles of SLNs and HP β CD-SLNs at all the time periods. The authors claim and the entire rationale becomes questionable at this point, if they are not significantly different in drug release.
21. Which equipment was used to determine the gelation time? The authors are advised to add the equipment details or sketch in the supplementary data.
22. Figure 8. Please indicate the level of significance among the data presented. In its current form, the formulations seems no significant difference.
23. This manuscript needs extensive revisions for grammatical and structural mistakes.

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L) --Manuscript Draft--

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Article Type:	Research Paper
Section/Category:	Carbohydrates, Natural Polyacids and Lignins
Keywords:	Safflower; solid lipid nanoparticles; floating gel in situ
Corresponding Author:	Andi Dian Permana, Ph.D Hasanuddin University Faculty of Pharmacy INDONESIA
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Order of Authors:	Andi Dian Permana, Ph.D Anwar Sam Ardiyah Nurul Fitri Marzaman Abdul Rahim Firzan Nainu Muh. Akbar Bahar Rangga Meidianto Asri
Abstract:	Safflower (<i>Carthamus tinctorius</i> L.) has been explored as source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as its bioactive compounds possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment were significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system was able to control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.
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Response to Reviewers:	Manuscript Number: IJBIMAC-D-22-09382 Combination approach of solid lipid nanoparticles and dry floating in situ delivery

systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

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Response to Reviewers

We are very thankful to the expert reviewers for taking the time to kindly review our manuscript and provide helpful comments for improvement and clarification. We have changed our title and abstract as per request from the editor and reviewer.

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☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Solid lipid nanoparticles-decorated cyclodextrin incorporated gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Abstract

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Keywords: Safflower, antioxidant, solid lipid nanoparticles, floating gel in situ, controlled release



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The Editor

International Journal of Biological Macromolecules

29th September 2022

Dear Sir/Madam,

I wish you to consider our manuscript entitled: **“Solid lipid nanoparticles-decorated cyclodextrin incorporated gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)”** for publication in International Journal of Biological Macromolecules.

Safflower (*Carthamus tinctorius* L.) is a potent natural antioxidant because of active compounds such as quercetin and luteolin. These components prevent damage to the skin caused by free radicals from UV rays. However, due to the poor solubility and transdermal permeation, the effectiveness of the compounds in showing their activity was limited. In this study, we develop solid lipid nanoparticles (SLNs)-based hydrogel formulations to increase penetration of bioactive compounds of safflower extract. The hot emulsification-ultrasonication method was used to produce SLN, and to obtain high antioxidant activity, 100% v/v ethanol was used in the extraction procedure. The results showed that this approach could encapsulate > 80% of both quercetin and luteolin. Moreover, FTIR, DSC, and PXRD spectra indicated that most of the drug was trapped in a lipid matrix and dispersed homogeneously at the molecular level, increasing the solubility. The formulations showed rapid release due to the large surface area of the small particles. Additionally, hydrogel application in SPE-loaded SLNs demonstrated increased skin retention and penetrability of the two primary compounds up to 19-folds. The results of the SPF test show a value above 15, showing the excellent promising application to prevent symptoms associated with photo-induced skin aging.



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We believe that this work will be of great interest to the readers of International Journal of Biological Macromolecules. The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in International Journal of Biological Macromolecules and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

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Manuscript Number: IJBIOMAC-D-22-09382

Combination approach of solid lipid nanoparticles and dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

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Response to Reviewers

We are very thankful to the expert reviewers for taking the time to kindly review our manuscript and provide helpful comments for improvement and clarification. We have changed our title and abstract as per request from the editor and reviewer.

Author Contributions: Conceptualization, A.D.P., A.R., F.N., and M.A.B.; methodology, A.D.P., A.S., and A.N.M.; software, A.D.P., A.S., A.N.M.; validation, A.D.P., A.R., and F.N.; formal analysis, A.S and A.N.M.; investigation, A.D.P., R.A.M., A.S., and A.N.M.; resources, A.D.P., A.R., R.A.M and F.N.; data curation, A.D.P., A.S., A.N.M.; writing—original draft preparation, A.D.P.; writing—review and editing, A.R., F.N., and M.A.B.; visualization, A.D.P., A.S., and A.N.M.; supervision, A.D.P.; project administration, A.D.P.; funding acquisition, A.D.P., A.R., and F.N. All authors have read and agreed to the published version of the manuscript.

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Andi Dian Permana*, Anwar Sam, Ardiyah Nurul Fitri Marzaman, Abdul Rahim, Firzan Nainu, Muh. Akbar Bahar, Rangga Meidianto Asri

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as its bioactive compounds possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment were significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system was able to control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, antioxidant, solid lipid nanoparticles, floating gel in situ, controlled release

1. Introduction

Recently, oxidative stress, caused by free radicals has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason of this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effect and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. Antioxidant properties of safflower is mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting its bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop delivery system to improve the solubility of these compounds.

Among several delivery system, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

been extensively applied to enhance the solubility and control the release of numerous hydrophobic compounds [22–24]. However, the poor stability and high propensity to aggregate in acid conditions limited their application through oral route [25]. Previously, hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs administered orally [26]. The decoration of SLNs containing amphotericin B, paramomycin and paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects [26,27]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated with HP β CD could be an appropriate strategy for improved solubility.

To facilitate the oral administration of the SLNs, it was important to develop a suitable system. One of delivery systems to control and sustain the release of drugs through oral administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of drugs would stay in the stomach for long period [28–30]. As one of GRDDS, in situ forming gel system has attracted the interest in drug delivery due to the transformation of the formulation from solution into floating gel when administered orally and reaching the gastric environment [31–33]. Numerous studies have explored the effectiveness of this system to control the release some drugs administered orally [29,34,35]. As one of macromolecules compounds, gellan gum has been widely used to develop GRDDS preparations, showing the controlled release pattern of the bioactive compounds [29,36–38].

In this study, we extracted the bioactive compounds from safflower and formulated the extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the dry form which would be dispersed in water prior to the oral administration. The floating gel in situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect of food intake on the dissolution profile was further investigated. The main finding of this investigation showed the potential of using natural product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvent, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used in this study room temperature. The mixtures were then filtered and the filtrate was subjected to rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoids content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [39], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used a standard compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [39], with a slight modification. The extract was initially

solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to mL) in volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as standard compound. The concentration of TPC were reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as control. The extraction solvents were utilized were used as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of extract were assessed using thiocyanate, with slight modification. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g) and 20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL)

was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1).

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [26]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

2.8. Determination of Particle Size, PDI and Zeta Potential

Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK) was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations. The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the SLNs was diluted in water accordingly.

2.9. Encapsulation Efficiency Determination

Indirect method was applied to determine the encapsulation efficiency of the compounds, by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes. The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-dissolved compounds were quantified by determining the compounds from the filtrate from the purification step to concentrate the SLNs using HPLC. The encapsulation efficiency of both compounds was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

2.10. FTIR study

To evaluate the possible intervention between the compounds in extract and excipients used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used. In this study, all samples were scanned between 4000 and 600 cm⁻¹ wavenumbers.

2.11. DSC evaluation

The thermal properties of the compounds in extract, as well as in SLNs formulation were evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments)). The samples were weighed of around 5 mg and were closed in aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min under N₂ flow (40 mL/min)

2.12. XRD study

X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state of the compounds in extract, as well as in SLNs formulation. All formulations were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min.

2.13. SEM evaluation

In this study, the morphologies of SLNs were detected using a scanning electron microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan).

2.14. Solubility Analysis

In order to investigate the solubility of both compounds in extract and SLN preparations, two different solvents, namely water and n-octanol were used. Briefly, extract and SLNs were added in excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from lipid matrix was carried out using dialysis method was used [40–43]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium were withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [44,45]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBC was prepared in the concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C

and spun for 10 min at 7000 rpm. The supernatant was collected and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of the hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	F1	F2	F3	F4	F5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSSGF media (100 mL) under the stirring at 50 rpm at 37 ± 0.5 °C. The formation of gel was observed and recorded as gelation time [46].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing time required by the gel formed to float in the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [46].

2.21. Measurement of Viscosity and Gel Strength

A brookfield Viscometer DV III (Brookfield Engineering Laboratories, MA) was used to determine the viscosity of the formulation after being dispersed in water. The study was carried using using spindle number 2 at 50 rpm. In an attempt to measure the gel strength, a Rheometer (TA Instrument, USA) was utilized. The study was conducted at 25 °C.

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. The pH of the mixture was adjusted to pH 5 using 1 M HCl [47].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the

formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [34]. The release profile of both compounds from crude extract loaded dry floating gel in situ was also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radical. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in

medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPD as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was statistical difference in IC₅₀ values of all extract ($p < 0.05$).

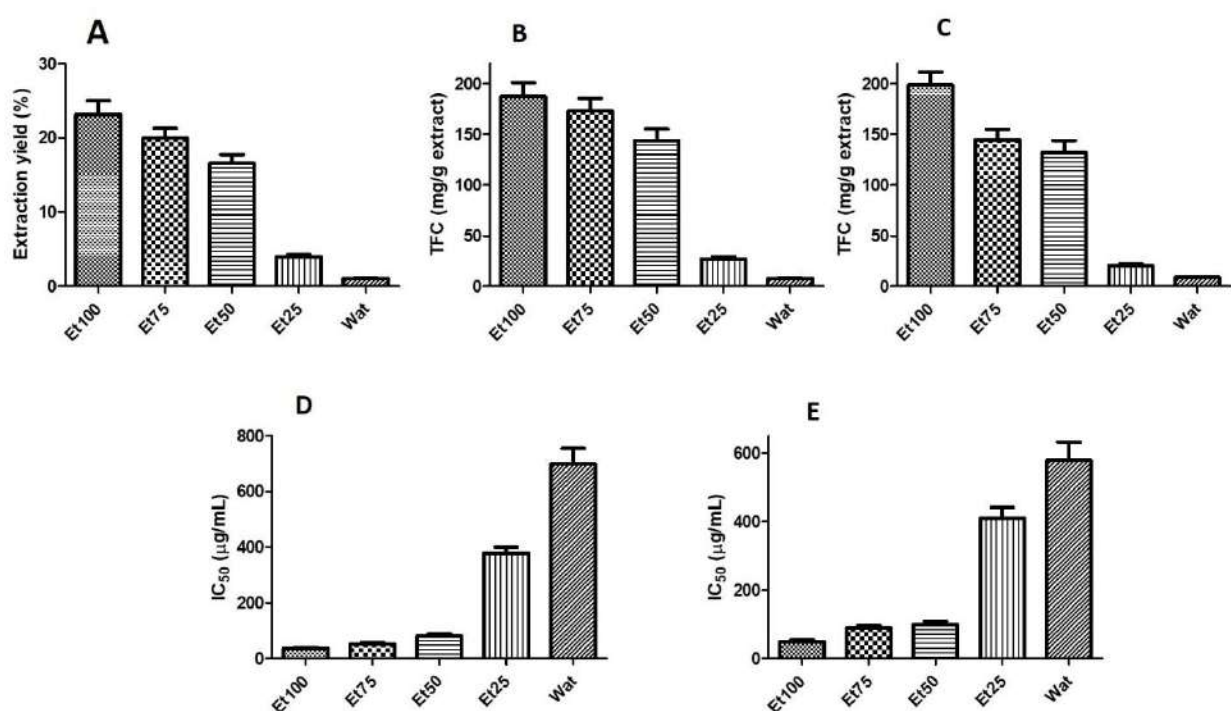


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPD (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, $n=3$)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extract were also investigated using lipid peroxidation method. As lipids are the major component of cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [39]. In this evaluation, it was found that the IC₅₀ against lipid peroxidation were of Et100, Et75,

Et50, Et25 and Wat are depicted in Figure X, showing the values of $48.75 \pm 3.42 \mu\text{g/mL}$, $89.04 \pm 6.56 \mu\text{g/mL}$, $98.97 \pm 8.91 \mu\text{g/mL}$, $409.51 \pm 31.67 \mu\text{g/mL}$ and $577.43 \pm 43.87 \mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC50 values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were $7.65 \pm 0.63 \text{ mg/g}$ for Et100, $4.32 \pm 0.32 \text{ mg/g}$ for Et75, $2.97 \pm 0.19 \text{ mg/g}$ for Et50, $0.74 \pm 0.05 \text{ mg/g}$ for Et25 and $0.02 \pm 0.001 \text{ mg/g}$ for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were $2.32 \pm 0.21 \text{ mg/g}$, $1.92 \pm 0.15 \text{ mg/g}$, $0.67 \pm 0.05 \text{ mg/g}$, $0.29 \pm 0.03 \text{ mg/g}$ and $0.01 \pm 0.001 \text{ mg/g}$, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

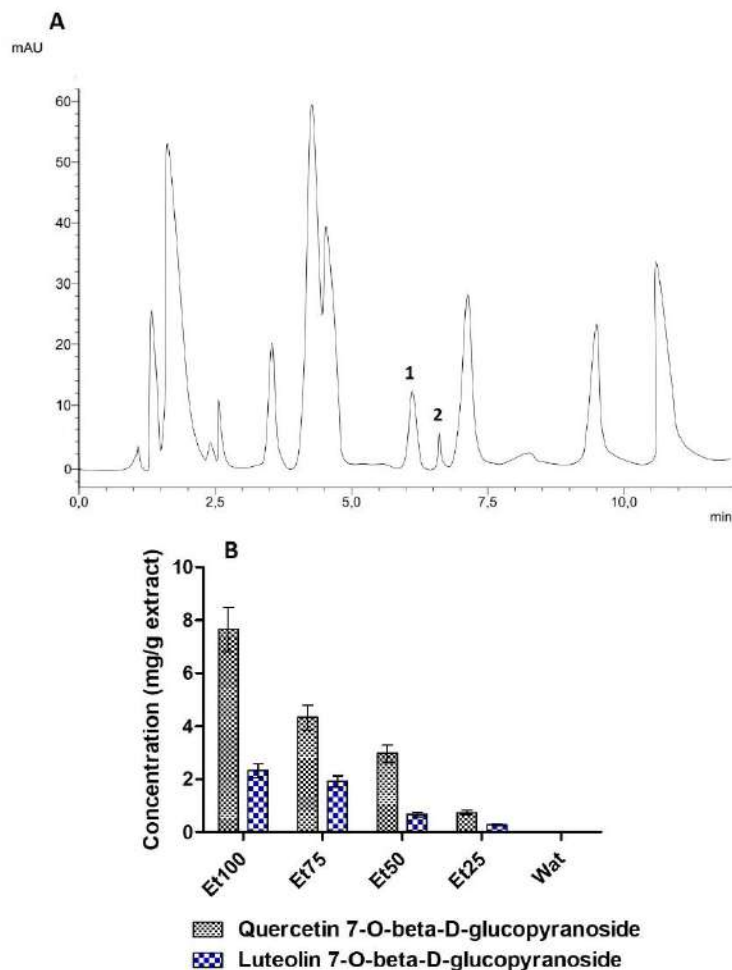


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [39]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [48]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [49]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency of both compounds in lipid matrix [39]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum

formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. Moreover, the encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [25,26]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

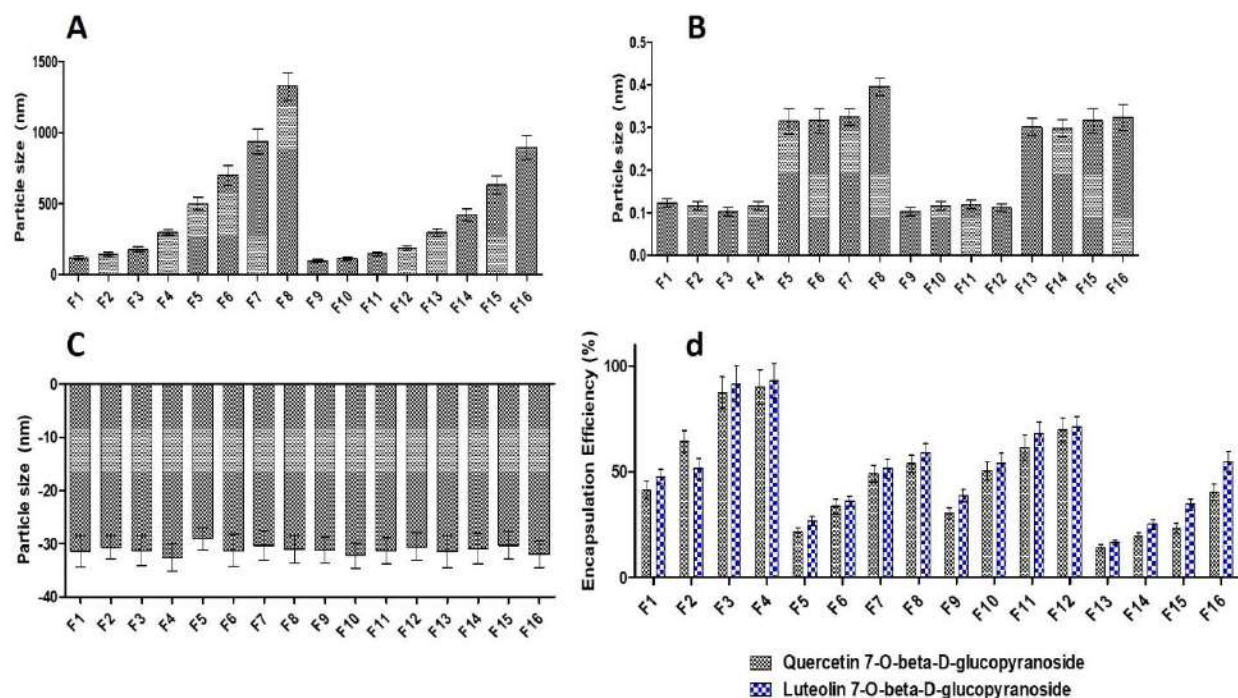


Figure 3. Particle size (A), PDI (B), zeta potential (C) and encapsulation efficiency results of SLNs prepared from safflower extract (mean $\pm$ SD, $n=3$)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both

compounds. Additionally, another peak was detected at 1684 cm⁻¹ which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm⁻¹ due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm⁻¹, 1363 cm⁻¹ and 1237 cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds.

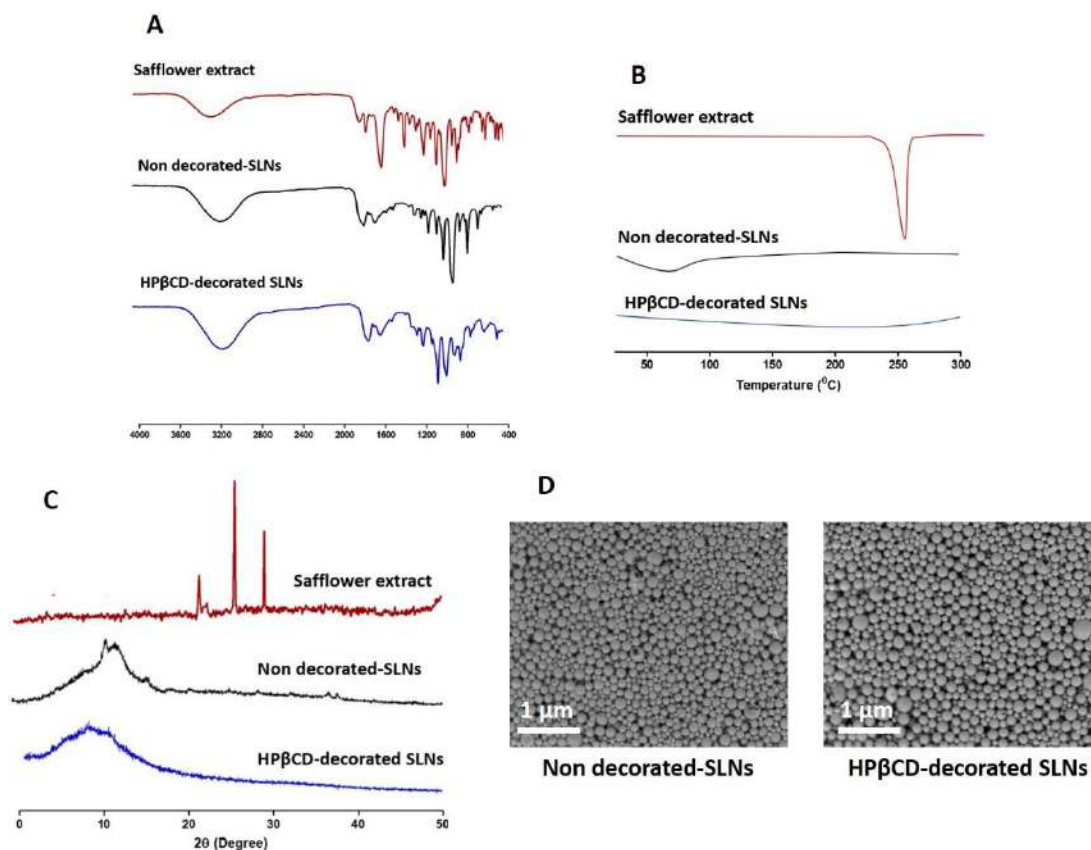


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4 shows the DSC thermograms of safflower extract and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252 °C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the SLN formulations, the sharp peak was not

observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21].

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were 18.23 ± 1.32 µg/mL and 16.54 ± 1.36 µg/mL for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely 408.45 ± 29.34 µg/mL and 434.31 ± 39.31 µg/mL. Following the incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HPβCD-SLNs (mean ± SD, n=3)

Compound		Samples	Aqueous solubility (µg/mL)	n-octanol solubility (µg/mL)
Quercetin glucopyranoside	7-O-beta-D-	Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HPβCD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin glucopyranoside	7-O-beta-D-	Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HPβCD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [50–54].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustain release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [55]. Following this, the slow-release patterns was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,44].

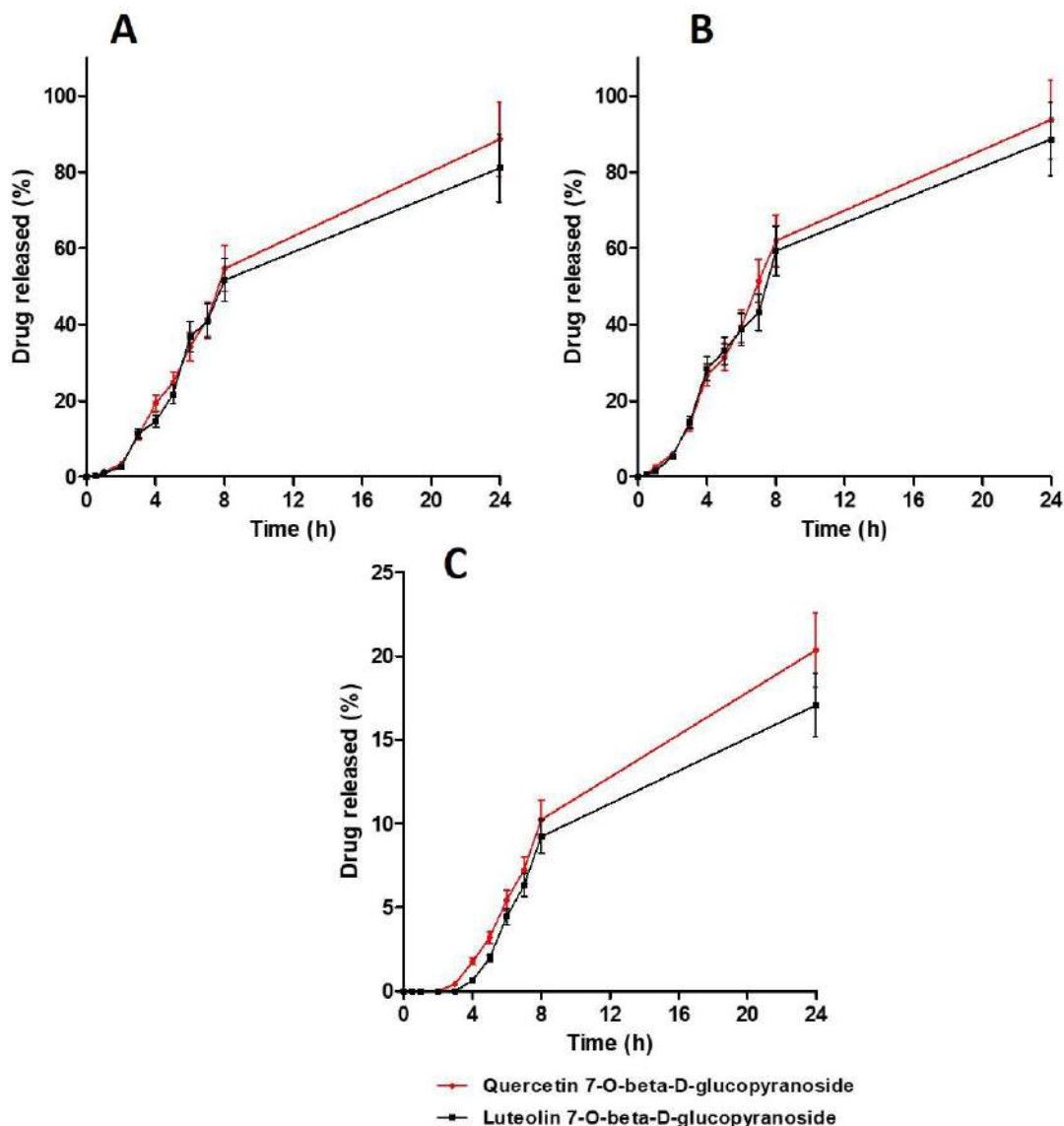


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD -SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6. As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [42,43].

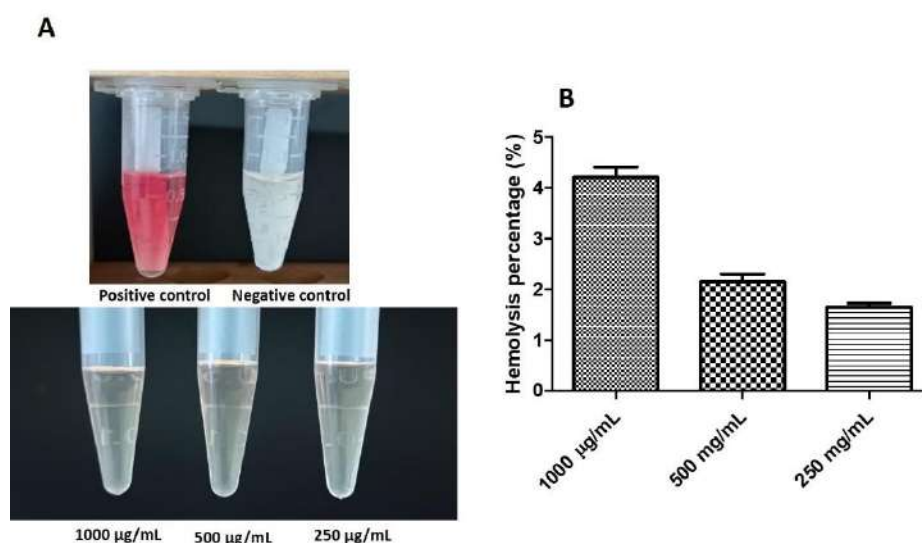


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporate HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [29]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [34]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [56]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [57,58]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for F1, F2, F3, F4 and F5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

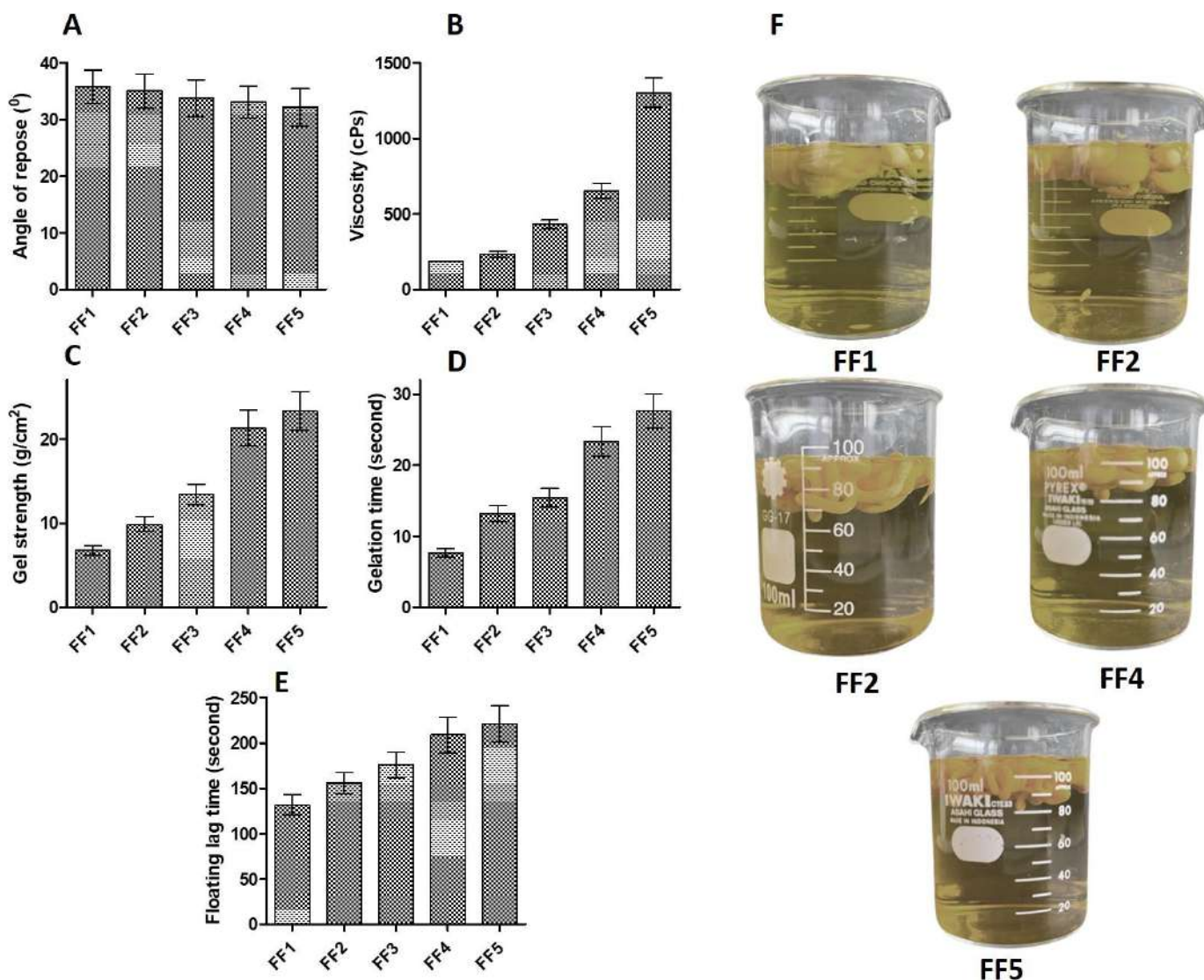


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, n=3). Representative images of floating gel in situ after the floating evaluation in FaSGGF, showing that all formulations were able to float (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The viscosity of the formulation was in the range of 187.98 ± 1.67 cps to 1302.62 ± 98.21 cps. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic

F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [57,58].

The strength values of the gel formulation are shown in Figure 7C. It was found that the increase of Pluronic F127 concentration from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [56]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [44].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled

formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which affected the release of CO₂ [59]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [60]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [61]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure X, below 7.5% of Pluronics (F1 and F2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (F3), 10% (F4) and 15% (F5) of Pluronics were capable of controlling the release of the compounds over 24 h.

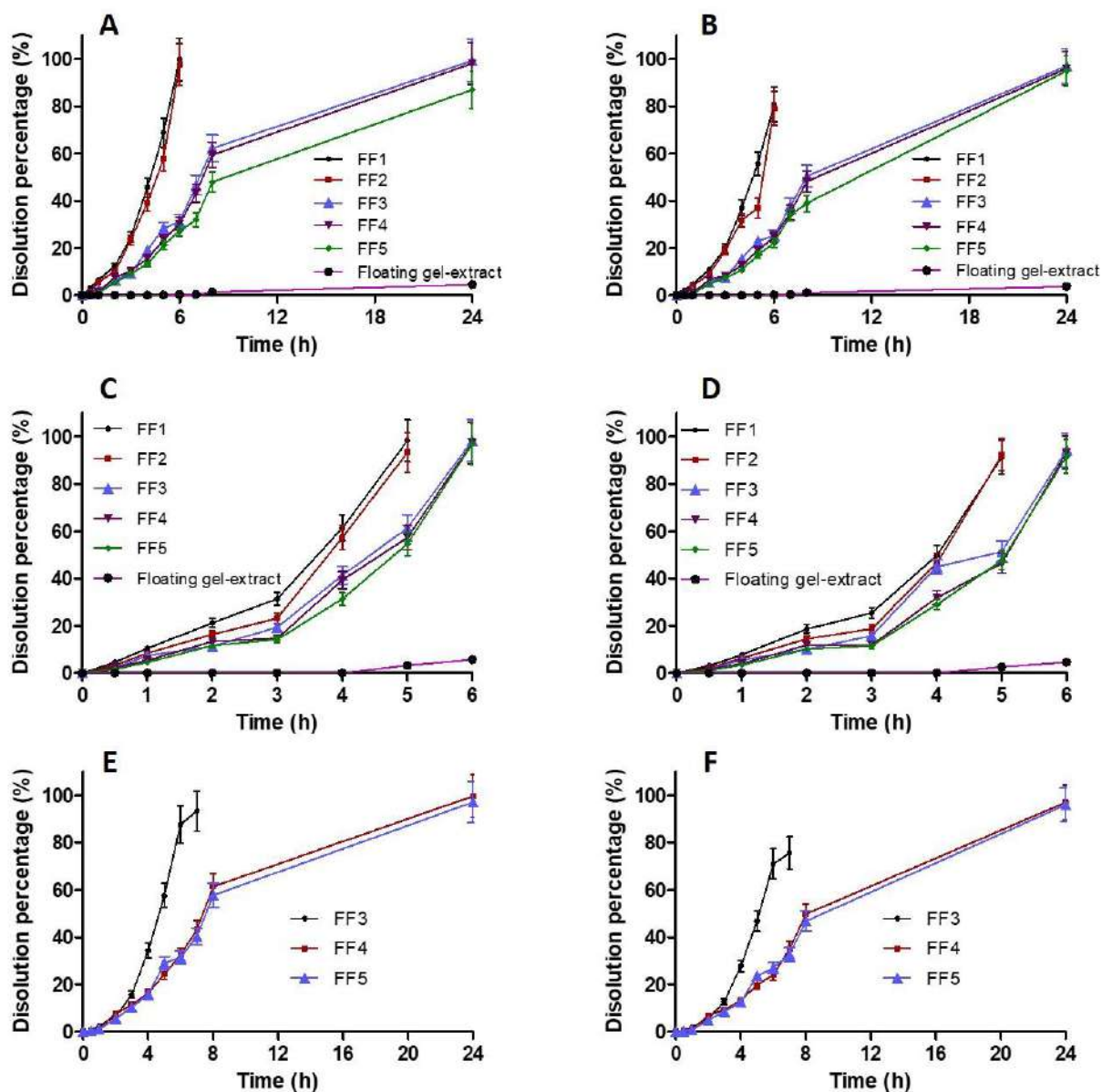


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (E) and luteolin 7-O-beta-D-glucopyranoside (F) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only F3, F4 and F5 were selected due to their ability to control the dissolution behavior for 24 h. After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, F4 and F4 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of F3 was significantly lower ($p < 0.05$) than F4 and F5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF. Thi study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. To further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 $\mu\text{g/mL}$ against DPPD and 48.75 ± 4.52 $\mu\text{g/mL}$ against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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Reply-To: International Journal of Biological Macromolecules <support@elsevier.com>

To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Manuscript Number: IJBIOMAC-D-22-09382R3

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

Dear Dr. Permana,

Thank you for submitting your manuscript to International Journal of Biological Macromolecules.

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International Journal of Biological Macromolecules values your contribution and I look forward to receiving your revised manuscript.

Kind regards,

Daniela Giacomazza

Editor

International Journal of Biological Macromolecules

Editor and Reviewer comments:

The title should be corrected as: **"Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media"**

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International Journal of Biological Macromolecules

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media --Manuscript Draft--

Manuscript Number:	IJBIMAC-D-22-09382R2
Article Type:	Research Paper
Section/Category:	Carbohydrates, Natural Polyacids and Lignins
Keywords:	Safflower; solid lipid nanoparticles; floating gel in situ
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Abstract:	Safflower (<i>Carthamus tinctorius</i> L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HPβCD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.
Suggested Reviewers:	Ryan Donnelly r.donnelly@qub.ac.uk Aaron Courtenay a.courtenay@ulster.ac.uk Eneko Larraneta e.larraneta@qub.ac.uk Ahmed Faheem ahmed.faheem@sunderland.ac.uk
Opposed Reviewers:	
Response to Reviewers:	Manuscript Number: IJBIMAC-D-22-09382R1 Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry

floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Response to Reviewers:

We thank the reviewer for reviewing the manuscript in detail and providing valuable suggestions for the improvement of our manuscript. We are pleased to submit the revision of the manuscript to

International Journal of Biological Macromolecules and addressed all comments, as follows:

Editor and Reviewer comments:

Please check the reference format

Response:

We thank the reviewer for pointing this out. We have corrected those misspelling in our revised manuscript.

Reviewer #1:

Comments

IJBIMAC-D-22-09382R1

1. The authors should performed in vivo pharmacokinetic analysis of the developed formulation to check the effect of the SLN-loaded gel in complex in vivo settings.

Response:

We thank the reviewer for the comment. Indeed, we agreed that in vivo pharmacokinetic analysis is crucial for the study. However, in study, we currently focused on the discovery of the bioactive compounds from Safflower, performed extensive analyses, optimized the formulation and perform proof of concept study. Specifically, to mimic the environment of the real condition, we evaluated the in vitro effect of food intake in the biorelevant media, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). Importantly, in this study, we have performed additional study to evaluate the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. We believe that our study could serve as a proof-of-concept study for the development of natural compounds in the combination of Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems to improve the oral bioavailability. Moreover, to represent our study, we have modified the title our study to "Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media". Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

2. What was the yield of safflower flowers in the process of extraction?

Response:

We thank the reviewer for the question. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. This has been included in the revised manuscript.

3. The authors should add references to all the methods reported in the manuscript.

Response:

	We thank the reviewer for pointing this out. We have included all references to all methods in our revised manuscript. 4. Please write the detailed methods of particle properties, FTIR, XRD, and SEM. Response: We thank the reviewer for the comment. We have written the detailed in the revised manuscript. 5. Why the authors used 2 methods (UV and HPLC) for quantification of drugs. Why not all the analysis were performed using HPLC? Response: We thank the reviewer for the question. In this study, we used HPLC to quantify the bioactive compounds in combination with UV detection. This method has been widely used in the formulation development (Al-Suwayeh et al., 2014; Ahmed et al., 2018; Anjani et al., 2022; Asma et al., 2022; Cedillo-Flores et al., 2022). 6. Did the authors develop HPLC method? What was the linearity, inter day & intraday variations?. Response: We thank the reviewer for the question. The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999. This has been included in the revised manuscript. 7. Beside the entrapment efficiency, the authors are advised to add drug content of the SLN. Response: We thank the reviewer for the suggestion. Following this, we have included the drug loading in the revised manuscript. 8. Why the in vitro dissolution study was performed at 100 rpm. Does it mimic stomach conditions? Response: We thank the reviewer for the question. As shown in 2.23, the in vitro dissolution study was carried out at 50 rpm to mimic the stomach conditions (Himawan et al., 2022). The 100 rpm was used in the release study. The release study was different to the dissolution study. In the release study, we evaluated the profile of bioactive compounds to release from the lipid matrix. This speed has been widely used to investigate the release profiles numerous drugs from different formulation matrix (Permana et al., 2019; Mir et al., 2020a, 2020b; Permana, Mir, et al., 2020; Tekko et al., 2020; Enggi et al., 2021). 9. Why the gel strength was determined at 25 C, when the formulation has to become gel in situ at physiological temperature? Please explain. Response: We thank the reviewer for pointing this out. Similar to other evaluations, we performed this at 37 °C. We have clarified this in the revised manuscript. 10. Why the pH of the dissolution media was adjusted to pH 5? Please explain Response: We thank the reviewer for pointing this out. The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M (Permana, Utami, et al., 2020a). Meanwhile, the preparation of FeSSGFF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl (Baxeavanis et al., 2020). We have clarified this in the revised manuscript. 11. Please add the details of statistical evaluation Response: We thank the reviewer for pointing this out. We have included this in the revised manuscript.

12. What is the difference between the in vitro drug release and in vitro drug dissolution study reported in the manuscript?

Response:

We thank the reviewer for the question. As shown in 2.23, the in vitro dissolution study was carried out at 50 rpm to mimic the stomach conditions (Himawan et al., 2022). The 100 rpm was used in the release study. The release study was different to the dissolution study. In the release study, we evaluated the profile of bioactive compounds to release from the lipid matrix. This speed has been widely used to investigate the release profiles numerous drugs from different formulation matrix (Permana et al., 2019; Mir et al., 2020a, 2020b; Permana, Mir, et al., 2020; Tekko et al., 2020; Enggi et al., 2021). In the in vitro dissolution study, we evaluated the dissolution behavior of bioactive compounds in the gastric environment.

13. What is the rational of incorporation of SLNs in HP β CD? If it is only stability, it can be achieved by simply lyophilizing the SLNs into dry powder? Please explain in the manuscript.

Response:

We thank the reviewer for the question. Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system (Permana et al., 2019). SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have been extensively applied to enhance the solubility and control the release of numerous hydrophobic compounds (Zur Mühlen, Schwarz and Mehnert, 1998; Triplett and Rathman, 2009; Mojahedian et al., 2013). With respect to the administration route, the oral route has been considered the most convenient and acceptable route for drug administration (McGuckin et al., 2022). However, the poor stability and high propensity to aggregate in acid conditions limited their application through the oral route (Wang et al., 2017). Several studies have shown that the oral administration of SLNs could results in instability of the particles in the gastric environment (Zimmermann and Müller, 2001; Qiu et al., 2018). Accordingly, it is crucial to investigate suitable approach to protect the SLNs from the gastric environment during oral administration. Previously, hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs administered orally (Parvez, Karole and Mudavath, 2022). The decoration of SLNs containing amphotericin B, paramomycin and paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects (Baek et al., 2012; Parvez, Karole and Mudavath, 2022). Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated with HP β CD could be an appropriate strategy for improved solubility. We have clarified this in the revised manuscript.

14. Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract is very confusing. There are so many peaks with high intensities, indicating the presence of other metabolites. I wonder how the authors justify the safety or no pharmacological effect of these metabolites. Do the authors have any evidences to rule out it?

Response:

We thank the reviewer for the comment. In this study, we used crude extract of Safflower. Therefore, in addition to the interest compounds, several compounds could be still contained in the extract. To ensure that we detected the correct compounds during the formulation development, we validated the HPLC method using UV detection. As mentioned previously, the HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 μ g/mL and 0.21 μ g/mL with limit of quantifications of 0.35 μ g/mL and 0.44 μ g/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999. Therefore, we believe that the analytical method could be used to identify both interest compounds in the presence of other compounds. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this

approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract. This has been included in the revised manuscript.

15. Did you check the viscosity of the SLNs with Geleol® as the main phenomenon of its selection is made on viscosity?

Response:

We thank the reviewer for the question. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution. In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids (Permana, Utami, et al., 2020b). This has been included in the revised manuscript.

16. FTIR analysis. Why the peak of safflower extract at 1100-1200 is absent in the corresponding SLNs spectra's?

Response:

We thank the reviewer for the question. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix. This has been included in the revised manuscript.

17. Why the authors didn't choose to perform the FTIR, DSC and XRD analysis of lipids, surfactants and their physical mixture?

Response:

We thank the reviewer for the question. We have performed additional experiments regarding this and the result has been included in the revised manuscript.

18. Moreover, the results of DSC and XRD very poorly properly discussed.

Response:

We thank the reviewer for the comment. We have added more discussion in the DSC and XRD parts in the revised manuscript.

19. Please explain with logical reasons the role of HPβCD-SLNs in enhancing the solubility of extract, even more than the SLNs?

Response:

We thank the reviewer for the question. As explained in our manuscript, the improvement of the solubility was due to the formation of nanoparticles. Furthermore, following the decoration using HPβCD, due to the solubilization enhancement ability of HPβCD (Anjani et al., 2022), the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values. We have included this in the revised manuscript.

20. Figure 5 shows no significant difference between the in vitro release profiles of SLNs and HPβCD-SLNs at all the time periods. The authors claim and the entire rational becomes questionable at this point, if they are not significantly different in drug release.

Response:

We thank the reviewer for pointing this out. Figure 5 shows the in vitro release profile of both compounds from SLNs in PBS media. In this study, we initially ensured that the decoration of SLNs using HPβCD did not change the release behaviour of both compounds. The main results are shown in Figure 8. In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the

dissolution behavior for 24 h. After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF. Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.. We have included this in the revised manuscript.

21. Which equipment was used to determine the gelation time? The authors are advised to add the equipment details or sketch in the supplementary data.

Response:

We thank the reviewer for pointing this out. The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The time required by the liquid formulation to form a gel was observed and recorded as gelation time (Singhavi, Pundkar and Khan, 2017). We have clarified this in the revised manuscript.

22. Figure 8. Please indicate the level of significance among the data presented. In its current form, the formulations seems no significant difference.

Response:

We thank the reviewer for pointing this out. We have added the level of significance among the data presented in the revised manuscript.

23. This manuscript needs extensive revisions for grammatical and structural mistakes.

Response:

We thank the reviewer for pointing this out. We have made a great effort to improve the English for grammatical and structural mistakes in the revised manuscript.

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This piece of the submission is being sent via mail.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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The Editor

International Journal of Biological Macromolecules

17 December 2022

Dear Sir/Madam,

I wish you to re-consider our manuscript entitled: **“Solid lipid nanoparticles-decorated cyclodextrin incorporated gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)”** for publication in International Journal of Biological Macromolecules. Moreover, to represent our study, we have modified the title our study to **“Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media**

We have made a few of changes to the manuscript as a result of the reviewers comments. We believe that the manuscript is now substantially improved. Importantly, we have made a great effort to addressed each of the reviewers' comments in the response to the reviewer file.

We believe that this work will be of great interest to the readers of International Journal of Biological Macromolecules. The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in International Journal of Biological Macromolecules and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

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Manuscript Number: IJBIOMAC-D-22-09382R1

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Response to Reviewers:

We thank the reviewer for reviewing the manuscript in detail and providing valuable suggestions for the improvement of our manuscript. We are pleased to submit the revision of the manuscript to

International Journal of Biological Macromolecules and addressed all comments, as follows:

Editor and Reviewer comments:

Please check the reference format

Response:

We thank the reviewer for pointing this out. We have corrected those misspelling in our revised manuscript.

Reviewer #1:

Comments

IJBIMAC-D-22-09382R1

1. The authors should performed *in vivo* pharmacokinetic analysis of the developed formulation to check the effect of the SLN-loaded gel in complex *in vivo* settings.

Response:

We thank the reviewer for the comment. Indeed, we agreed that *in vivo* pharmacokinetic analysis is crucial for the study. However, in study, we currently focused on the discovery of the bioactive compounds from Safflower, performed extensive analyses, optimized the formulation and perform proof of concept study. Specifically, to mimic the environment of the real condition, we evaluated the *in vitro* effect of food intake in the biorelevant media, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). Importantly, in this study, we have performed additional study to evaluate the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. We believe that our study could serve as a proof-of-concept study for the development of natural compounds in the combination of Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating *in situ* delivery systems to improve the oral bioavailability. Moreover, to represent our study, we have modified the title our study to “**Solid lipid nanoparticles-decorated**

cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media". Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

2. What was the yield of safflower flowers in the process of extraction?

Response:

We thank the reviewer for the question. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. This has been included in the revised manuscript.

3. The authors should add references to all the methods reported in the manuscript.

Response:

We thank the reviewer for pointing this out. We have included all references to all methods in our revised manuscript.

4. Please write the detailed methods of particle properties, FTIR, XRD, and SEM.

Response:

We thank the reviewer for the comment. We have written the detailed in the revised manuscript.

5. Why the authors used 2 methods (UV and HPLC) for quantification of drugs. Why not all the analysis were performed using HPLC?

Response:

We thank the reviewer for the question. In this study, we used HPLC to quantify the bioactive compounds in combination with UV detection. This method has been widely used in the formulation development (Al-Suwayeh *et al.*, 2014; Ahmed *et al.*, 2018; Anjani *et al.*, 2022; Asma *et al.*, 2022; Cedillo-Flores *et al.*, 2022).

6. Did the authors develop HPLC method? What was the linearity, inter day & intraday variations?.

Response:

We thank the reviewer for the question. The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-

glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999. This has been included in the revised manuscript.

7. Beside the entrapment efficiency, the authors are advised to add drug content of the SLN.

Response:

We thank the reviewer for the suggestion. Following this, we have included the drug loading in the revised manuscript.

8. Why the *in vitro* dissolution study was performed at 100 rpm. Does it mimic stomach conditions?

Response:

We thank the reviewer for the question. As shown in 2.23, the *in vitro* dissolution study was carried out at 50 rpm to mimic the stomach conditions (Himawan *et al.*, 2022). The 100 rpm was used in the release study. The release study was different to the dissolution study. In the release study, we evaluated the profile of bioactive compounds to release from the lipid matrix. This speed has been widely used to investigate the release profiles numerous drugs from different formulation matrix (Permana *et al.*, 2019; Mir *et al.*, 2020a, 2020b; Permana, Mir, *et al.*, 2020; Tekko *et al.*, 2020; Enggi *et al.*, 2021).

9. Why the gel strength was determined at 25 °C, when the formulation has to become gel in situ at physiological temperature? Please explain.

Response:

We thank the reviewer for pointing this out. Similar to other evaluations, we performed this at 37 °C. We have clarified this in the revised manuscript.

10. Why the pH of the dissolution media was adjusted to pH 5? Please explain

Response:

We thank the reviewer for pointing this out. The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M (Permana, Utami, *et al.*, 2020a). Meanwhile, the preparation of FeSSGFF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl (Baxevanis *et al.*, 2020). We have clarified this in the revised manuscript.

11. Please add the details of statistical evaluation

Response:

We thank the reviewer for pointing this out. We have included this in the revised manuscript.

12. What is the difference between the in vitro drug release and in vitro drug dissolution study reported in the manuscript?

Response:

We thank the reviewer for the question. As shown in 2.23, the *in vitro* dissolution study was carried out at 50 rpm to mimic the stomach conditions (Himawan *et al.*, 2022). The 100 rpm was used in the release study. The release study was different to the dissolution study. In the release study, we evaluated the profile of bioactive compounds to release from the lipid matrix. This speed has been widely used to investigate the release profiles numerous drugs from different formulation matrix (Permana *et al.*, 2019; Mir *et al.*, 2020a, 2020b; Permana, Mir, *et al.*, 2020; Tekko *et al.*, 2020; Enggi *et al.*, 2021). In the *in vitro* dissolution study, we evaluated the dissolution behavior of bioactive compounds in the gastric environment.

13. What is the rational of incorporation of SLNs in HP β CD? If it is only stability, it can be achieved by simply lyophilizing the SLNs into dry powder? Please explain in the manuscript.

Response:

We thank the reviewer for the question. Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system (Permana *et al.*, 2019). SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have been extensively applied to enhance the solubility and

control the release of numerous hydrophobic compounds (Zur Mühlen, Schwarz and Mehnert, 1998; Triplett and Rathman, 2009; Mojahedian *et al.*, 2013). With respect to the administration route, the oral route has been considered the most convenient and acceptable route for drug administration (McGuckin *et al.*, 2022). However, the poor stability and high propensity to aggregate in acid conditions limited their application through the oral route (Wang *et al.*, 2017). Several studies have shown that the oral administration of SLNs could results in instability of the particles in the gastric environment (Zimmermann and Müller, 2001; Qiu *et al.*, 2018). Accordingly, it is crucial to investigate suitable approach to protect the SLNs from the gastric environment during oral administration. Previously, hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs administered orally (Parvez, Karole and Mudavath, 2022). The decoration of SLNs containing amphotericin B, paramomycin and paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects (Baek *et al.*, 2012; Parvez, Karole and Mudavath, 2022). Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated with HP β CD could be an appropriate strategy for improved solubility. We have clarified this in the revised manuscript.

14. Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract is very confusing. There are so many peaks with high intensities, indicating the presence of other metabolites. I wonder how the authors justify the safety or no pharmacological effect of these metabolites. Do the authors have any evidences to rule out it?

Response:

We thank the reviewer for the comment. In this study, we used crude extract of Safflower. Therefore, in addition to the interest compounds, several compounds could be still contained in the extract. To ensure that we detected the correct compounds during the formulation development, we validated the HPLC method using UV detection. As mentioned previously, the HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999. Therefore, we believe that the analytical method could be used to identify both interest compounds in the presence of other compounds. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract. This has been included in the revised manuscript.

15. Did you check the viscosity of the SLNs with Geleol® as the main phenomenon of its selection is made on viscosity?

Response:

We thank the reviewer for the question. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution. In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids (Permana, Utami, *et al.*, 2020b). This has been included in the revised manuscript.

16. FTIR analysis. Why the peak of safflower extract at 1100-1200 is absent in the corresponding SLNs spectra's?

Response:

We thank the reviewer for the question. It should be noted that the spectrums observed in the extract between 1100-1200 cm^{-1} were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix. This has been included in the revised manuscript.

17. Why the authors didn't choose to perform the FTIR, DSC and XRD analysis of lipids, surfactants and their physical mixture?

Response:

We thank the reviewer for the question. We have performed additional experiments regarding this and the result has been included in the revised manuscript.

18. Moreover, the results of DSC and XRD very poorly properly discussed.

Response:

We thank the reviewer for the comment. We have added more discussion in the DSC and XRD parts in the revised manuscript.

19. Please explain with logical reasons the role of HP β CD-SLNs in enhancing the solubility of extract, even more than the SLNs?

Response:

We thank the reviewer for the question. As explained in our manuscript, the improvement of the solubility was due to the formation of nanoparticles. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD (Anjani *et al.*, 2022), the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values. We have included this in the revised manuscript.

20. Figure 5 shows no significant difference between the *in vitro* release profiles of SLNs and HP β CD-SLNs at all the time periods. The authors claim and the entire rationale becomes questionable at this point, if they are not significantly different in drug release.

Response:

We thank the reviewer for pointing this out. Figure 5 shows the *in vitro* release profile of both compounds from SLNs in PBS media. In this study, we initially ensured that the decoration of SLNs using HP β CD did not change the release behaviour of both compounds. The main results are shown in Figure 8. In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h. After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related to the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF. Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution

percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.. We have included this in the revised manuscript.

21. Which equipment was used to determine the gelation time? The authors are advised to add the equipment details or sketch in the supplementary data.

Response:

We thank the reviewer for pointing this out. The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The time required by the liquid formulation to form a gel was observed and recorded as gelation time (Singhavi, Pundkar and Khan, 2017). We have clarified this in the revised manuscript.

22. Figure 8. Please indicate the level of significance among the data presented. In its current form, the formulations seems no significant difference.

Response:

We thank the reviewer for pointing this out. We have added the level of significance among the data presented in the revised manuscript.

23. This manuscript needs extensive revisions for grammatical and structural mistakes.

Response:

We thank the reviewer for pointing this out. We have made a great effort to improve the English for grammatical and structural mistakes in the revised manuscript.

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Author Contributions: Conceptualization, A.D.P., A.R., F.N., and M.A.B.; methodology, A.D.P., A.S., L.C., and A.N.M.; software, A.D.P., A.S., A.N.M.; validation, A.D.P., A.R., and F.N.; formal analysis, A.S and A.N.M.; investigation, A.D.P., A.S., and A.N.M.; resources, A.D.P., A.R., and F.N.; data curation, A.D.P., A.S., A.N.M.; writing—original draft preparation, A.D.P.; writing—review and editing, A.R., F.N., L.C., and M.A.B.; visualization, A.D.P., A.S., and A.N.M.; supervision, A.D.P.; project administration, A.D.P.; funding acquisition, A.D.P., A.R., and F.N. All authors have read and agreed to the published version of the manuscript.

Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard

compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 μ L of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and

20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was

found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed with the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

215 2.8. Determination of Particle Size, PDI and Zeta Potential

216 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 217 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 218 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 219 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 220 particle size, Z-average (d.nm) with intensity (%) was used.

221 2.9. Encapsulation Efficiency and Drug Loading Determination

222 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 223 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 224 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 225 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 226 dissolved compounds were quantified by determining the compounds from the filtrate from the
 227 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 228 loading of both compounds were calculated using the following equation:

$$229 \text{ Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

230 Drug loading (%) = $\frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$

233 **2.10. FTIR study**

234 To evaluate the possible intervention between the compounds in extract and excipients
235 used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this
236 study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹
237 wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s
238 and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

240 **2.11. DSC evaluation**

241 The thermal properties of the compounds in extract, as well as in SLNs formulation were
242 evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21].
243 Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in
244 aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min
245 under N₂ flow (40 mL/min).

247 **2.12. XRD study**

248 X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state
249 of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical
250 mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples
251 were measured at a sampling width of 0.02°.

253 **2.13. SEM evaluation**

254 In this study, the morphologies of SLNs were detected using a scanning electron
255 microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

256 **2.14. Solubility Analysis**

257 In order to investigate the solubility of both compounds in extract and SLN preparations,
258 two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were
259 added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature

at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The

time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

A brookfield Viscometer DV III (Brookfield Engineering Laboratories, MA) was used to determine the viscosity of the formulation after being dispersed in water. The study was carried using spindle number 2 at 50 rpm. In an attempt to measure the gel strength, a Rheometer (TA Instrument, USA) was utilized [37]. The study was conducted at 37 °C.

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGFF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [52].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed

using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPD as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

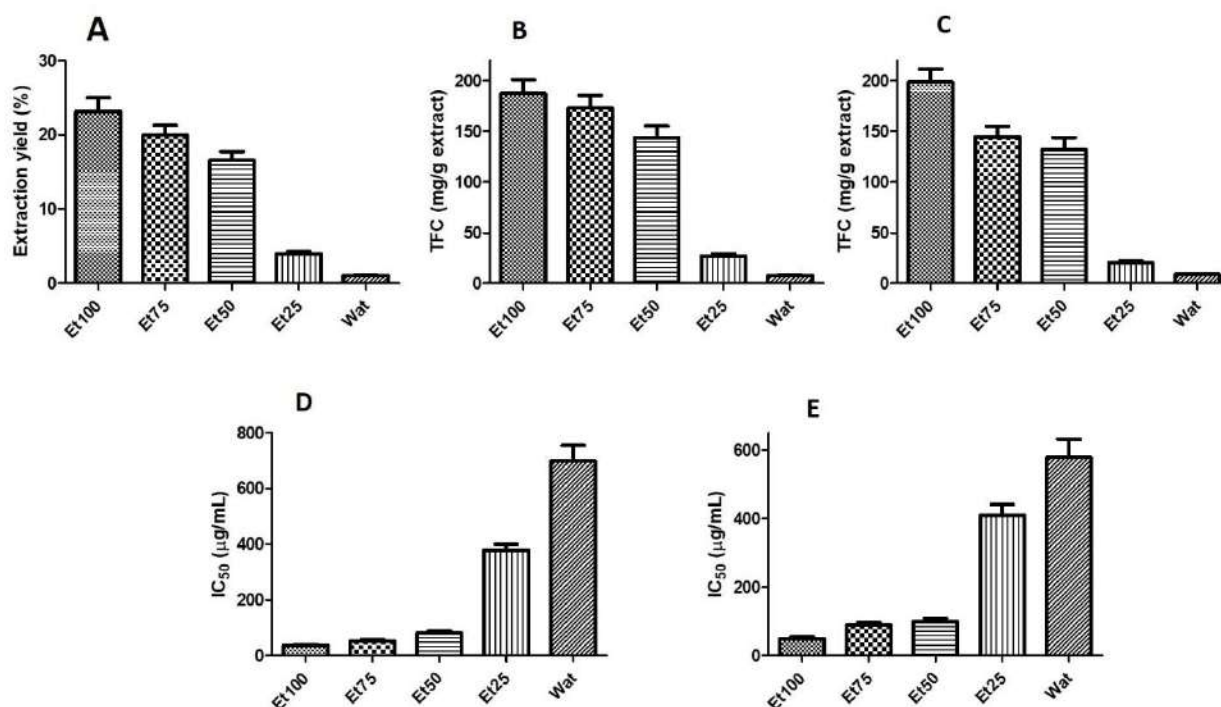


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPD (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, n=3)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were 48.75 ± 3.42 $\mu\text{g/mL}$, 89.04 ± 6.56 $\mu\text{g/mL}$, 98.97 ± 8.91 $\mu\text{g/mL}$, 409.51 ± 31.67 $\mu\text{g/mL}$ and 577.43 ± 43.87 $\mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were 7.65 ± 0.63 mg/g for Et100, 4.32 ± 0.32 mg/g for Et75, 2.97 ± 0.19 mg/g for Et50, 0.74 ± 0.05 mg/g for Et25 and 0.02 ± 0.001 mg/g for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were 2.32 ± 0.21 mg/g, 1.92 ± 0.15 mg/g, 0.67 ± 0.05 mg/g, 0.29 ± 0.03 mg/g and 0.01 ± 0.001 mg/g, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

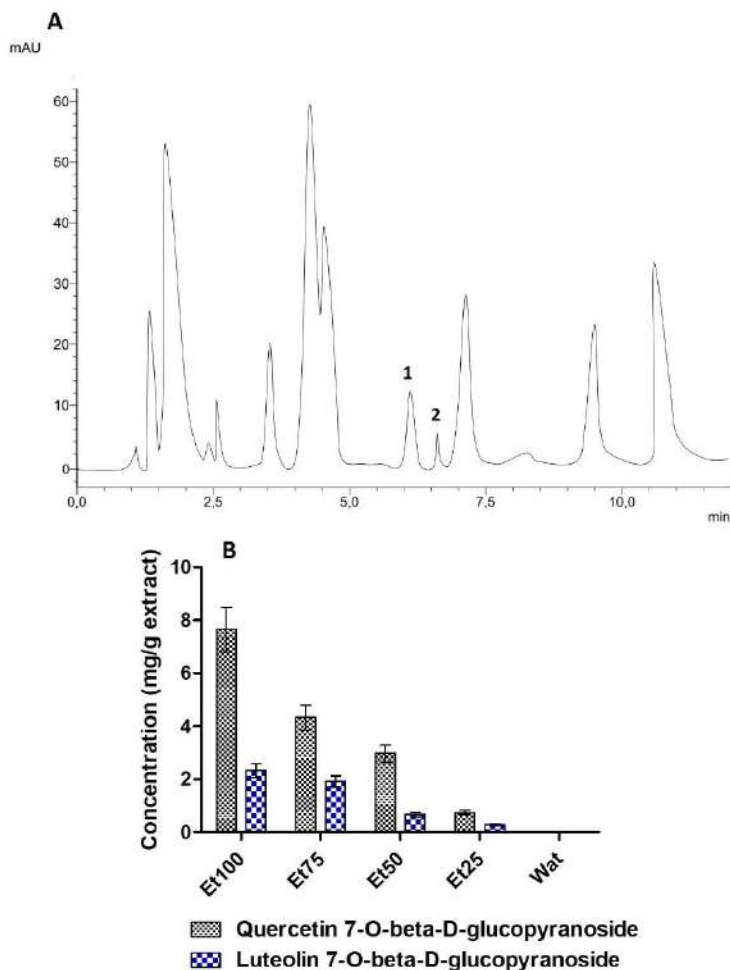


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [53]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [54]. As the HLB of Tween80 and PVA were > 24 and 15, respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

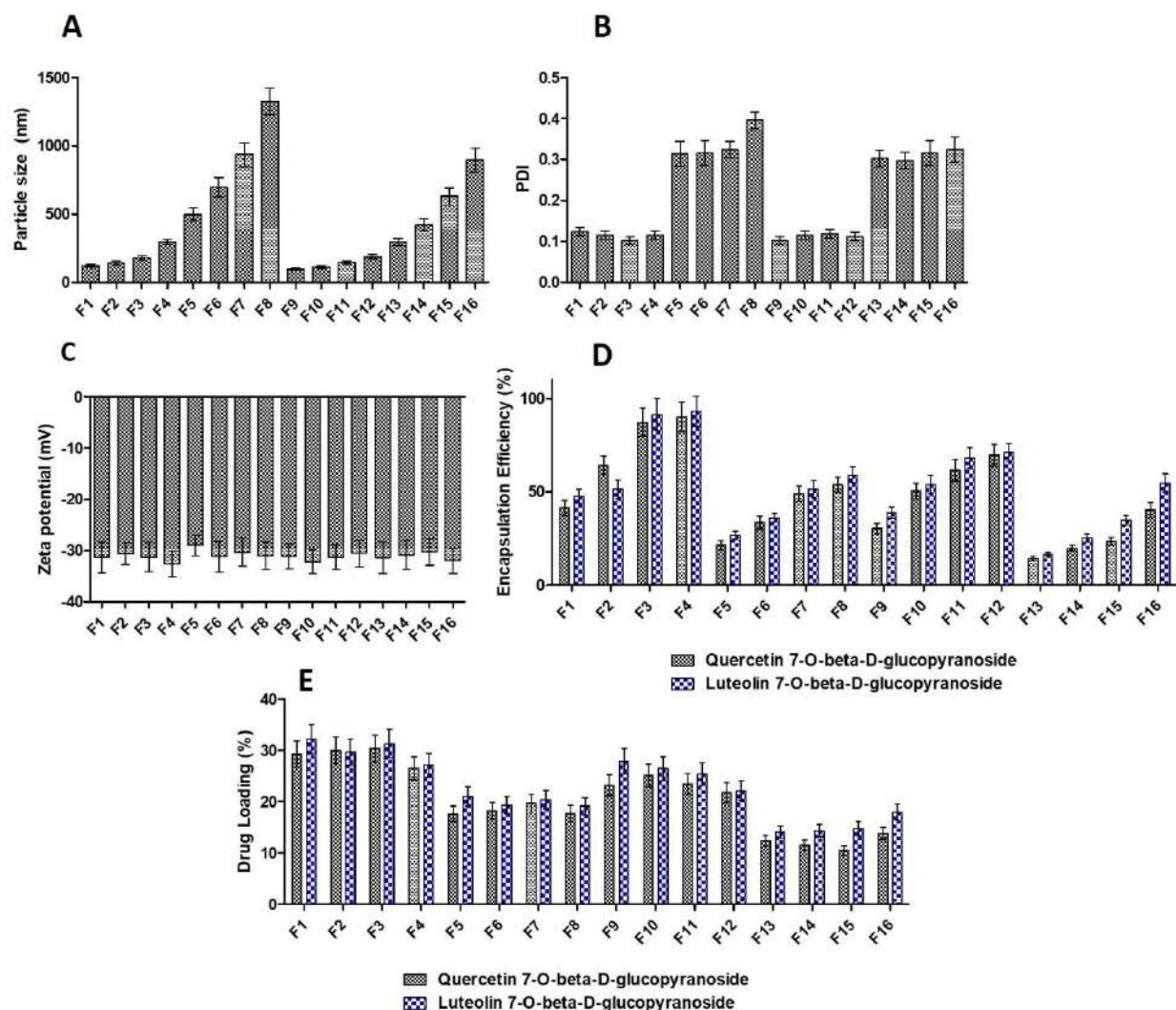


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

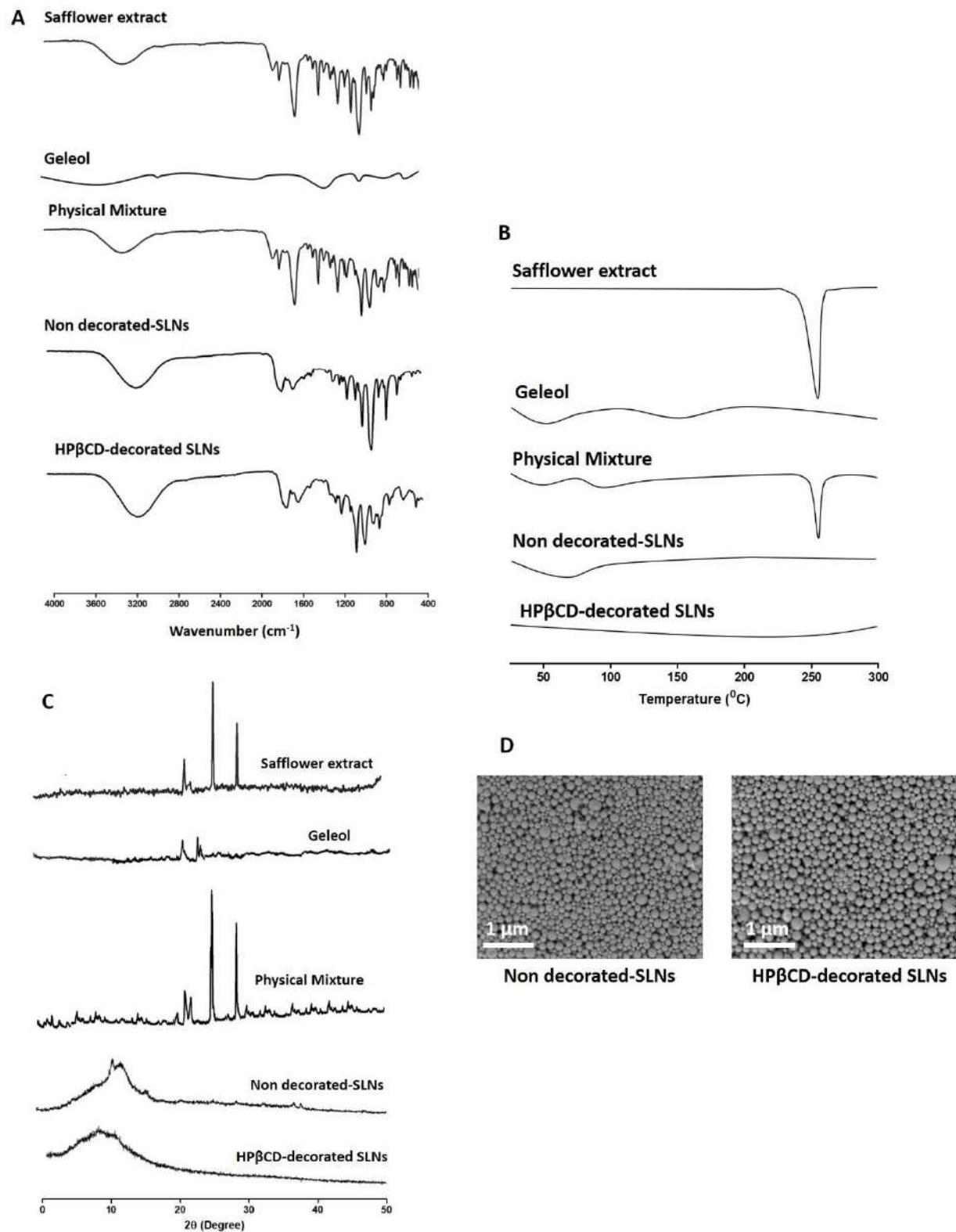


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [55], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [56–60].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustained release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of the compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [61]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

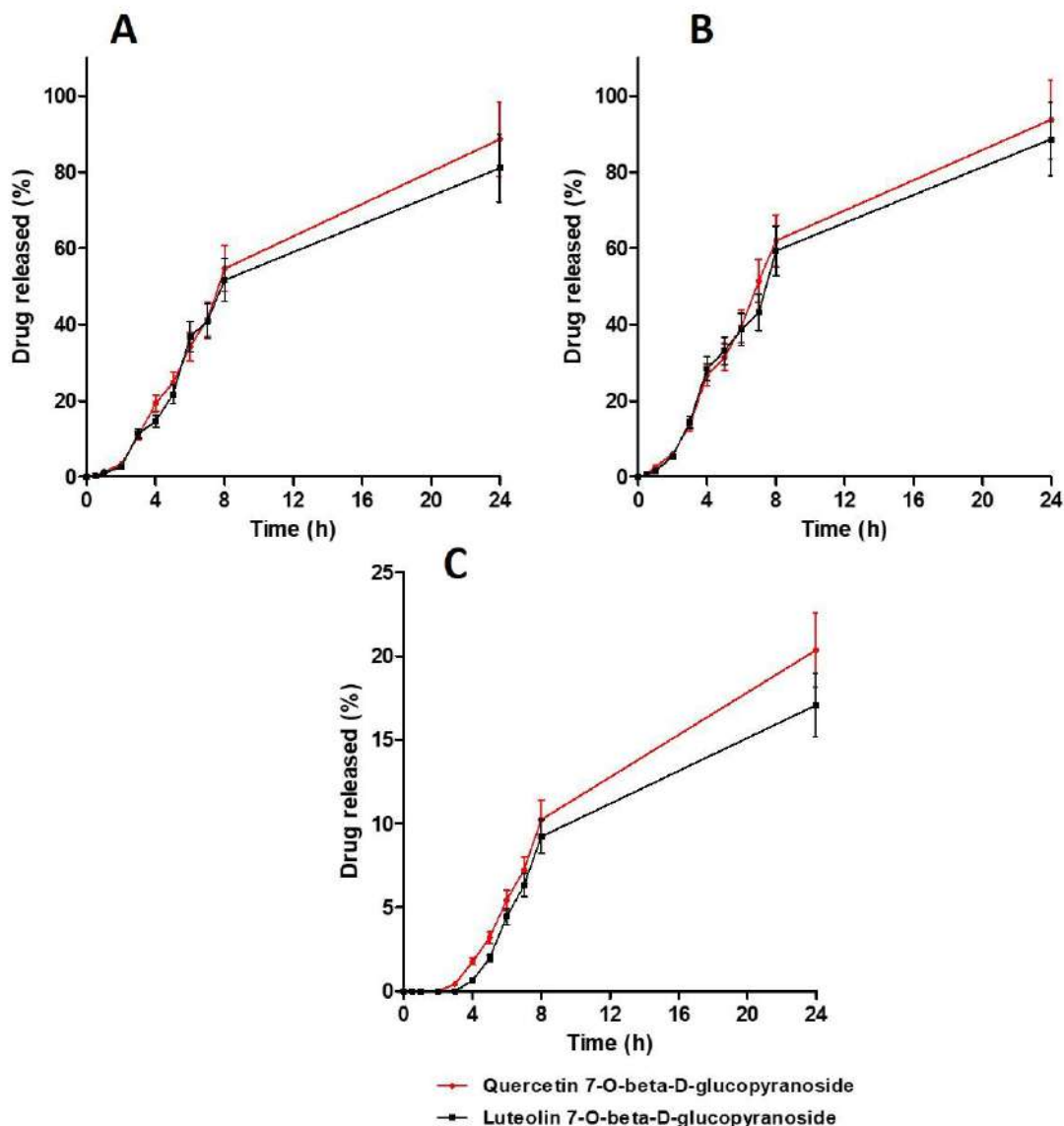


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, $n=3$)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

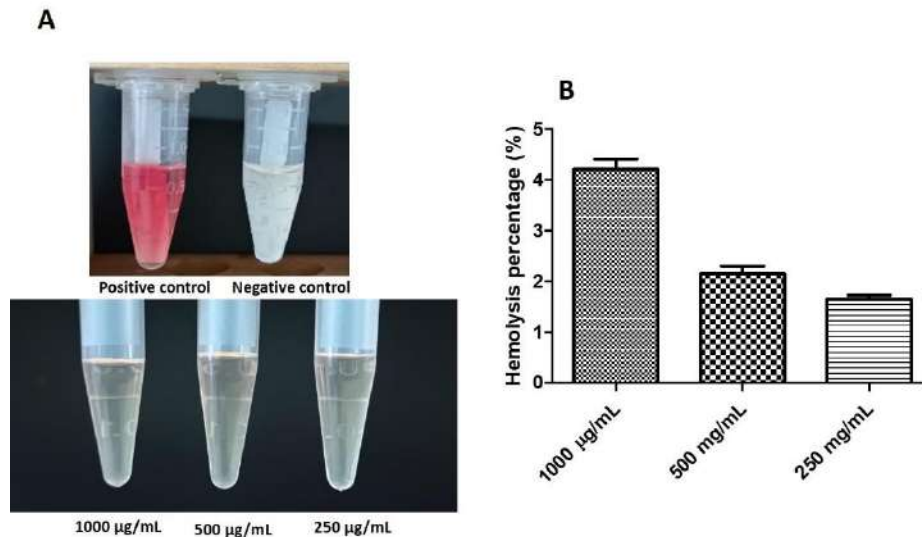


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, $n=3$).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [62]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [63,64]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

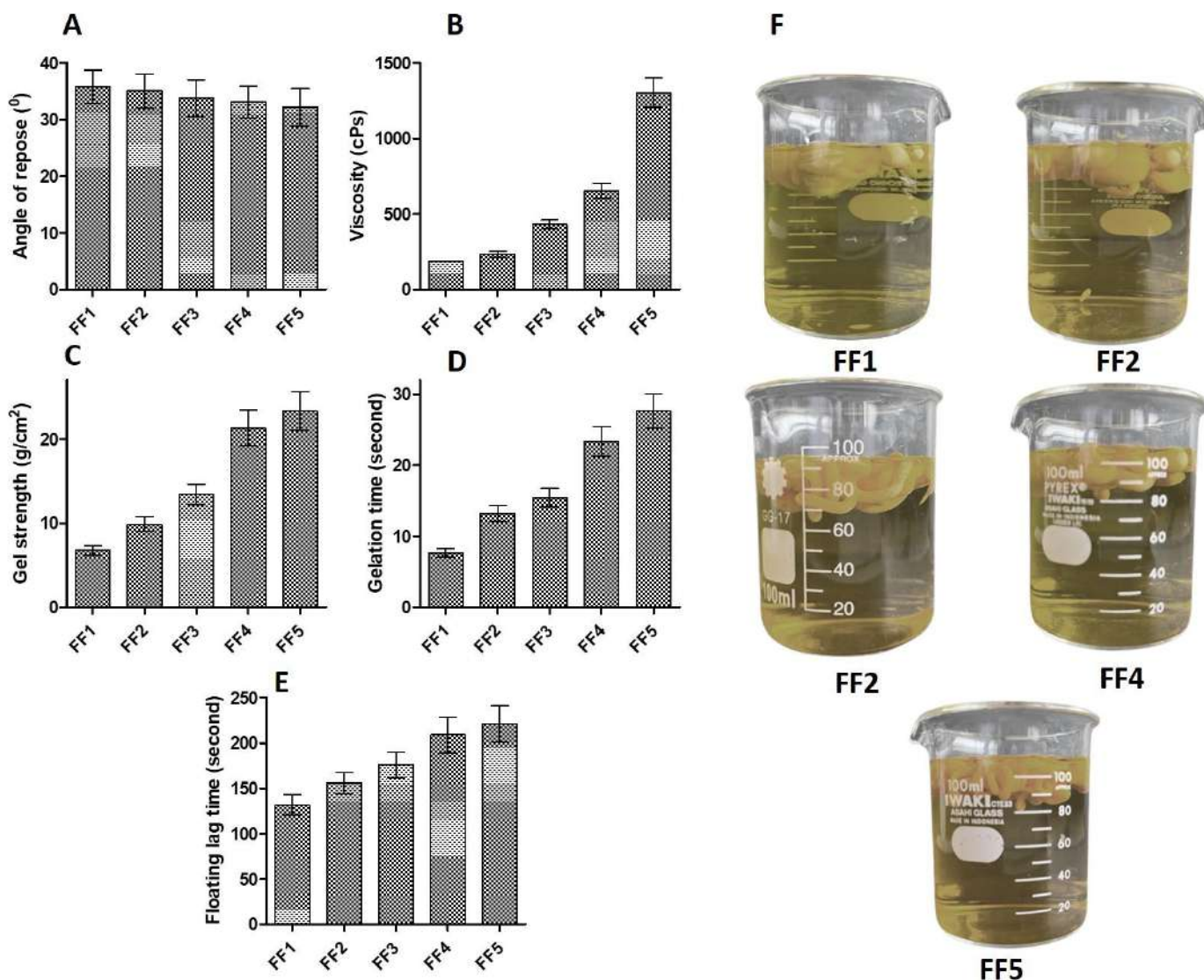


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, n=3). Representative images of floating gel in situ after the floating evaluation in FaSGGF, showing that all formulations were able to float (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The viscosity of the formulation was in the range of 187.98 ± 1.67 cps to 1302.62 ± 98.21 cps. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic

F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [63,64].

The strength values of the gel formulation are shown in Figure 7C. It was found that the increase of Pluronic F127 concentration from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [62]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled

667 formulation to float also increased. This might be associated with the increase of the viscosity of
668 the formulation when the concentration of Pluronics increased. As a result, the penetration of the
669 gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which
670 affected the release of CO₂ [65]. A previous study has shown the same phenomenon showing that
671 the floating lag time increased following the increase of the polymeric material in the system [66].
672 It should be noted that all formulations remained floating after 24 h (Figure 7F).

674 3.17. In Vitro Drug Dissolution Study

675 The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-
676 glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three
677 different conditions. To ensure that the formulation could control the dissolution of both
678 compounds, the burst release should be avoided [67]. In this study, initially we evaluated the
679 dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-
680 glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are
681 depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ
682 could potentially control the dissolution profile of active compounds from safflower extract.
683 Without the SLNs formulation, the dissolution percentages were found to be significant lower (p
684 < 0.05) in all cases. Specifically, the difference in Pluronic concentrations resulted in various
685 control release patterns. As shown in Figure 8, below 7.5% of Pluronics (FF1 and FF2), the
686 sustained release patterns were only observed for 6 h. On the other hand, formulations containing
687 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the
688 compounds over 24 h.

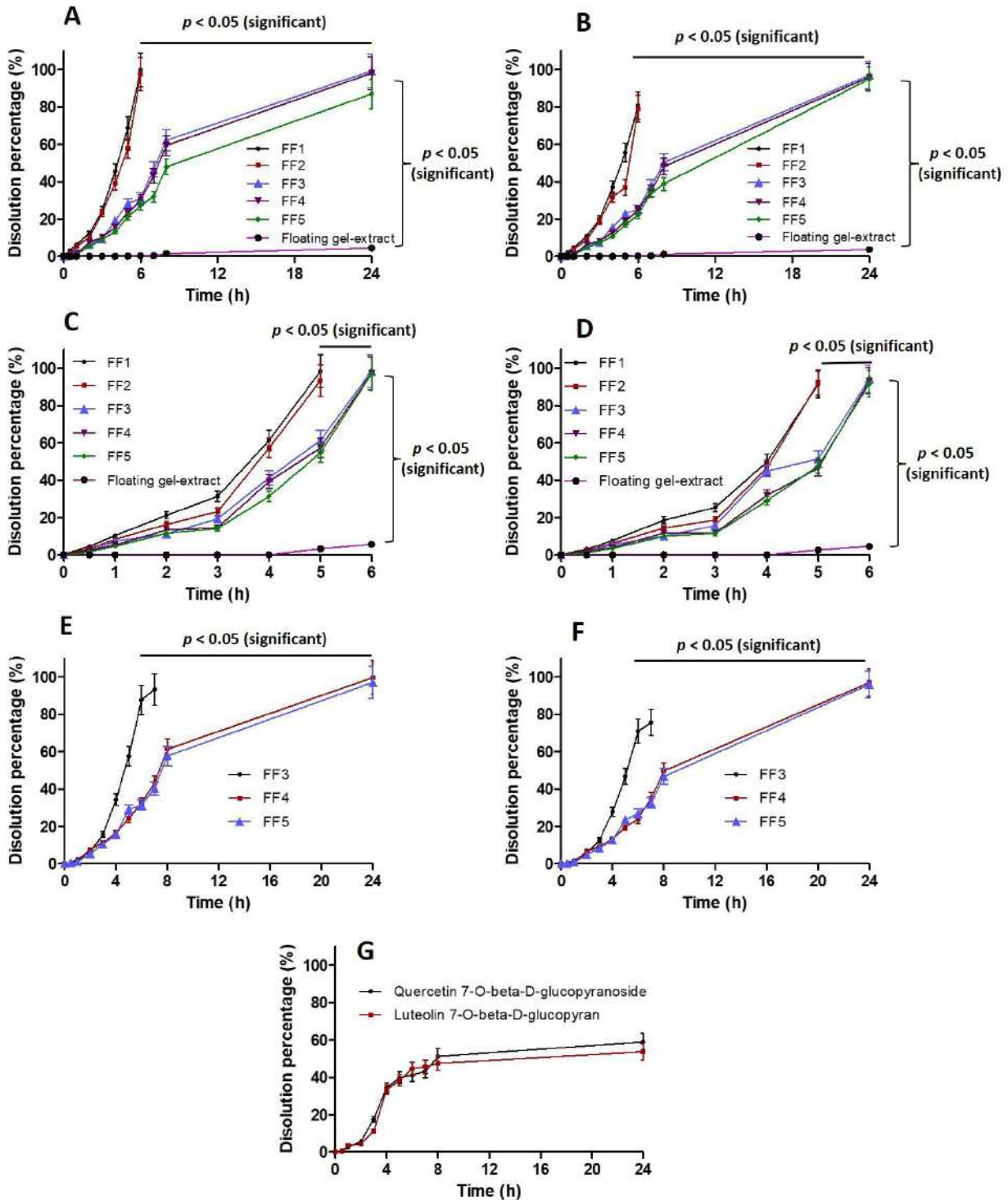


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF; in vitro dissolution

profiles of quercetin 7-O-beta-D-glucopyranoside (E) and luteolin 7-O-beta-D-glucopyranoside (F) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (G) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h. After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF. Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach. Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 $\mu\text{g/mL}$ against DPPD and 48.75 ± 4.52 $\mu\text{g/mL}$ against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard

compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and

20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was

found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed with the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

215 2.8. Determination of Particle Size, PDI and Zeta Potential

216 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 217 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 218 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 219 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 220 particle size, Z-average (d.nm) with intensity (%) was used.

221 2.9. Encapsulation Efficiency and Drug Loading Determination

222 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 223 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 224 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 225 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 226 dissolved compounds were quantified by determining the compounds from the filtrate from the
 227 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 228 loading of both compounds were calculated using the following equation:

$$229 \text{ Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

230
$$\text{Drug loading (\%)} = \frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$$

231

232

233 2.10. FTIR study

234 To evaluate the possible intervention between the compounds in extract and excipients
235 used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this
236 study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹
237 wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s
238 and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

239

240 2.11. DSC evaluation

241 The thermal properties of the compounds in extract, as well as in SLNs formulation were
242 evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21].
243 Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in
244 aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min
245 under N₂ flow (40 mL/min).

246

247 2.12. XRD study

248 X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state
249 of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical
250 mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples
251 were measured at a sampling width of 0.02°.

252

253 2.13. SEM evaluation

254 In this study, the morphologies of SLNs were detected using a scanning electron
255 microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

256 2.14. Solubility Analysis

257 In order to investigate the solubility of both compounds in extract and SLN preparations,
258 two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were
259 added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature

at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The

time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

A brookfield Viscometer DV III (Brookfield Engineering Laboratories, MA) was used to determine the viscosity of the formulation after being dispersed in water. The study was carried using using spindle number 2 at 50 rpm. In an attempt to measure the gel strength, a Rheometer (TA Instrument, USA) was utilized [37]. The study was conducted at 37 °C.

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGFF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [52].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed

using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPD as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

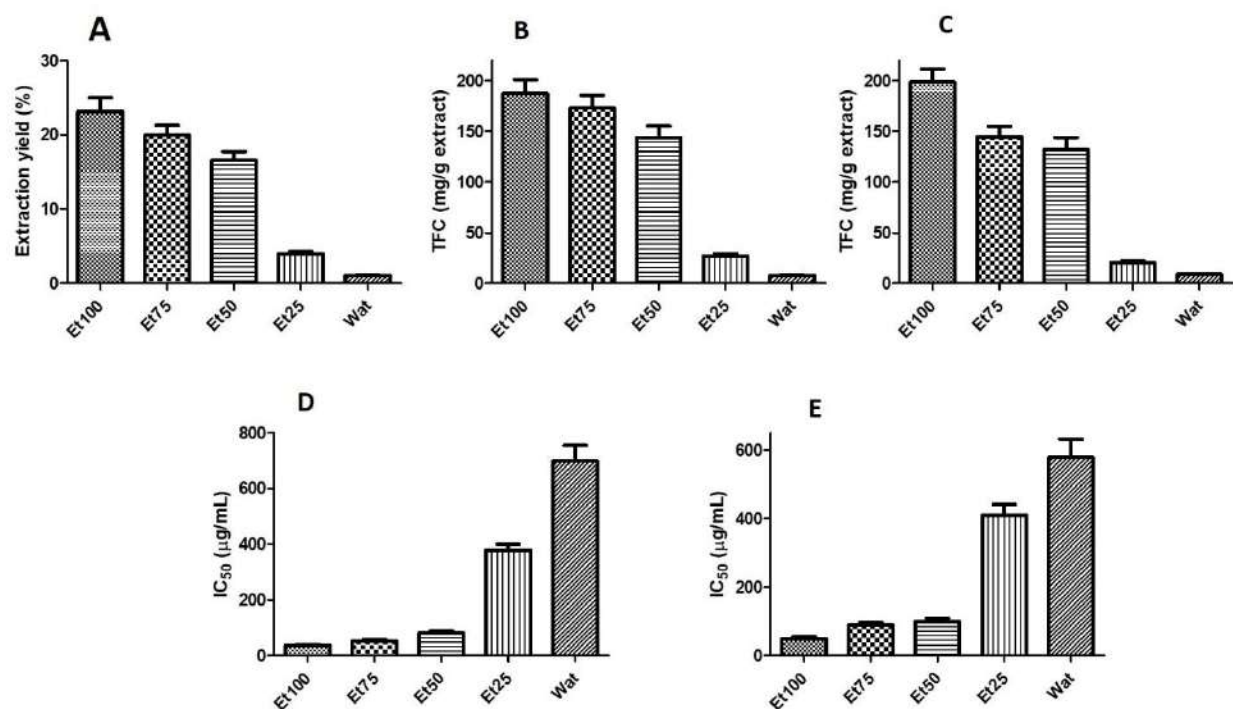


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPD (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, $n=3$)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were 48.75 ± 3.42 $\mu\text{g/mL}$, 89.04 ± 6.56 $\mu\text{g/mL}$, 98.97 ± 8.91 $\mu\text{g/mL}$, 409.51 ± 31.67 $\mu\text{g/mL}$ and 577.43 ± 43.87 $\mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were 7.65 ± 0.63 mg/g for Et100, 4.32 ± 0.32 mg/g for Et75, 2.97 ± 0.19 mg/g for Et50, 0.74 ± 0.05 mg/g for Et25 and 0.02 ± 0.001 mg/g for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were 2.32 ± 0.21 mg/g, 1.92 ± 0.15 mg/g, 0.67 ± 0.05 mg/g, 0.29 ± 0.03 mg/g and 0.01 ± 0.001 mg/g, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

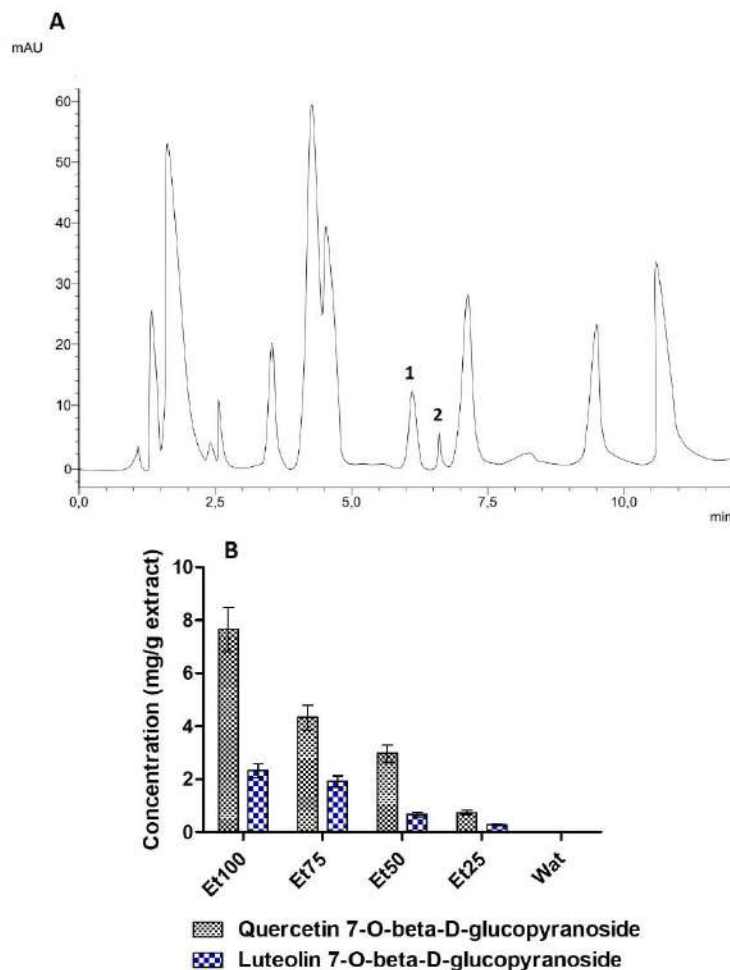


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively.

The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [53]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [54]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

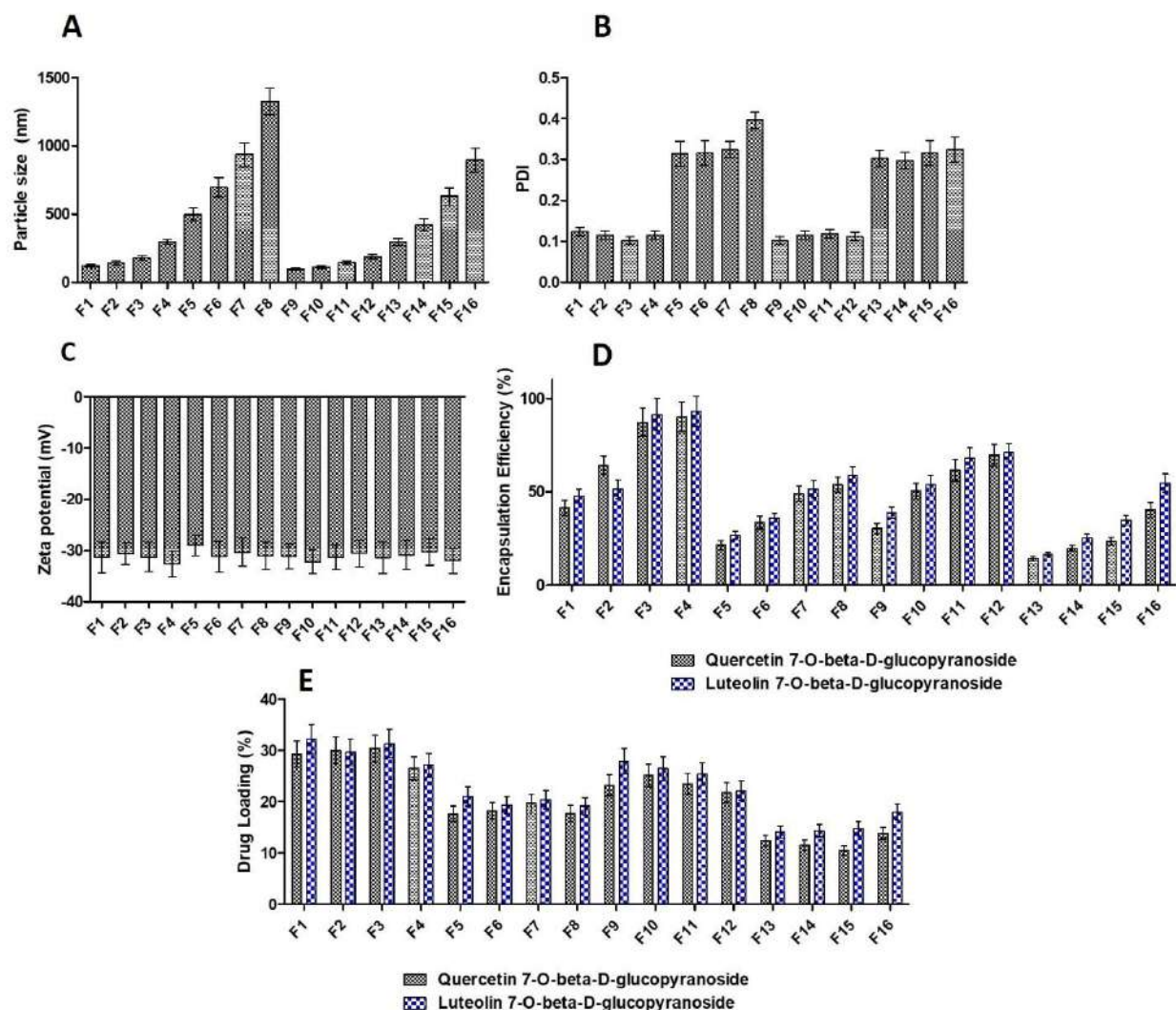


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

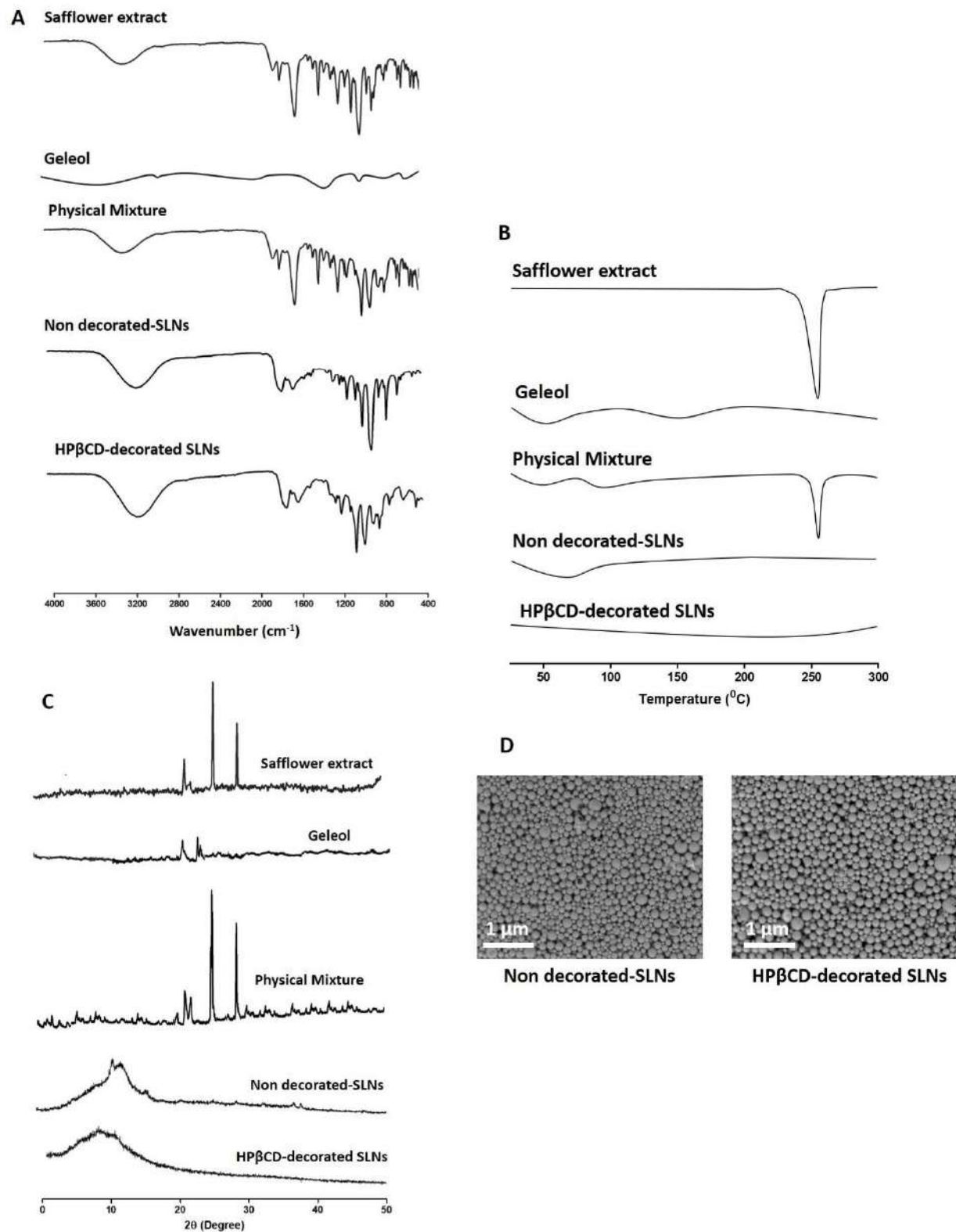


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [55], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [56–60].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, **sustained** release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of **the** compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [61]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

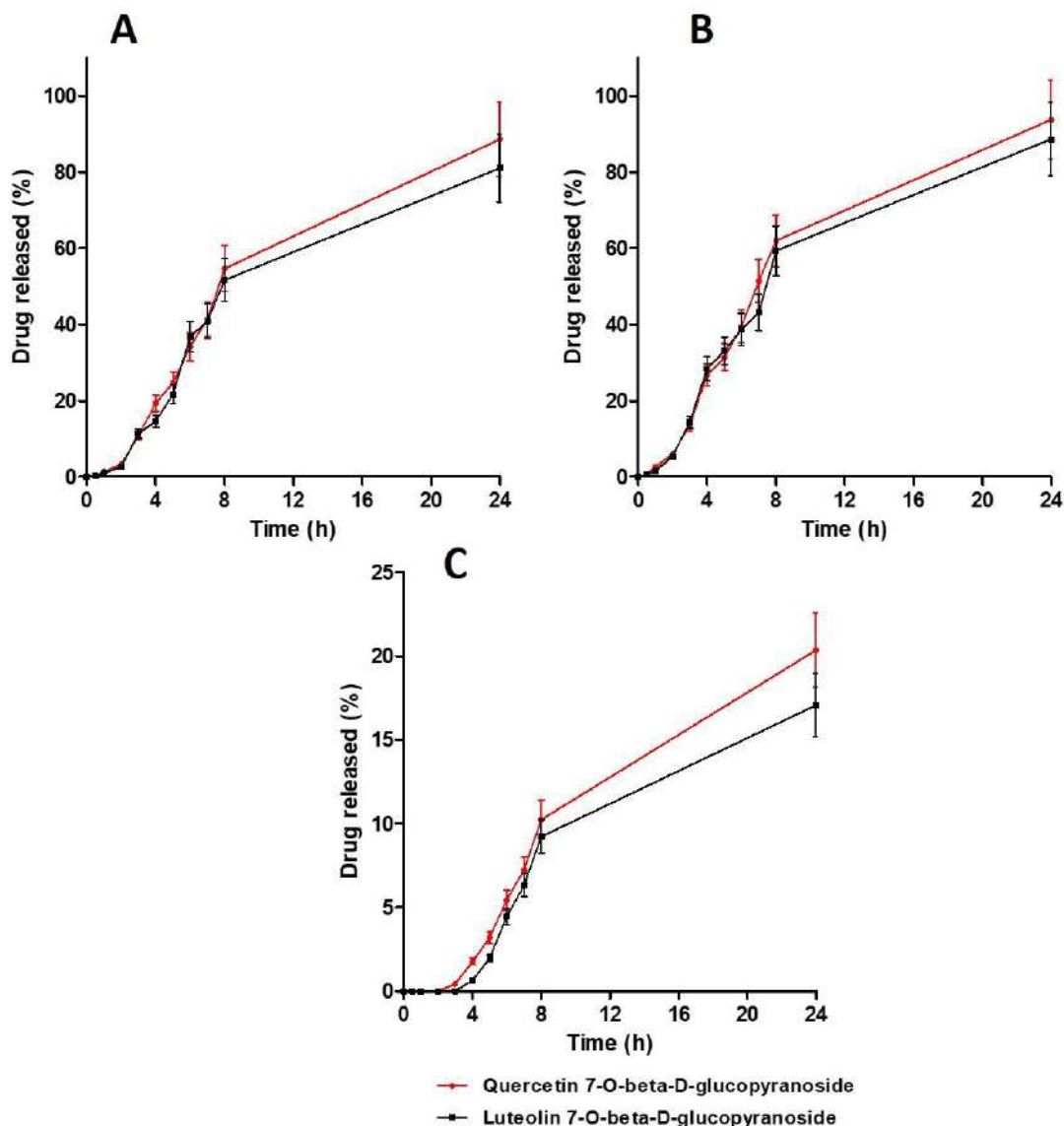


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

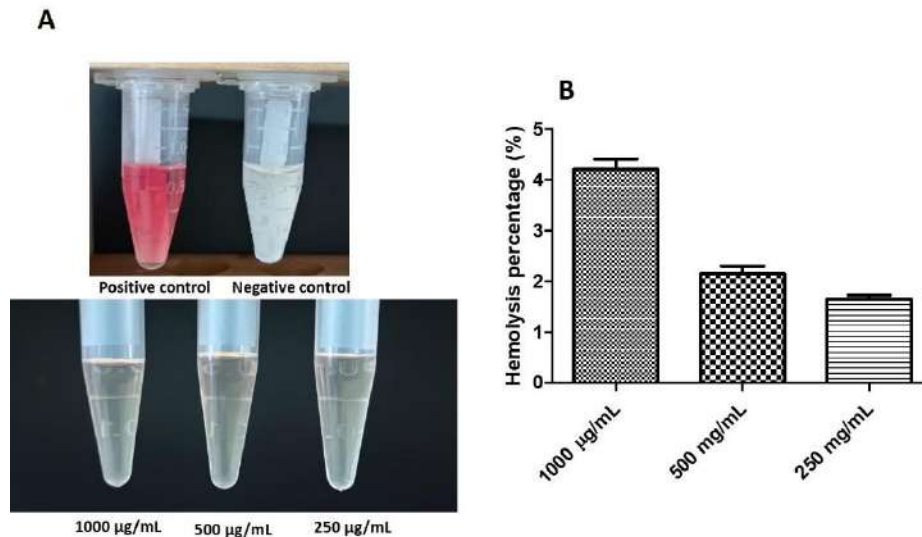


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [62]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [63,64]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

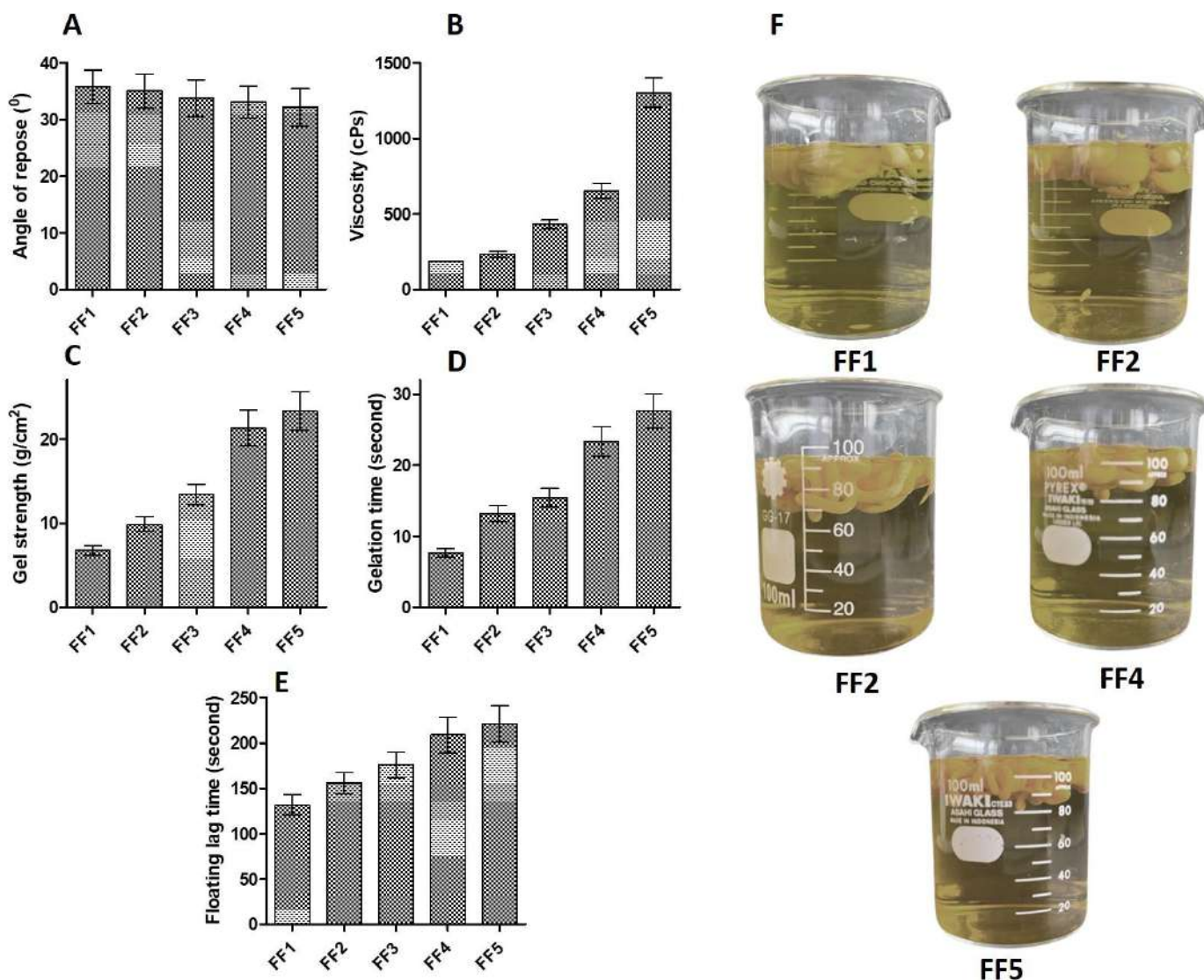


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, n=3). Representative images of floating gel in situ after the floating evaluation in FaSGGF, showing that all formulations were able to float (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The viscosity of the formulation was in the range of 187.98 ± 1.67 cps to 1302.62 ± 98.21 cps. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic

F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [63,64].

The strength values of the gel formulation are shown in Figure 7C. It was found that the increase of Pluronic F127 concentration from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [62]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled

formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which affected the release of CO₂ [65]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [66]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [67]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure 8, below 7.5% of Pluronics (FF1 and FF2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the compounds over 24 h.

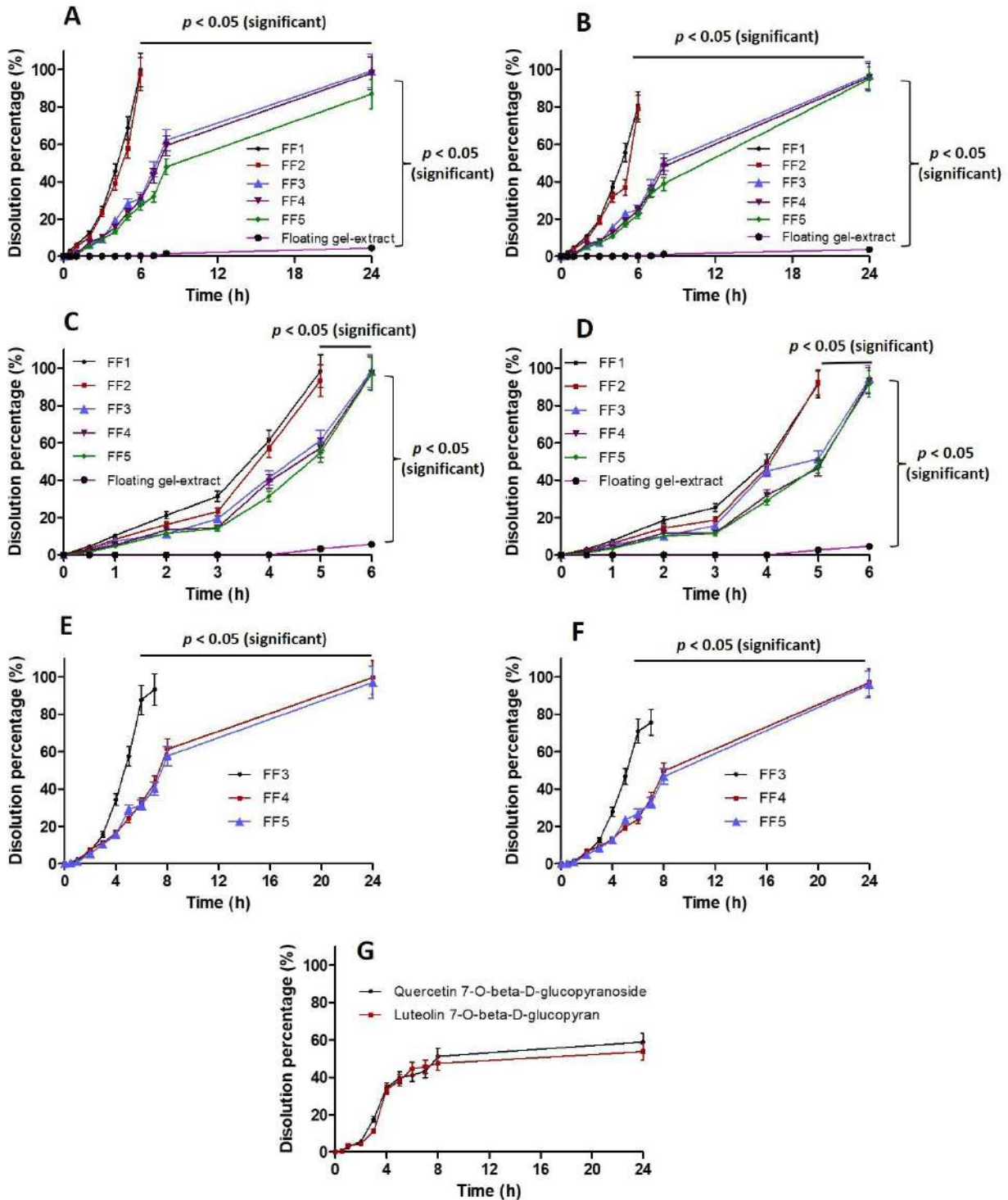


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF; in vitro dissolution

profiles of quercetin 7-O-beta-D-glucopyranoside (E) and luteolin 7-O-beta-D-glucopyranoside (F) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; *in vitro* dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (G) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h. After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF. Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 8G, the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach. Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, *in vivo* studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 $\mu\text{g/mL}$ against DPPD and 48.75 ± 4.52 $\mu\text{g/mL}$ against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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International Journal of Biological Macromolecules

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media --Manuscript Draft--

Manuscript Number:	IJBIMAC-D-22-09382R3
Article Type:	Research Paper
Section/Category:	Carbohydrates, Natural Polyacids and Lignins
Keywords:	Safflower; solid lipid nanoparticles; floating gel in situ
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Abstract:	Safflower (<i>Carthamus tinctorius</i> L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HPβCD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.
Suggested Reviewers:	Ryan Donnelly r.donnelly@qub.ac.uk Aaron Courtenay a.courtenay@ulster.ac.uk Eneko Larraneta e.larraneta@qub.ac.uk Ahmed Faheem ahmed.faheem@sunderland.ac.uk
Opposed Reviewers:	
Response to Reviewers:	Manuscript Number: IJBIMAC-D-22-09382R2 Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry

floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Response to Reviewers:

We thank the reviewer for reviewing the manuscript in detail and providing valuable suggestions for the improvement of our manuscript. We are pleased to submit the revision of the manuscript to International Journal of Biological Macromolecules and addressed all comments, as follows:

Editor comments:

The method concerning the rheological measurements is very incomplete: gap, strain and measurement type is completely missing. How did you calculate the gel strength? Did you perform mechanical spectra? If so, at what frequency the values shown in the figure 7C were chosen? I suppose that the dispersion had a non-Newtonian behaviour. If so, at what shear strain/stress the values shown in the figure 7B were chosen? It is important that the Authors report the rheological plot of the measurements done to obtain the data reported in Figure 7B and 7C. Most important: Rheological measurements give information about the gelation time using methods reported in literature. Why the Authors used a such empirical method having done the rheological measurements?

Response:

We thank the reviewer for the comment. Following this, we have repeated the determination and included the complete information in the revised manuscript. Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSGGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was decreased using the trim gap. The determination was carried out at 25°C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value). We believe that our revised manuscript is now clear and providing complete information.

Reviewer's Responses to Questions

Note: In order to effectively convey your recommendations for improvement to the author(s), and help editors make well-informed and efficient decisions, we ask you to answer the following specific questions about the manuscript and provide additional suggestions where appropriate.

1. Are the objectives and the rationale of the study clearly stated?

Please provide suggestions to the author(s) on how to improve the clarity of the objectives and rationale of the study. Please number each suggestion so that author(s) can more easily respond.

Reviewer #2: yes

Reviewer #3: Yes the objectives and rational of study is mentioned clearly

Response:

We thank the reviewer for the comment.

2. If applicable, is the method/study reported in sufficient detail to allow for its replicability and/or reproducibility?

Please provide suggestions to the author(s) on how to improve the replicability/reproducibility of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:
Yes ☒ No ☐ N/A ☐

Provide further comments here:

Reviewer #3: Mark as appropriate with an X:
Yes ☒ No ☐ N/A ☐

Provide further comments here:

Response:

We thank the reviewer for the comment.

3. If applicable, are statistical analyses, controls, sampling mechanism, and statistical reporting (e.g., P-values, CIs, effect sizes) appropriate and well described?

Please clearly indicate if the manuscript requires additional peer review by a statistician. Kindly provide suggestions to the author(s) on how to improve the statistical analyses, controls, sampling mechanism, or statistical reporting. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:
Yes ☐ No ☒ N/A ☐
Provide further comments here:

Reviewer #3: Mark as appropriate with an X:
Yes ☒ No ☐ N/A ☐
Provide further comments here:
Response:
We thank the reviewer for the comment.

4. Could the manuscript benefit from additional tables or figures, or from improving or removing (some of the) existing ones?

Please provide specific suggestions for improvements, removals, or additions of figures or tables. Please number each suggestion so that author(s) can more easily respond.

Reviewer #2: no need to add extra data table/figure. modification of figure caption is needed, make it shorter.
Response:
We thank the reviewer for the suggestion. We have made the captions shorter. Additionally, we divided Figure 8 to two different figures to reduce the words number of the caption.

Reviewer #3: The data presented are adequate
Response:
We thank the reviewer for the comment.

5. If applicable, are the interpretation of results and study conclusions supported by the data?

Please provide suggestions (if needed) to the author(s) on how to improve, tone down, or expand the study interpretations/conclusions. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:
Yes ☒ No ☐ N/A ☐
Provide further comments here:

Reviewer #3: Mark as appropriate with an X:
Yes ☒ No ☐ N/A ☐
Provide further comments here:
Response:
We thank the reviewer for the comment.

6. Have the authors clearly emphasized the strengths of their study/methods?

Please provide suggestions to the author(s) on how to better emphasize the strengths of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: yes
Reviewer #3: The novelty of the study and the methodology section is clear
Response:
We thank the reviewer for the comment.

7. Have the authors clearly stated the limitations of their study/methods?

Please list the limitations that the author(s) need to add or emphasize. Please number each limitation so that author(s) can more easily respond.

Reviewer #2: no
Response:
We thank the reviewer for the comment. In our manuscript, we have explained as follows:
As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-

glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.
Reviewer #3: Yes, the information provided is adequate
Response:
We thank the reviewer for the comment.

8. Does the manuscript structure, flow or writing need improving (e.g., the addition of subheadings, shortening of text, reorganization of sections, or moving details from one section to another)?

Please provide suggestions to the author(s) on how to improve the manuscript structure and flow. Please number each suggestion so that author(s) can more easily respond.
Reviewer #2: Now after revision manuscript is alright.
Reviewer #3: Good
Response:
We thank the reviewer for the comment.

9. Could the manuscript benefit from language editing?
Reviewer #2: Yes
Reviewer #3: No
Response:
We thank the reviewer for the comment. We have now improved the English of the manuscript.

Reviewer #2: Paper can be accepted with following minor revision
Line 133 methanol (up to ?????mL)—amount is mssing.
Response:
We have corrected this.

Figure captions are very lengthy, reduce the caption.
Response:
We thank the reviewer for the suggestion. We have made the captions shorter. Additionally, we divided Figure 8 to two different figures to reduce the words number of the caption.

In conclusion line 730 it is DPPH not DPPD.
Response:
We have corrected this.
Reviewer #3: None
Response:
We thank the reviewer for the comment.

This piece of the submission is being sent via mail.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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The Editor

International Journal of Biological Macromolecules

17 December 2022

Dear Sir/Madam,

I wish you to re-consider our manuscript entitled: **“Solid lipid nanoparticles-decorated cyclodextrin incorporated gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)”** for publication in International Journal of Biological Macromolecules. Moreover, to represent our study, we have modified the title our study to **“Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media**

We have made a few of changes to the manuscript as a result of the reviewers comments. We believe that the manuscript is now substantially improved. Importantly, we have made a great effort to addressed each of the reviewers' comments in the response to the reviewer file.

We believe that this work will be of great interest to the readers of International Journal of Biological Macromolecules. The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in International Journal of Biological Macromolecules and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Response to Reviewers:

We thank the reviewer for reviewing the manuscript in detail and providing valuable suggestions for the improvement of our manuscript. We are pleased to submit the revision of the manuscript to International Journal of Biological Macromolecules and addressed all comments, as follows:

Editor comments:

The method concerning the rheological measurements is very incomplete:

gap, strain and measurement type is completely missing. How did you calculate the gel strength? Did you perform mechanical spectra? If so, at what frequency the values shown in the figure 7C were chosen? I suppose that the dispersion had a non-Newtonian behaviour. If so, at what shear strain/stress the values shown in the figure 7B were chosen? It is important that the Authors report the rheological plot of the measurements done to obtain the data reported in Figure 7B and 7C. Most important: Rheological measurements give information about the gelation time using methods reported in literature. Why the Authors used a such empirical method having done the rheological measurements?

Response:

We thank the reviewer for the comment. Following this, we have repeated the determination and included the complete information in the revised manuscript. Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSGGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was

decreased using the trim gap. The determination was carried out at 25 °C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value). We believe that our revised manuscript is now clear and providing complete information.

Reviewer's Responses to Questions

Note: In order to effectively convey your recommendations for improvement to the author(s), and help editors make well-informed and efficient decisions, we ask you to answer the following specific questions about the manuscript and provide additional suggestions where appropriate.

1. Are the objectives and the rationale of the study clearly stated?

Please provide suggestions to the author(s) on how to improve the clarity of the objectives and rationale of the study. Please number each suggestion so that author(s) can more easily respond.

Reviewer #2: yes

Reviewer #3: Yes the objectives and rational of study is mentioned clearly

Response:

We thank the reviewer for the comment.

2. If applicable, is the method/study reported in sufficient detail to allow for its replicability and/or reproducibility?

Please provide suggestions to the author(s) on how to improve the replicability/reproducibility of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:

Yes ☒ No ☐ N/A ☐

Provide further comments here:

Reviewer #3: Mark as appropriate with an X:

Yes ☒ No ☐ N/A ☐

Provide further comments here:

Response:

[We thank the reviewer for the comment.](#)

3. If applicable, are statistical analyses, controls, sampling mechanism, and statistical reporting (e.g., P-values, CIs, effect sizes) appropriate and well described?

Please clearly indicate if the manuscript requires additional peer review by a statistician. Kindly provide suggestions to the author(s) on how to improve the statistical analyses, controls, sampling mechanism, or statistical reporting. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:

Yes ☐ No ☒ N/A ☐

Provide further comments here:

Reviewer #3: Mark as appropriate with an X:

Yes ☒ No ☐ N/A ☐

Provide further comments here:

Response:

[We thank the reviewer for the comment.](#)

4. Could the manuscript benefit from additional tables or figures, or from improving or removing (some of the) existing ones?

Please provide specific suggestions for improvements, removals, or additions of figures or tables. Please number each suggestion so that author(s) can more easily respond.

Reviewer #2: no need to add extra data table/figure. modification of figure caption is needed, make it shorter.

Response:

We thank the reviewer for the suggestion. We have made the captions shorter. Additionally, we divided Figure 8 to two different figures to reduce the words number of the caption.

Reviewer #3: The data presented are adequate

Response:

We thank the reviewer for the comment.

5. If applicable, are the interpretation of results and study conclusions supported by the data?

Please provide suggestions (if needed) to the author(s) on how to improve, tone down, or expand the study interpretations/conclusions. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: Mark as appropriate with an X:

Yes ☒ No ☐ N/A ☐

Provide further comments here:

Reviewer #3: Mark as appropriate with an X:

Yes ☒ No ☐ N/A ☐

Provide further comments here:

Response:

We thank the reviewer for the comment.

6. Have the authors clearly emphasized the strengths of their study/methods?

Please provide suggestions to the author(s) on how to better emphasize the strengths of their study. Please number each suggestion so that the author(s) can more easily respond.

Reviewer #2: yes

Reviewer #3: The novelty of the study and the methodology section is clear

Response:

We thank the reviewer for the comment.

7. Have the authors clearly stated the limitations of their study/methods?

Please list the limitations that the author(s) need to add or emphasize. Please number each limitation so that author(s) can more easily respond.

Reviewer #2: no

Response:

We thank the reviewer for the comment. In our manuscript, we have explained as follows:
As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

Reviewer #3: Yes, the information provided is adequate

Response:

We thank the reviewer for the comment.

8. Does the manuscript structure, flow or writing need improving (e.g., the addition of subheadings, shortening of text, reorganization of sections, or moving details from one section to another)?

Please provide suggestions to the author(s) on how to improve the manuscript structure and flow. Please number each suggestion so that author(s) can more easily respond.

Reviewer #2: Now after revision manuscript is alright.

Reviewer #3: Good

Response:

We thank the reviewer for the comment.

9. Could the manuscript benefit from language editing?

Reviewer #2: Yes

Reviewer #3: No

Response:

We thank the reviewer for the comment. We have now improved the English of the manuscript.

Reviewer #2: Paper can be accepted with following minor revision

Line 133 methanol (up to ?????mL)—amount is mssing.

Response:

We have corrected this.

Figure captions are very lengthy, reduce the caption.

Response:

We thank the reviewer for the suggestion. We have made the captions shorter. Additionally, we divided Figure 8 to two different figures to reduce the words number of the caption.

In conclusion line 730 it is DPPH not DPPD.

Response:

We have corrected this.

Reviewer #3: None

Response:

We thank the reviewer for the comment.

Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard

compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 μ L of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to 10 mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and

20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was

found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed with the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

215 2.8. Determination of Particle Size, PDI and Zeta Potential

216 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 217 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 218 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 219 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 220 particle size, Z-average (d.nm) with intensity (%) was used.

221 2.9. Encapsulation Efficiency and Drug Loading Determination

222 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 223 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 224 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 225 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 226 dissolved compounds were quantified by determining the compounds from the filtrate from the
 227 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 228 loading of both compounds were calculated using the following equation:

$$229 \text{ Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

230 Drug loading (%) = $\frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$

233 **2.10. FTIR study**

234 To evaluate the possible intervention between the compounds in extract and excipients
235 used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this
236 study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹
237 wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s
238 and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

240 **2.11. DSC evaluation**

241 The thermal properties of the compounds in extract, as well as in SLNs formulation were
242 evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21].
243 Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in
244 aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min
245 under N₂ flow (40 mL/min).

247 **2.12. XRD study**

248 X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state
249 of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical
250 mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples
251 were measured at a sampling width of 0.02°.

253 **2.13. SEM evaluation**

254 In this study, the morphologies of SLNs were detected using a scanning electron
255 microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

256 **2.14. Solubility Analysis**

257 In order to investigate the solubility of both compounds in extract and SLN preparations,
258 two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were
259 added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature

at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The

time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSGGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSGGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was decreased using the trim gap. The determination was carried out at 25 °C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value) [52,53].

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [54].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type

could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPH as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

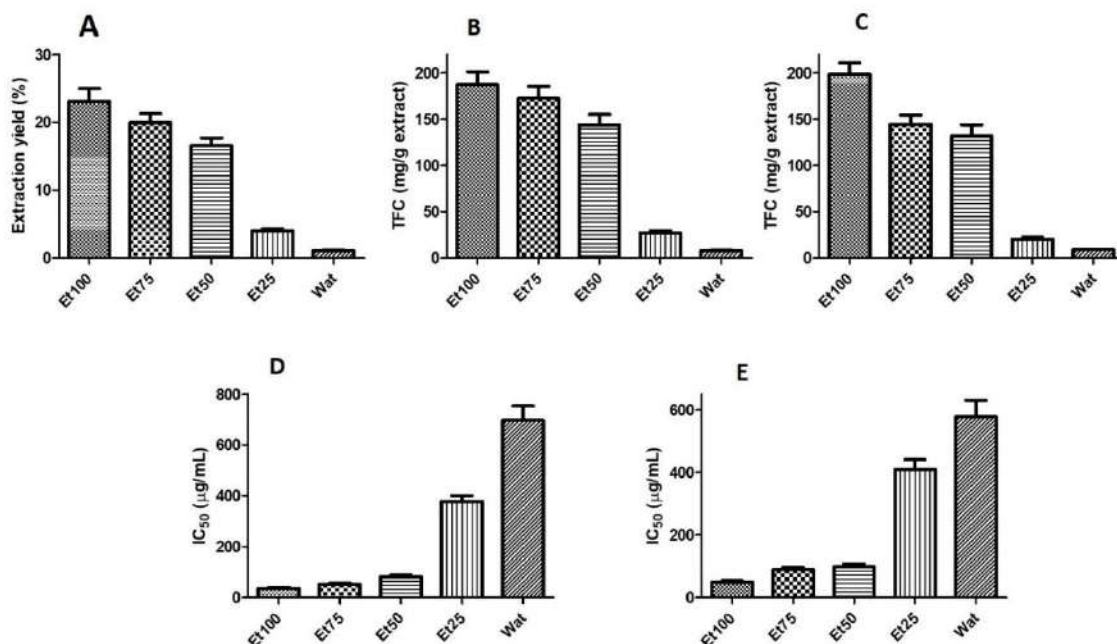


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPH (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, $n=3$)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were $48.75 \pm 3.42 \mu\text{g/mL}$, $89.04 \pm 6.56 \mu\text{g/mL}$, $98.97 \pm 8.91 \mu\text{g/mL}$, $409.51 \pm 31.67 \mu\text{g/mL}$ and $577.43 \pm 43.87 \mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were $7.65 \pm 0.63 \text{ mg/g}$ for Et100, $4.32 \pm 0.32 \text{ mg/g}$ for Et75, $2.97 \pm 0.19 \text{ mg/g}$ for Et50, $0.74 \pm 0.05 \text{ mg/g}$ for Et25 and $0.02 \pm 0.001 \text{ mg/g}$ for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were $2.32 \pm 0.21 \text{ mg/g}$, $1.92 \pm 0.15 \text{ mg/g}$, $0.67 \pm 0.05 \text{ mg/g}$, $0.29 \pm 0.03 \text{ mg/g}$ and $0.01 \pm 0.001 \text{ mg/g}$, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

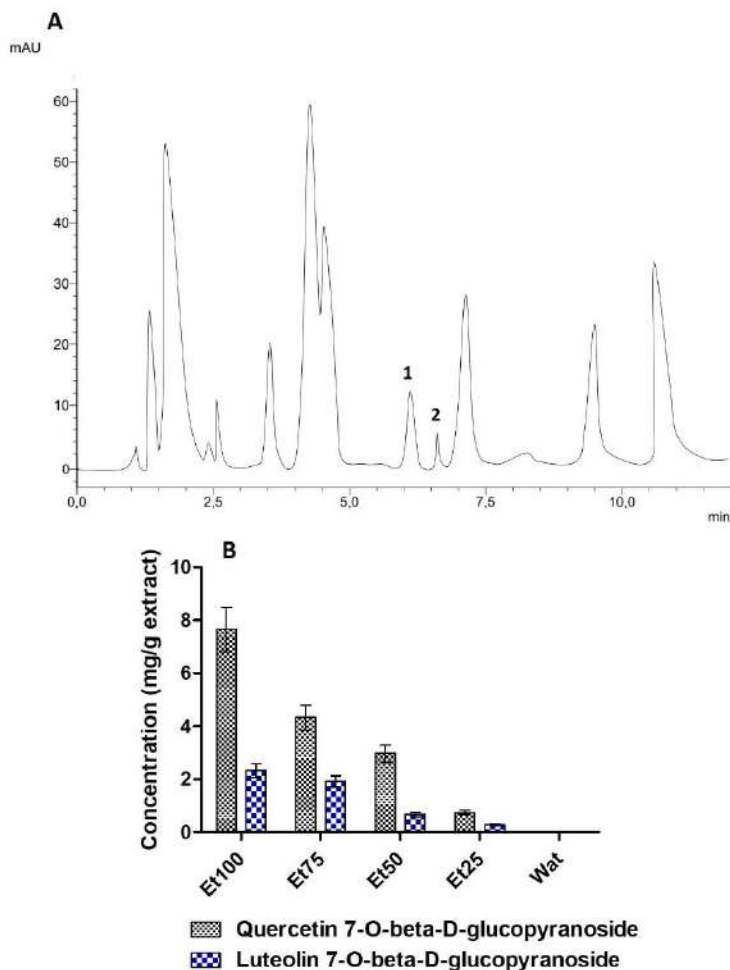


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [55]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [56]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

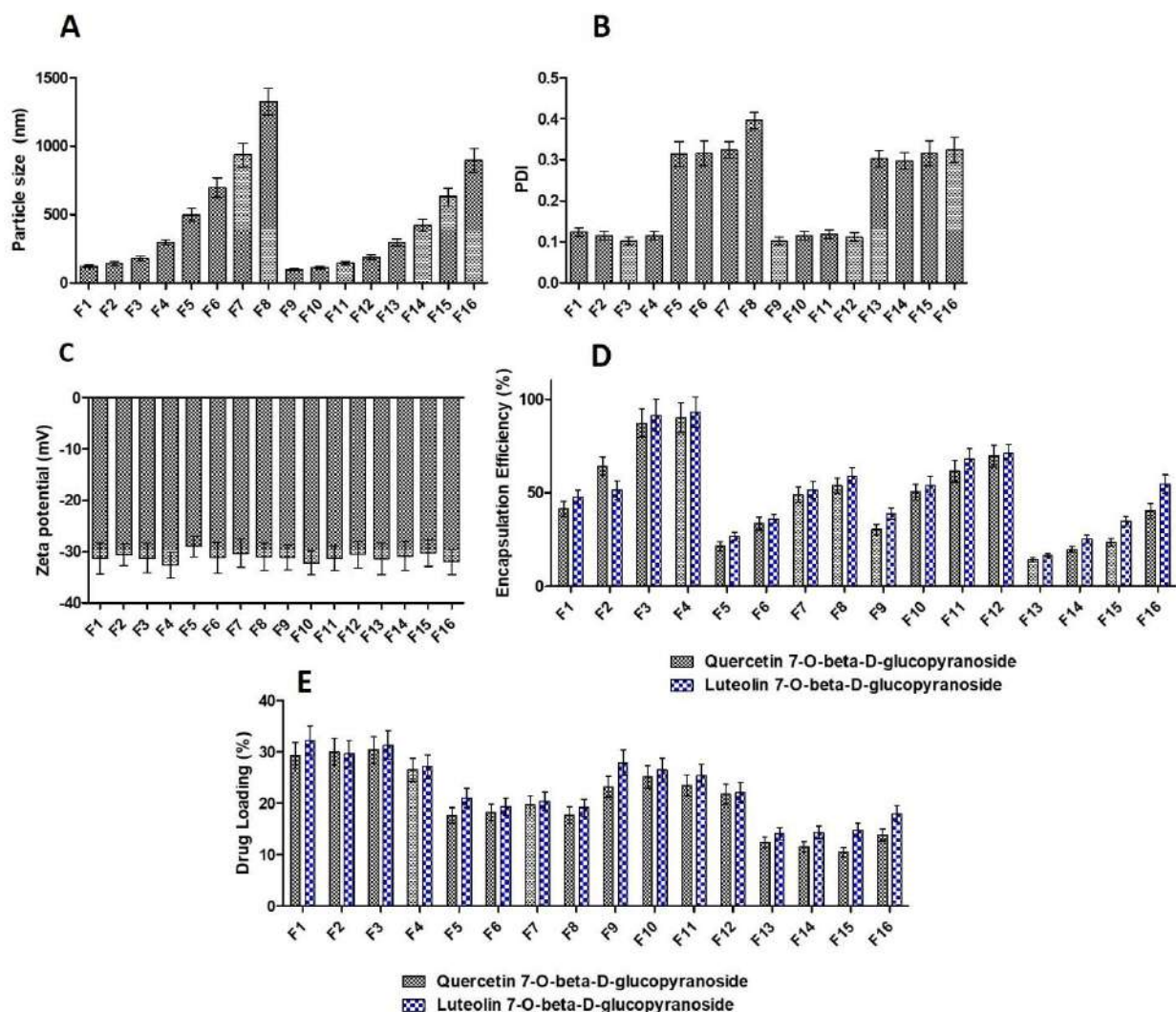


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

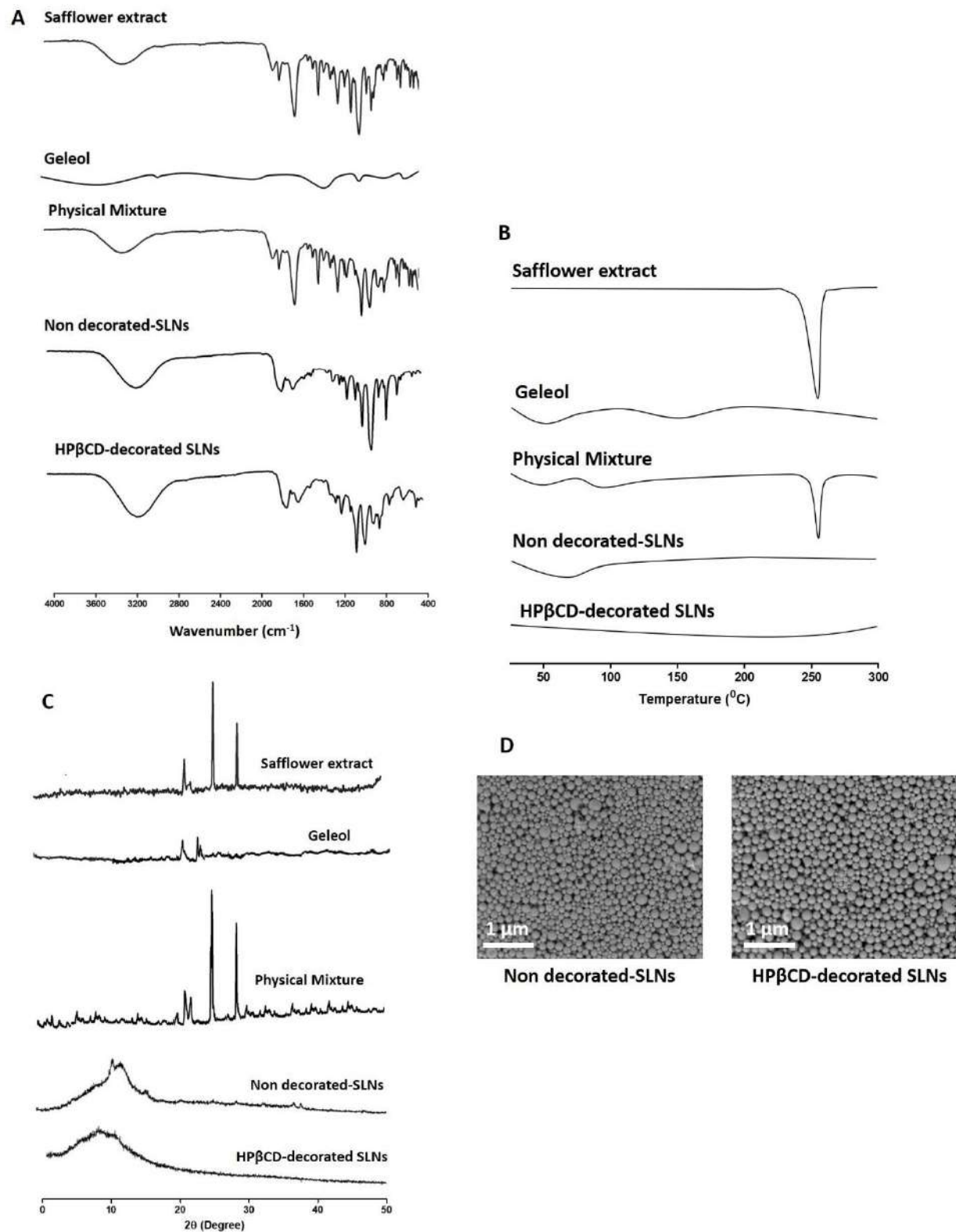


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [57], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [58–62].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustained release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of the compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [63]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

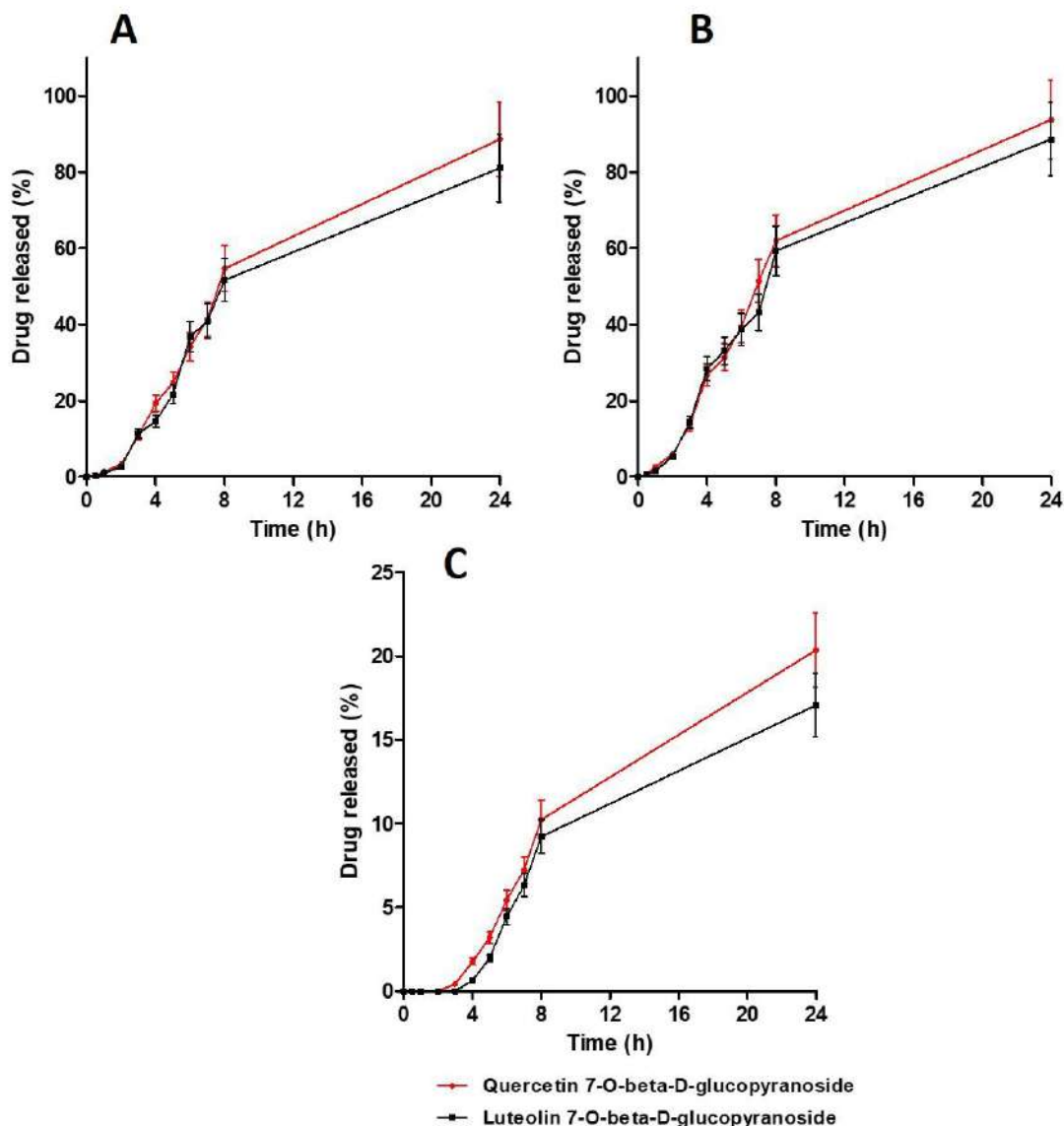


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

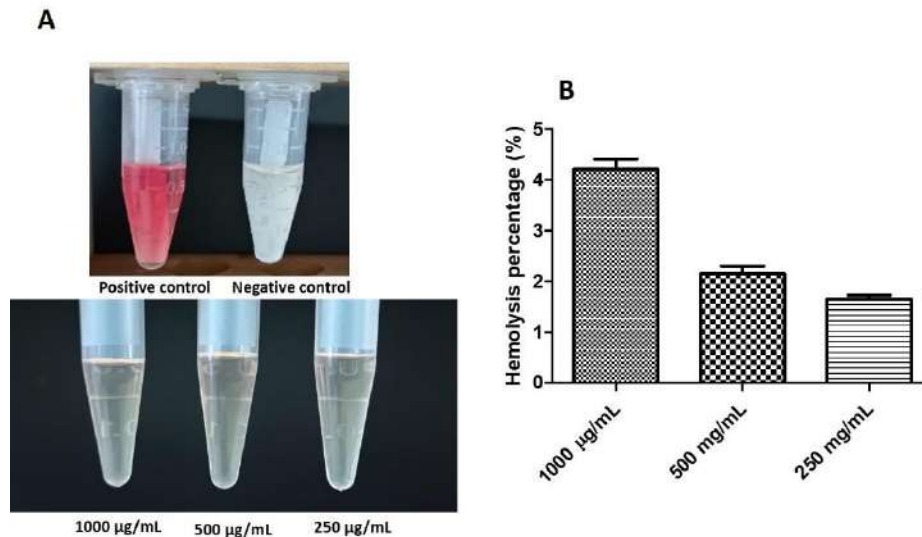


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [64]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [65,66]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

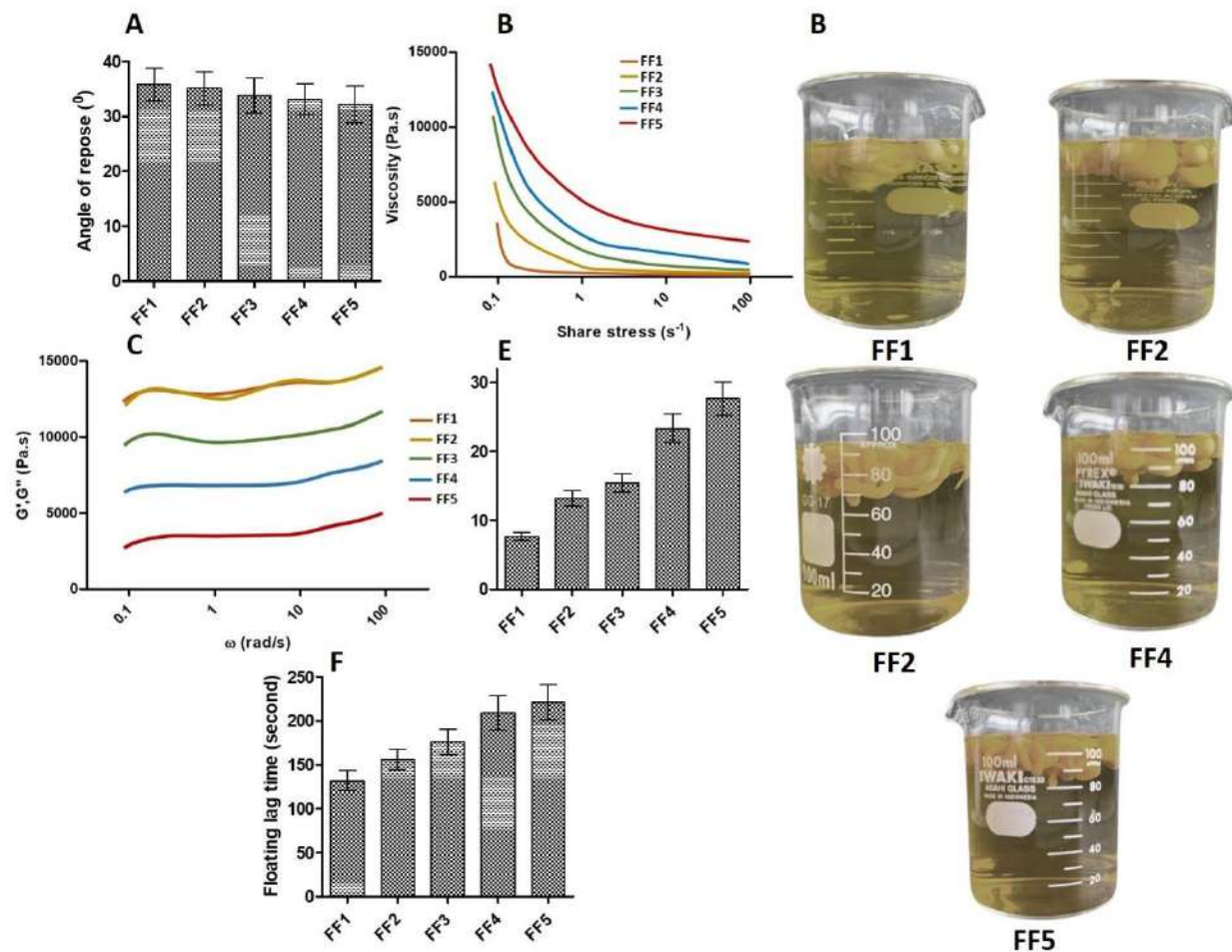


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, $n=3$). Representative images of floating gel in situ (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The rheogram showed that all the formulations exhibited non-Newtonian behavior. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [65,66].

The strength values of the gel formulation are shown in Figure 7C. The value of $G'G''$ represented the strength of the gels. It was found that the increase of Pluronic F127 concentration

from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [64]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which

affected the release of CO₂ [67]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [68]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [69]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure 8A and 8B, below 7.5% of Pluronics (FF1 and FF2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the compounds over 24 h.

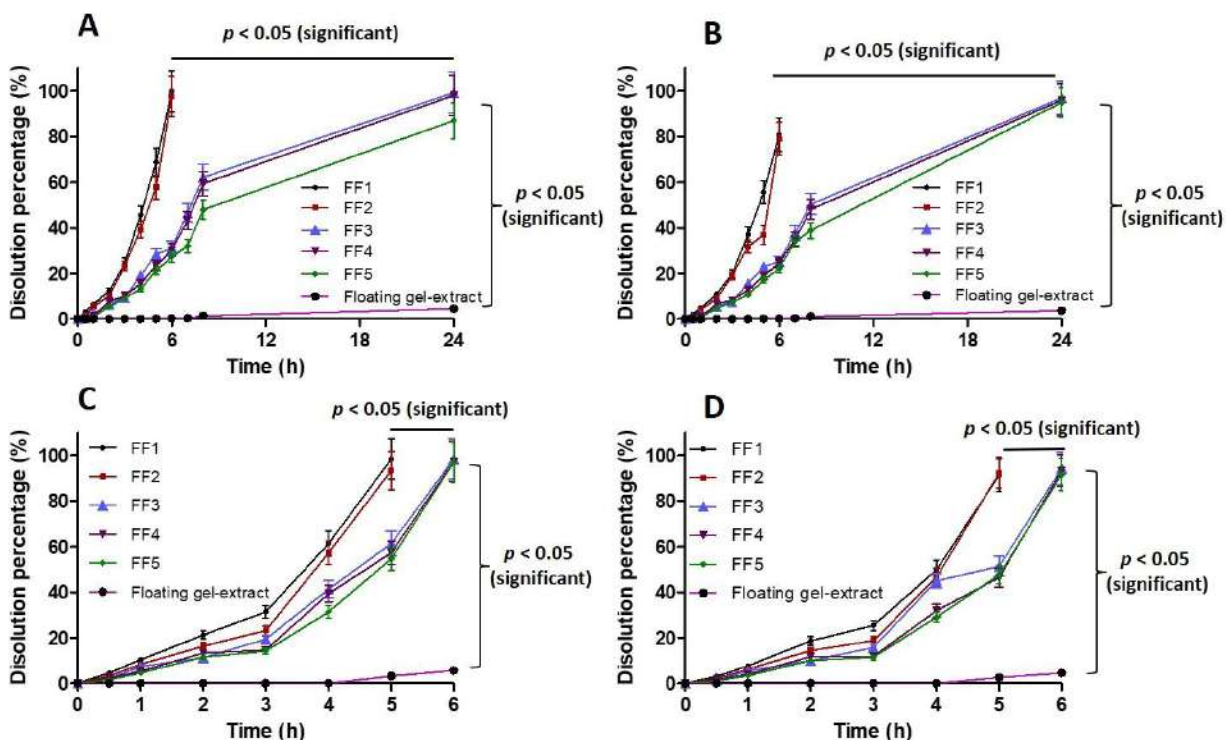


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF (mean $\pm$ SD, $n=3$).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8C and 8D, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h (Figure 9A and 9B). After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF.

Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 9C the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.

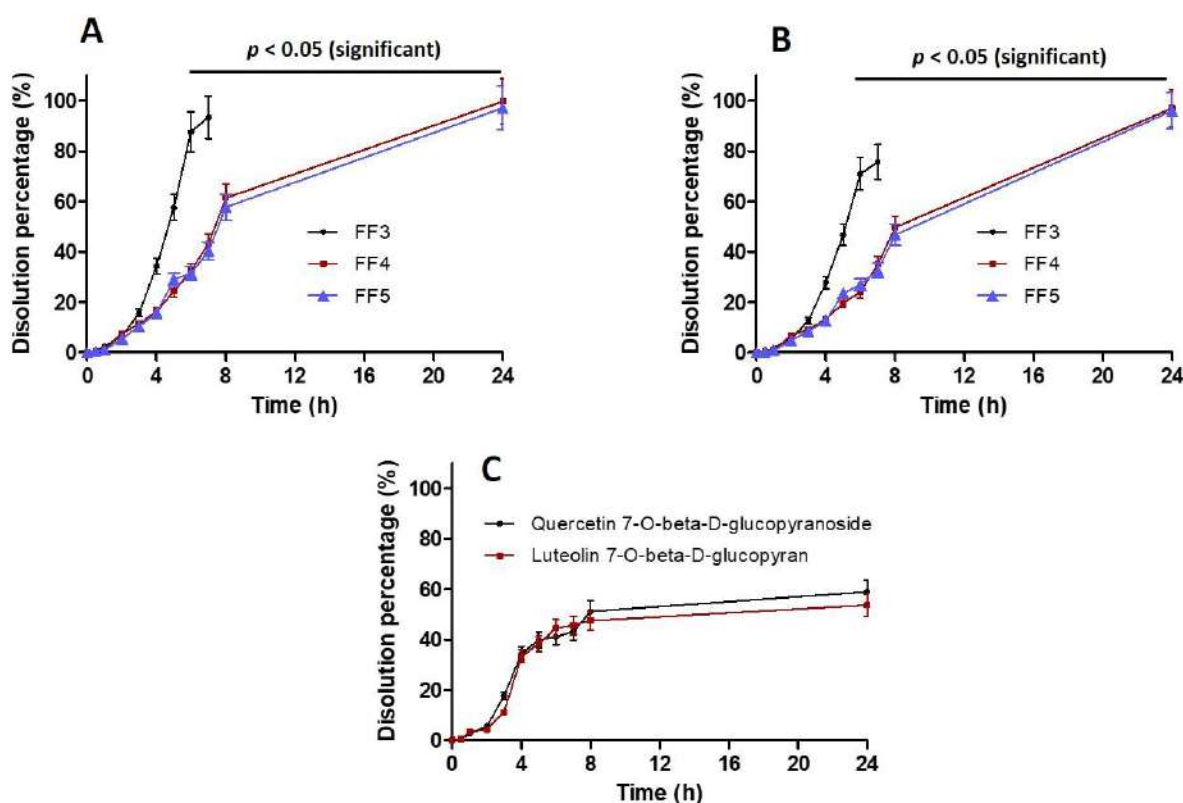


Figure 9. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (C) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate

its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 µg/mL against DPPH and 48.75 ± 4.52 µg/mL against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard

compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to 10 mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and

20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was

found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HP β CD, the SLNs dispersion was mixed with the same volume of HP β -CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

215 2.8. Determination of Particle Size, PDI and Zeta Potential

216 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 217 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 218 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 219 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 220 particle size, Z-average (d.nm) with intensity (%) was used.

221 2.9. Encapsulation Efficiency and Drug Loading Determination

222 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 223 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 224 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 225 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 226 dissolved compounds were quantified by determining the compounds from the filtrate from the
 227 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 228 loading of both compounds were calculated using the following equation:

$$229 \text{ Encapsulation efficiency (\%)} = \frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$$

230 Drug loading (%) = $\frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$

233 **2.10. FTIR study**

234 To evaluate the possible intervention between the compounds in extract and excipients
235 used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this
236 study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹
237 wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s
238 and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

240 **2.11. DSC evaluation**

241 The thermal properties of the compounds in extract, as well as in SLNs formulation were
242 evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21].
243 Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in
244 aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min
245 under N₂ flow (40 mL/min).

247 **2.12. XRD study**

248 X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state
249 of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical
250 mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples
251 were measured at a sampling width of 0.02°.

253 **2.13. SEM evaluation**

254 In this study, the morphologies of SLNs were detected using a scanning electron
255 microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

256 **2.14. Solubility Analysis**

257 In order to investigate the solubility of both compounds in extract and SLN preparations,
258 two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were
259 added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature

at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into FaSGGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The

time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSGGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSGGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was decreased using the trim gap. The determination was carried out at 25 °C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value) [52,53].

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [54].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin) and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type

could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPH as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of $36.87 \pm 2.81 \mu\text{g/mL}$, $52.91 \pm 4.69 \mu\text{g/mL}$, $81.98 \pm 7.53 \mu\text{g/mL}$, $378.28 \pm 21.17 \mu\text{g/mL}$ and $698.43 \pm 54.93 \mu\text{g/mL}$, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

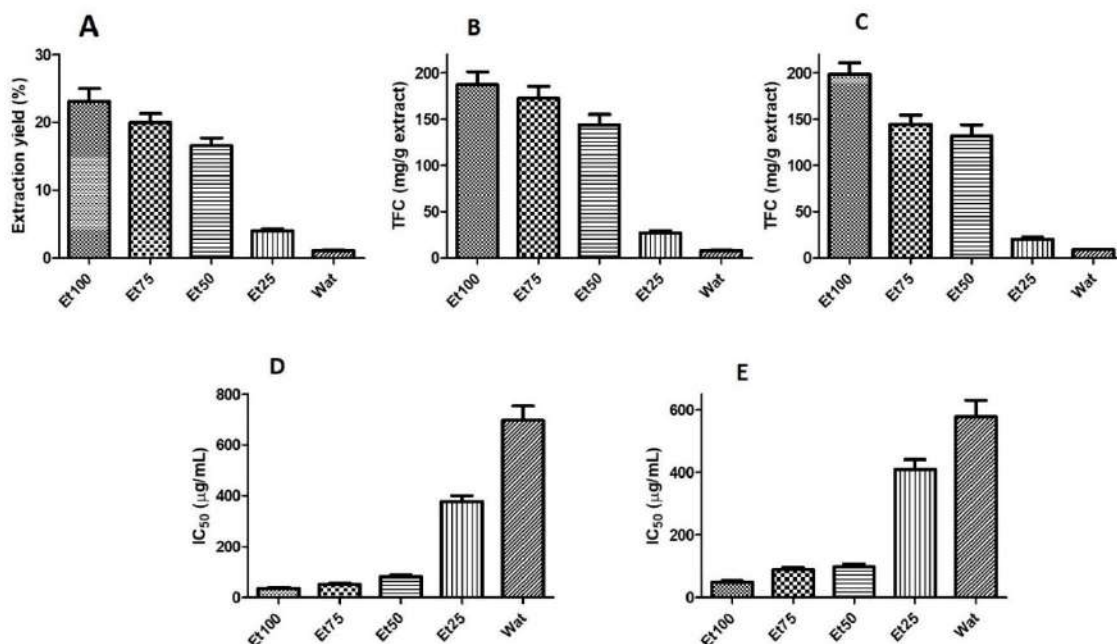


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPH (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, $n=3$)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were $48.75 \pm 3.42 \mu\text{g/mL}$, $89.04 \pm 6.56 \mu\text{g/mL}$, $98.97 \pm 8.91 \mu\text{g/mL}$, $409.51 \pm 31.67 \mu\text{g/mL}$ and $577.43 \pm 43.87 \mu\text{g/mL}$, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were $7.65 \pm 0.63 \text{ mg/g}$ for Et100, $4.32 \pm 0.32 \text{ mg/g}$ for Et75, $2.97 \pm 0.19 \text{ mg/g}$ for Et50, $0.74 \pm 0.05 \text{ mg/g}$ for Et25 and $0.02 \pm 0.001 \text{ mg/g}$ for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were $2.32 \pm 0.21 \text{ mg/g}$, $1.92 \pm 0.15 \text{ mg/g}$, $0.67 \pm 0.05 \text{ mg/g}$, $0.29 \pm 0.03 \text{ mg/g}$ and $0.01 \pm 0.001 \text{ mg/g}$, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

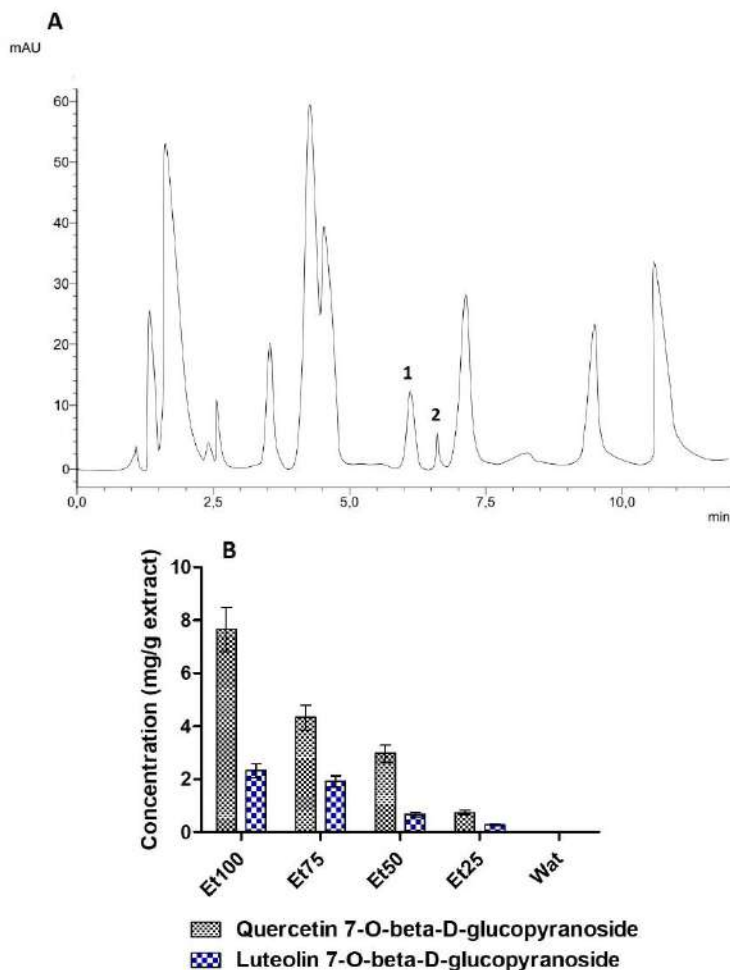


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [55]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [56]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

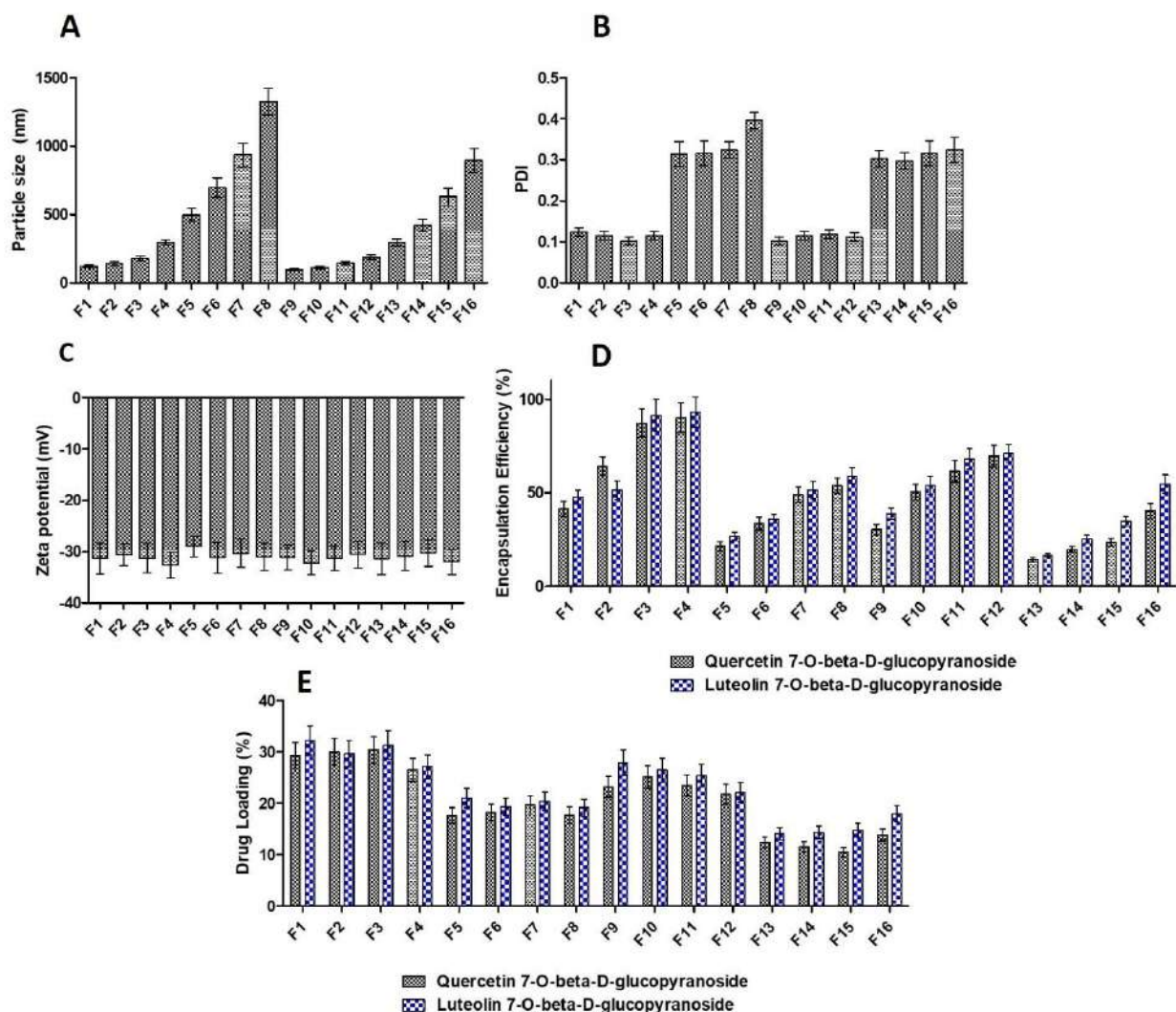


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

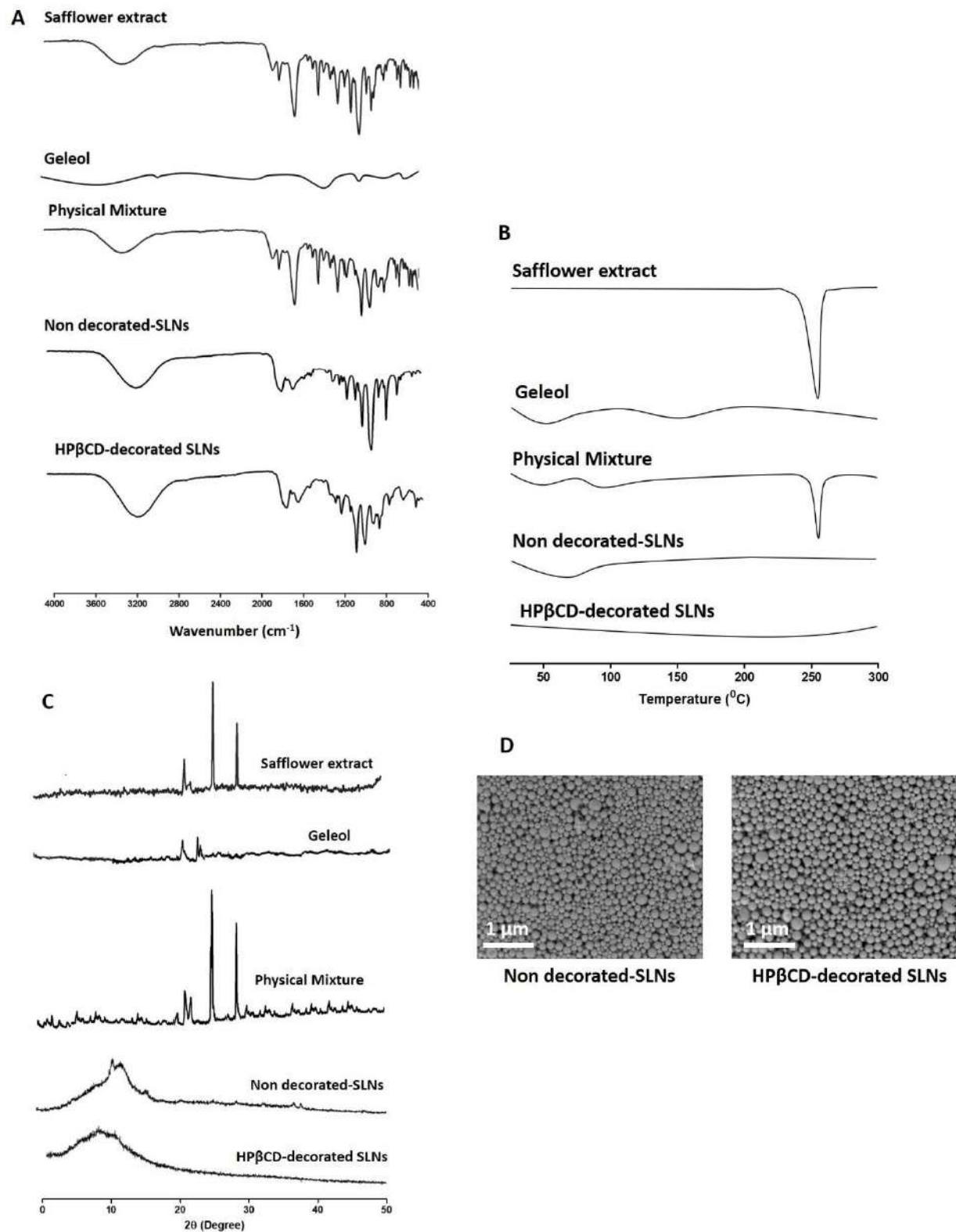


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [57], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [58–62].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustained release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of the compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [63]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

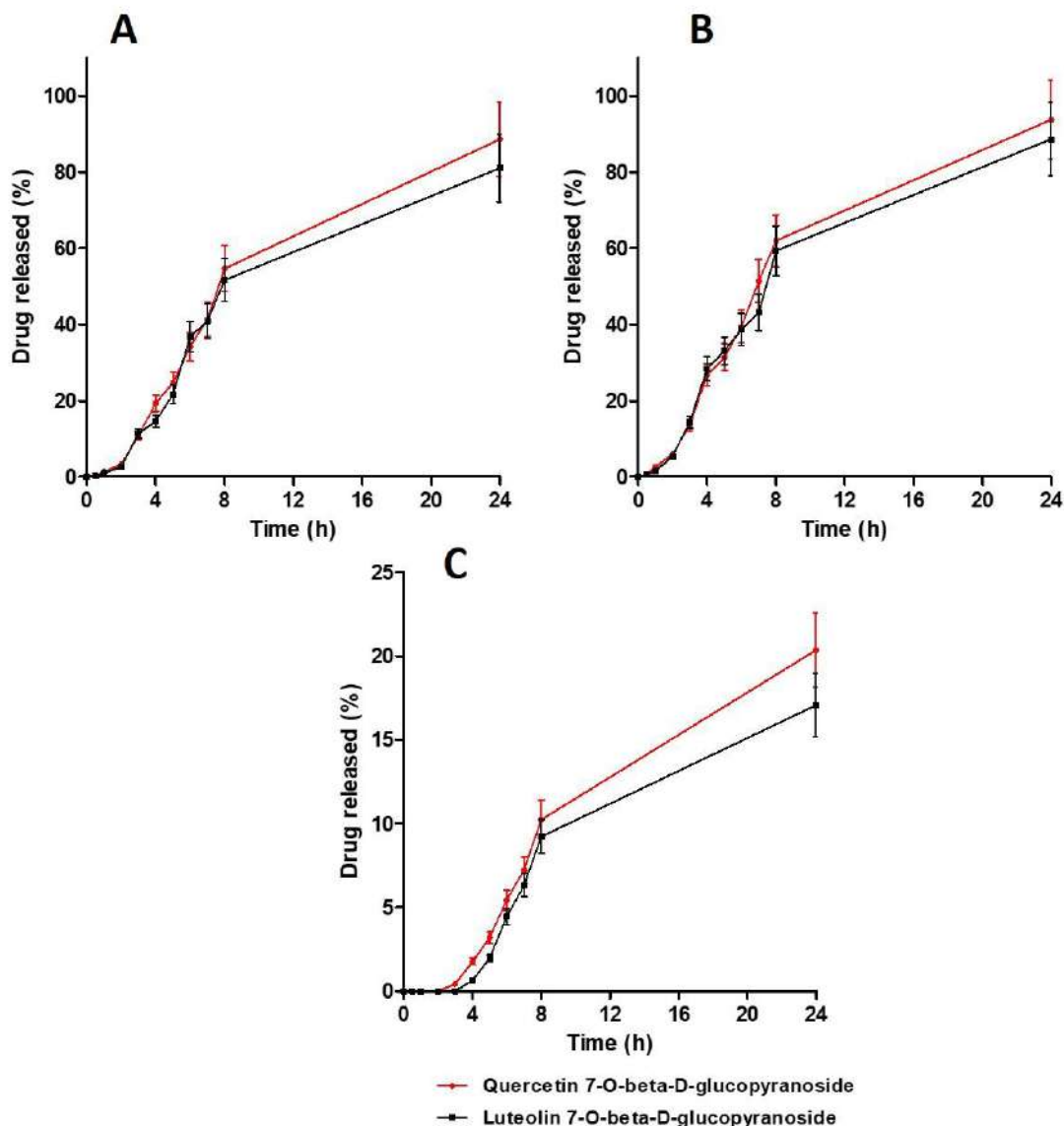


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean ± SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

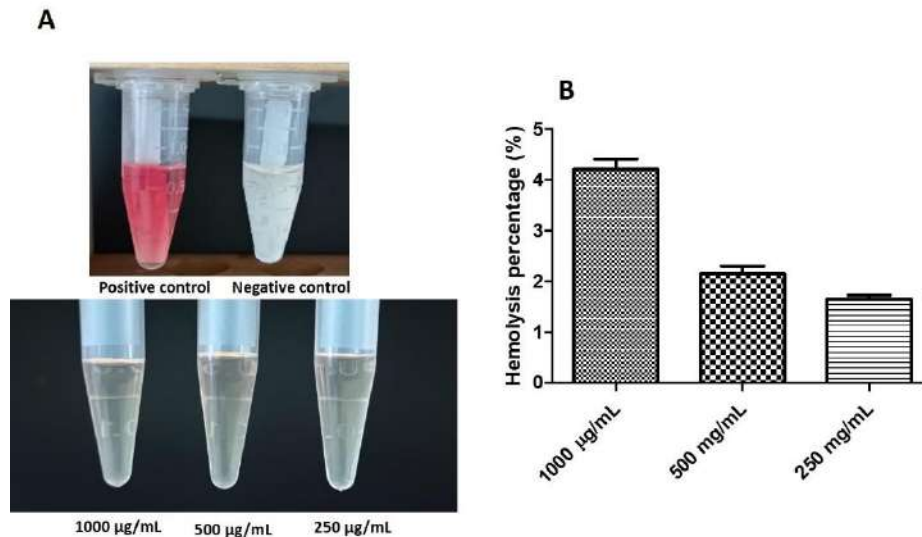


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [64]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [65,66]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

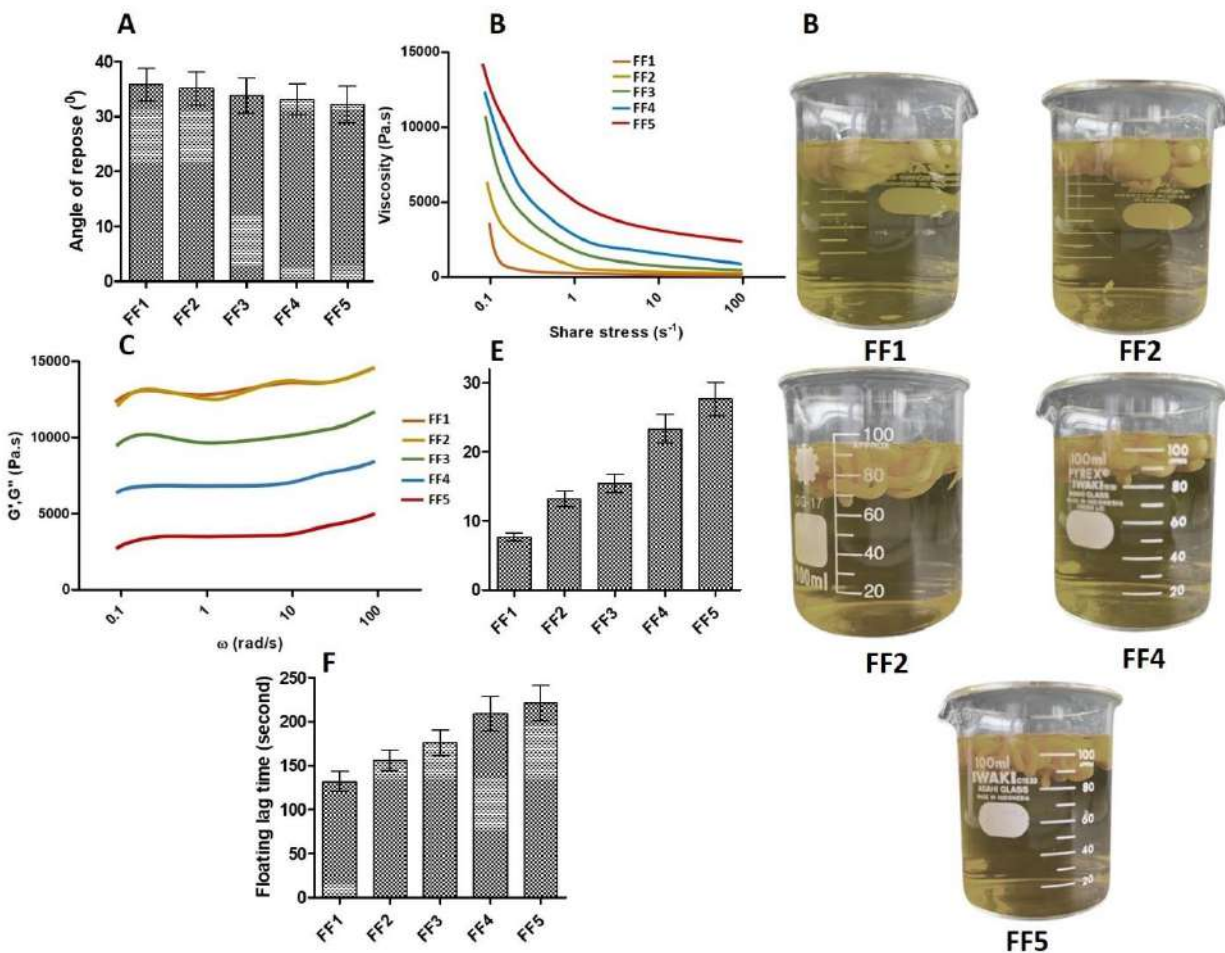


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, n=3). Representative images of floating gel in situ (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The rheogram showed that all the formulations exhibited non-Newtonian behavior. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [65,66].

The strength values of the gel formulation are shown in Figure 7C. The value of G' and G'' represented the strength of the gels. It was found that the increase of Pluronic F127 concentration

from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [64]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which

affected the release of CO₂ [67]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [68]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [69]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure 8A and 8B, below 7.5% of Pluronics (FF1 and FF2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the compounds over 24 h.

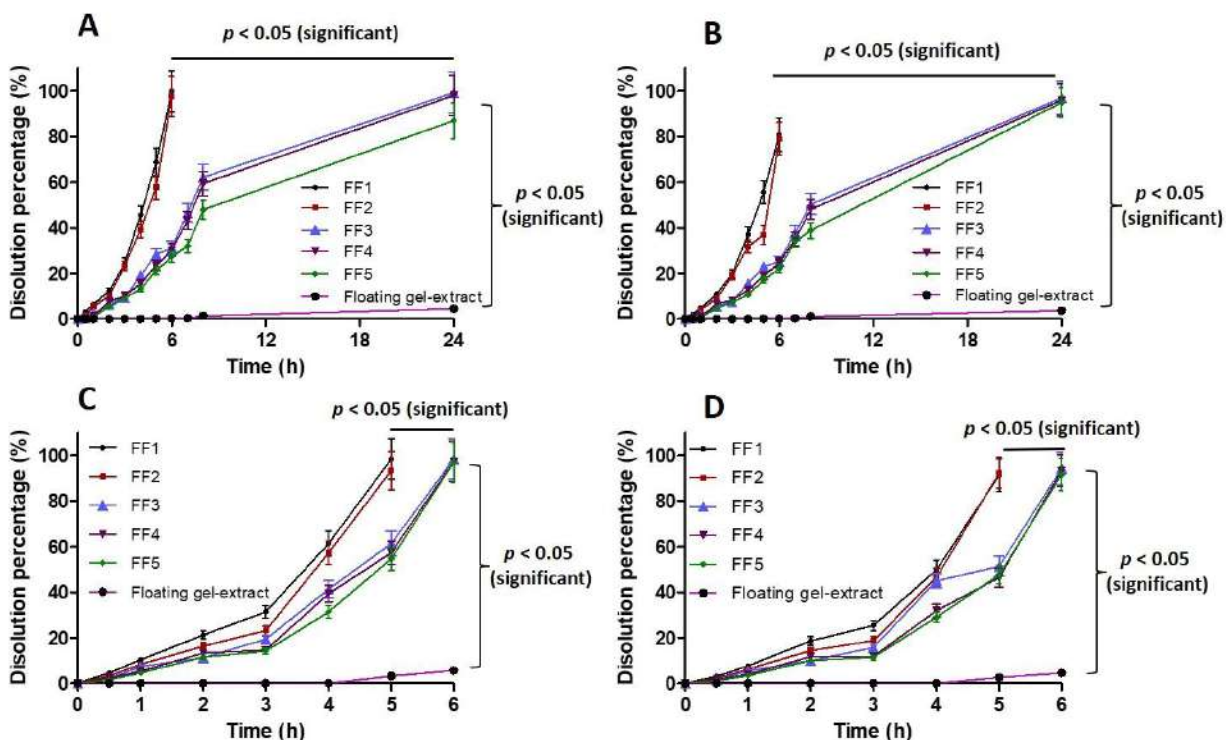


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF (mean $\pm$ SD, $n=3$).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8C and 8D, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h (Figure 9A and 9B). After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF.

Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 9C the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.

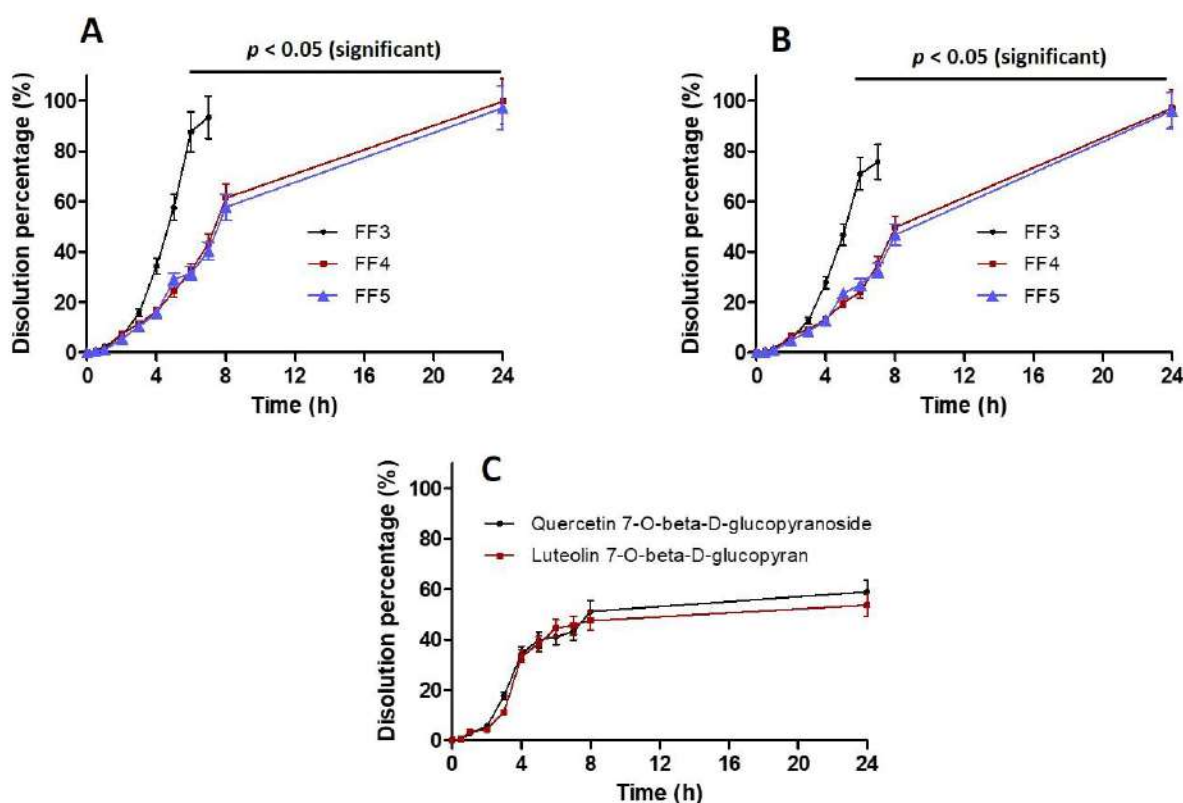


Figure 9. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (C) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate

its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 µg/mL against DPPH and 48.75 ± 4.52 µg/mL against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media --Manuscript Draft--

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Abstract:	Safflower (<i>Carthamus tinctorius</i> L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HPβCD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.
Suggested Reviewers:	Ryan Donnelly r.donnelly@qub.ac.uk Aaron Courtenay a.courtenay@ulster.ac.uk Eneko Larraneta e.larraneta@qub.ac.uk Ahmed Faheem ahmed.faheem@sunderland.ac.uk
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	floating in situ delivery systems for controlled release of bioactive compounds of Safflower (<i>Carthamus tinctorius</i>. L) Editor and Reviewer comments: The title should be corrected as: "Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (<i>Carthamus tinctorius</i>. L): A proof of concept study in biorelevant media" Response: We thank the Editor and Reviewer for the suggestion. We have corrected the title in the revised manuscript

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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The Editor

International Journal of Biological Macromolecules

17 December 2022

Dear Sir/Madam,

I wish you to re-consider our manuscript entitled: **“Solid lipid nanoparticles-decorated cyclodextrin incorporated gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)”** for publication in International Journal of Biological Macromolecules. Moreover, to represent our study, we have modified the title our study to **“Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media**

We have made a few of changes to the manuscript as a result of the reviewers comments. We believe that the manuscript is now substantially improved. Importantly, we have made a great effort to addressed each of the reviewers' comments in the response to the reviewer file.

We believe that this work will be of great interest to the readers of International Journal of Biological Macromolecules. The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in International Journal of Biological Macromolecules and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia
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Author Contributions: Conceptualization, A.D.P., A.R., F.N., and M.A.B.; methodology, A.D.P., A.S., L.C., and A.N.M.; software, A.D.P., A.S., A.N.M.; validation, A.D.P., A.R., and F.N.; formal analysis, A.S and A.N.M.; investigation, A.D.P., A.S., and A.N.M.; resources, A.D.P., A.R., and F.N.; data curation, A.D.P., A.S., A.N.M.; writing—original draft preparation, A.D.P.; writing—review and editing, A.R., F.N., L.C., and M.A.B.; visualization, A.D.P., A.S., and A.N.M.; supervision, A.D.P.; project administration, A.D.P.; funding acquisition, A.D.P., A.R., and F.N. All authors have read and agreed to the published version of the manuscript.

Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

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Solid lipid nanoparticles-decorated cyclodextrin incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L)

Editor and Reviewer comments:

The title should be corrected as: "**Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media**"

Response:

We thank the Editor and Reviewer for the suggestion. We have corrected the title in the revised manuscript

Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogenous antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogenous antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogenous antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Hydroxypropyl beta-cyclodextrin was kindly gifted by Cyclolab Ltd., Budapest, Hungary. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a

spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to 10 mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and 20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with

limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HPβCD, the SLNs dispersion was mixed with the same volume of HPβ-CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212

213 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

214

215

216 **2.8. Determination of Particle Size, PDI and Zeta Potential**

217 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 218 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 219 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 220 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 221 particle size, Z-average (d.nm) with intensity (%) was used.

222 **2.9. Encapsulation Efficiency and Drug Loading Determination**

223 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 224 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 225 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 226 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 227 dissolved compounds were quantified by determining the compounds from the filtrate from the
 228 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 229 loading of both compounds were calculated using the following equation:

Encapsulation efficiency (%) = $\frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$

Drug loading (%) = $\frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$

2.10. FTIR study

To evaluate the possible intervention between the compounds in extract and excipients used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹ wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

2.11. DSC evaluation

The thermal properties of the compounds in extract, as well as in SLNs formulation were evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21]. Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min under N₂ flow (40 mL/min).

2.12. XRD study

X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples were measured at a sampling width of 0.02°.

2.13. SEM evaluation

In this study, the morphologies of SLNs were detected using a scanning electron microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

2.14. Solubility Analysis

In order to investigate the solubility of both compounds in extract and SLN preparations, two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were

added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into

FaSSGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSSGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was decreased using the trim gap. The determination was carried out at 25 °C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value) [52,53].

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [54].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin)

and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPH as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of 36.87 ± 2.81 μ g/mL, 52.91 ± 4.69 μ g/mL, 81.98 ± 7.53 μ g/mL, 378.28 ± 21.17 μ g/mL and 698.43 ± 54.93 μ g/mL, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

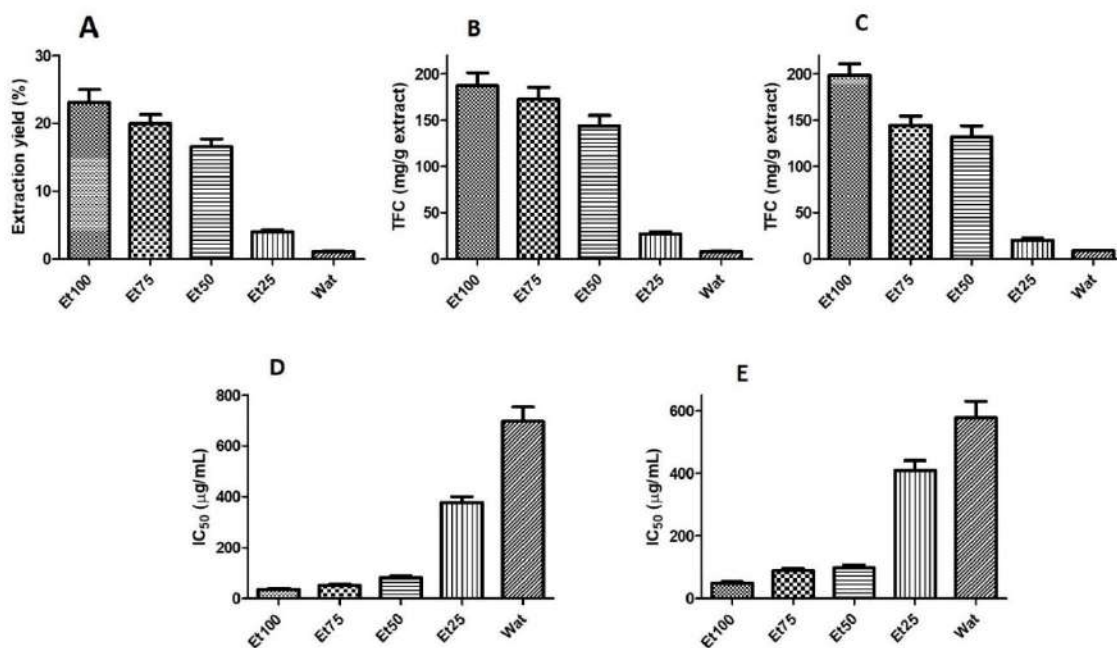


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPH (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, n=3)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were 48.75 ± 3.42 μ g/mL, 89.04 ± 6.56 μ g/mL, 98.97 ± 8.91 μ g/mL, 409.51 ± 31.67 μ g/mL and 577.43 ± 43.87 μ g/mL, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were 7.65 ± 0.63 mg/g for Et100, 4.32 ± 0.32 mg/g for Et75, 2.97 ± 0.19 mg/g for Et50, 0.74 ± 0.05 mg/g for Et25 and 0.02 ± 0.001 mg/g for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were 2.32 ± 0.21 mg/g, 1.92 ± 0.15 mg/g, 0.67 ± 0.05 mg/g, 0.29 ± 0.03 mg/g and 0.01 ± 0.001 mg/g, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

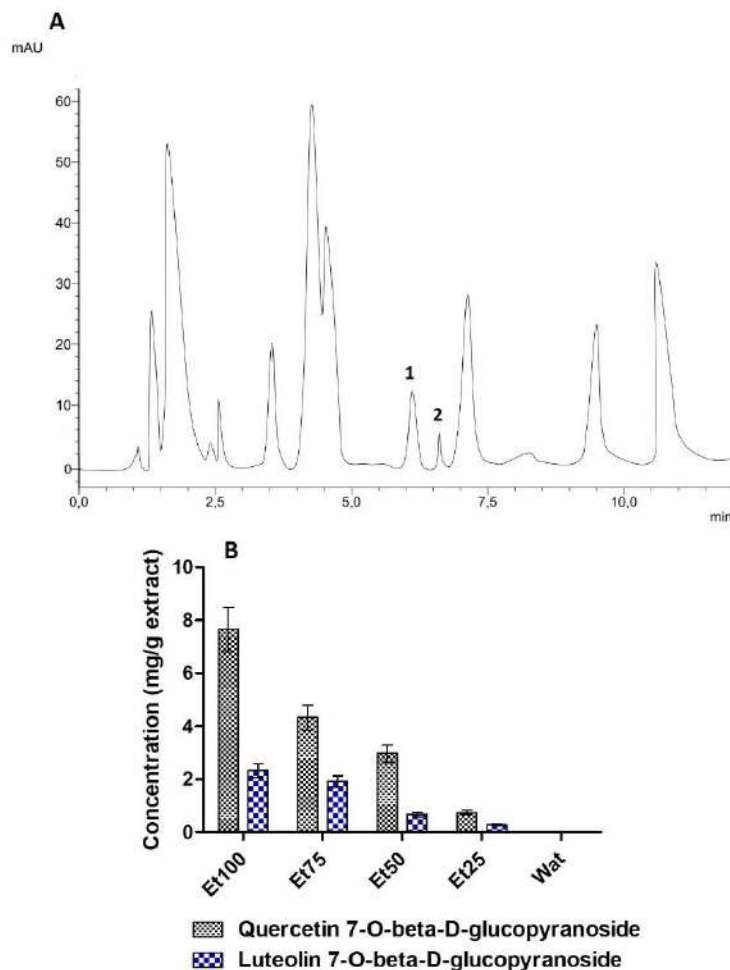


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [55]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [56]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

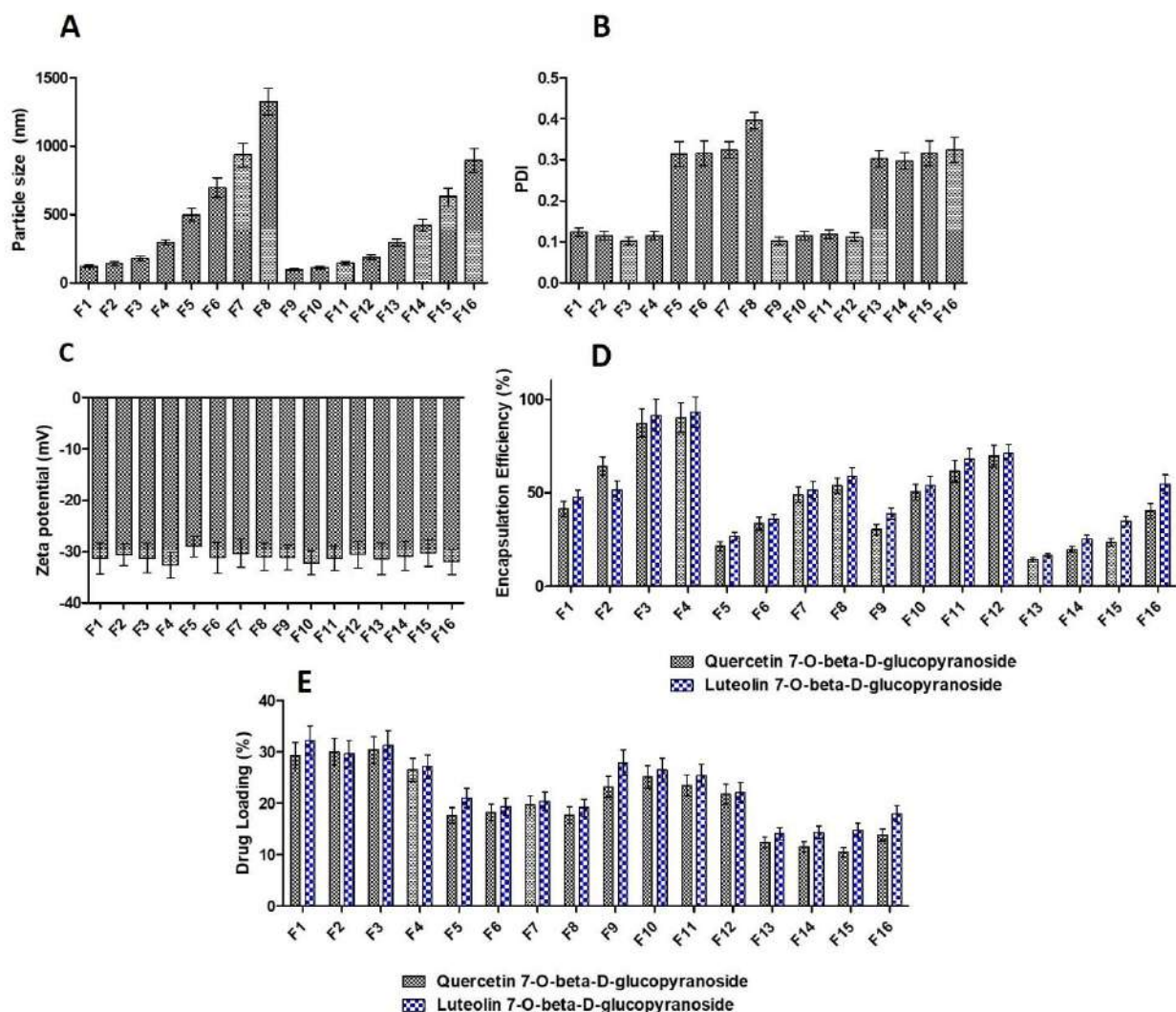


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

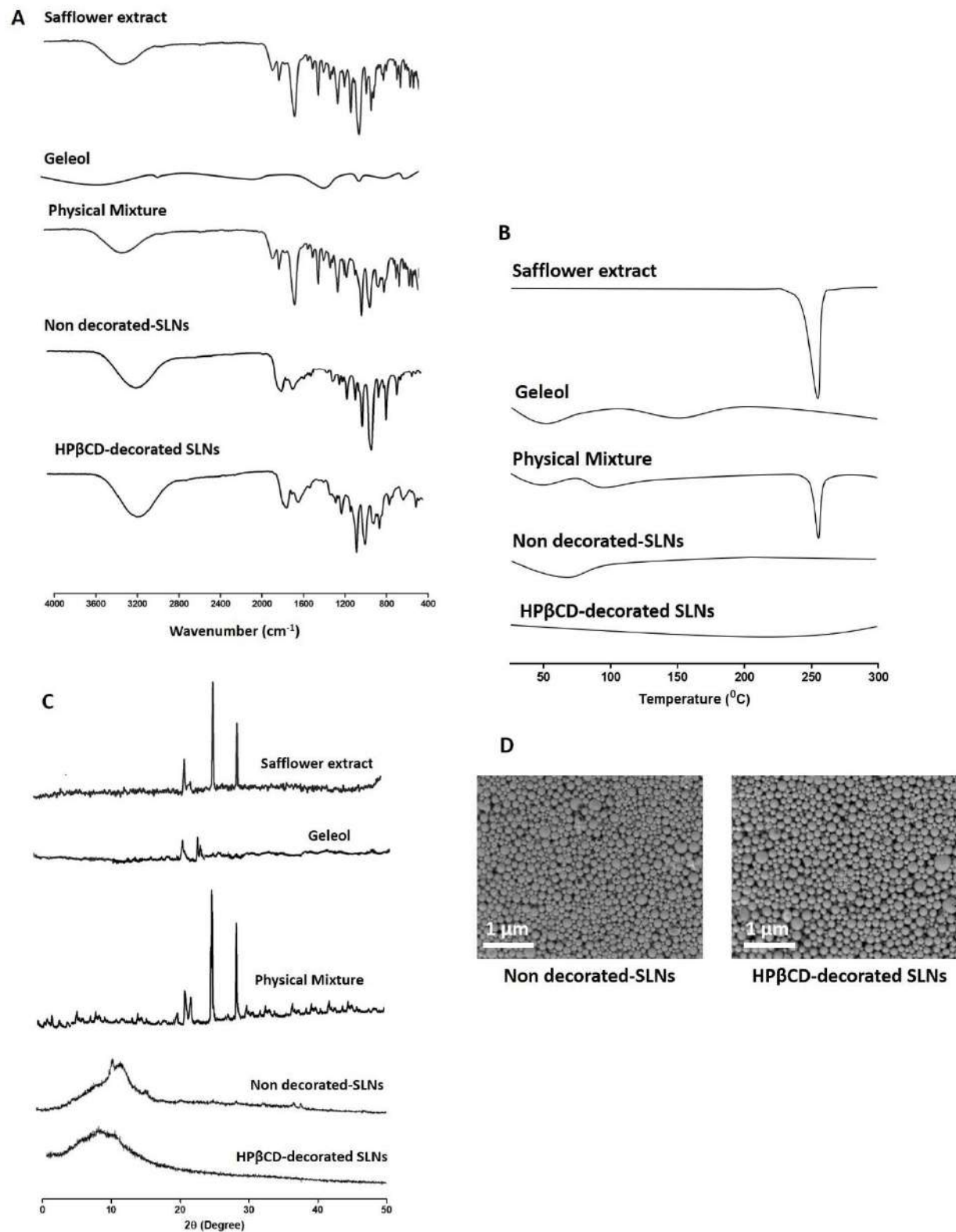


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [57], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [58–62].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustained release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of the compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [63]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

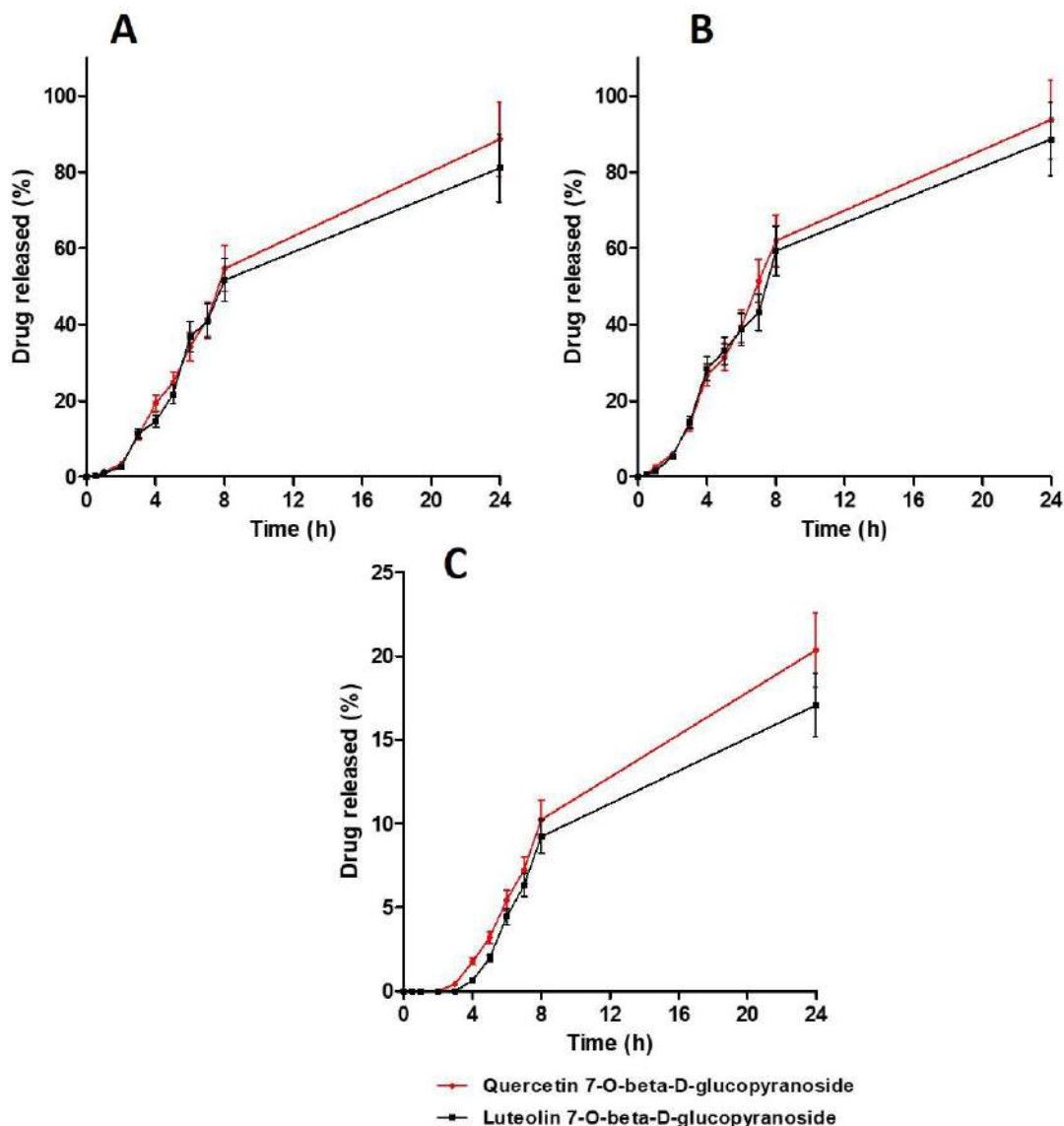


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

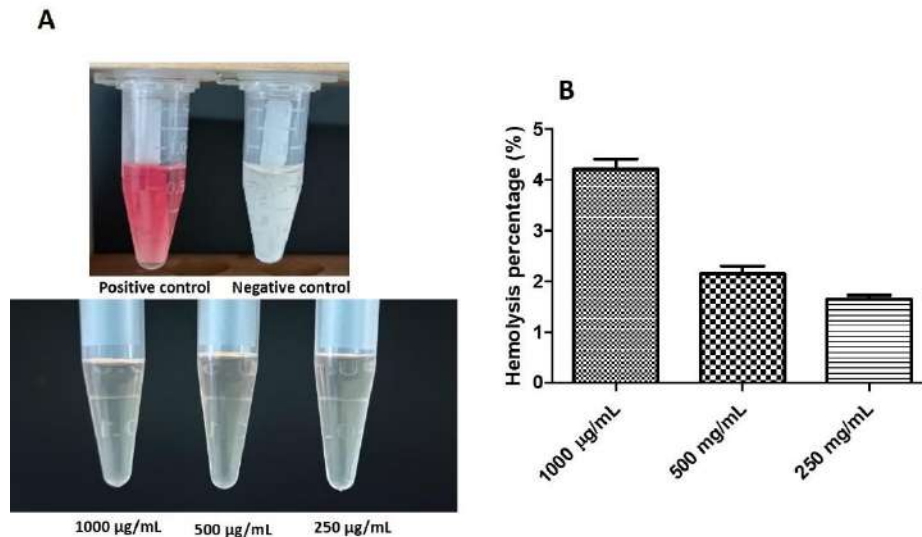


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [64]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [65,66]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

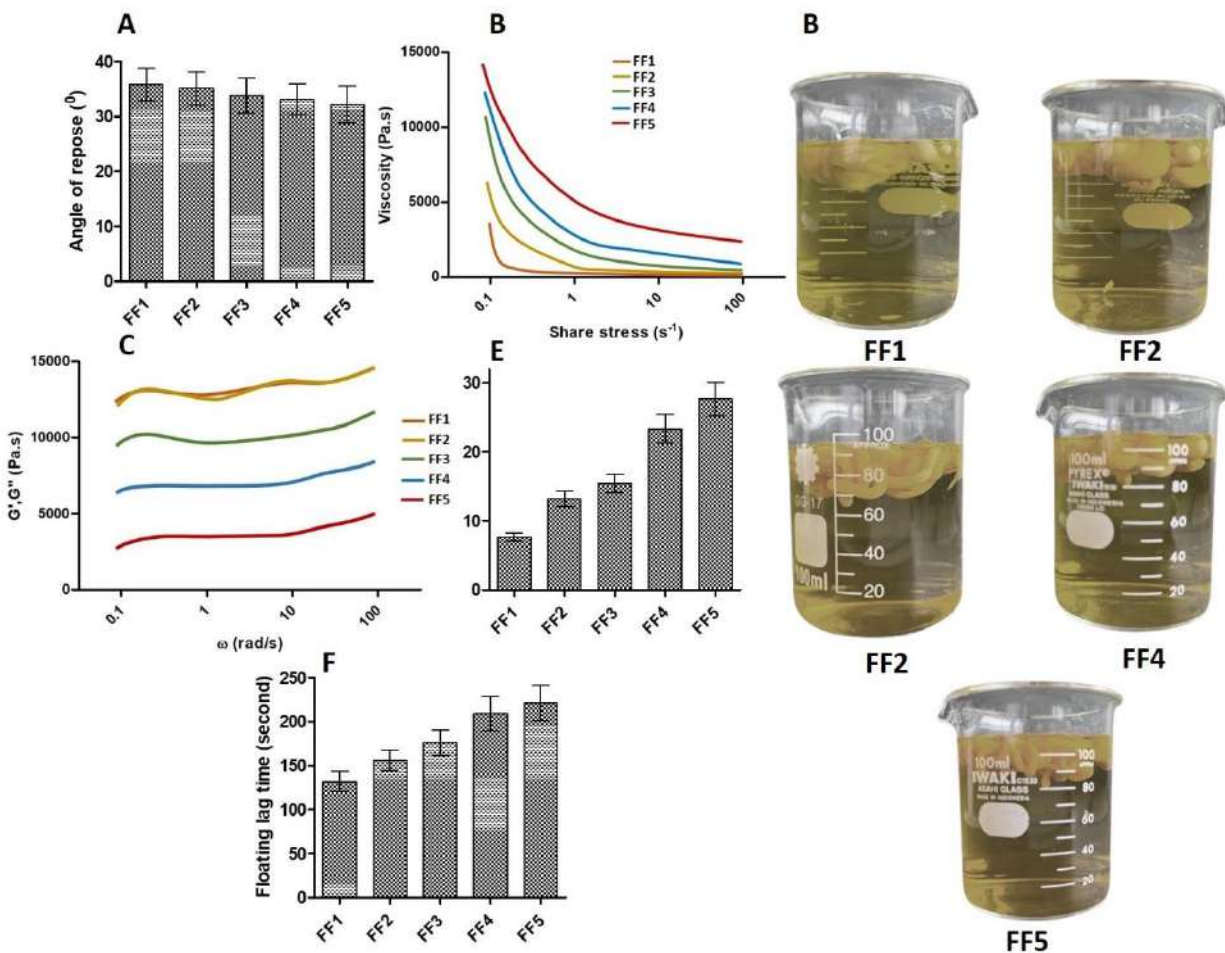


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, $n=3$). Representative images of floating gel in situ (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The rheogram showed that all the formulations exhibited non-Newtonian behavior. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [65,66].

The strength values of the gel formulation are shown in Figure 7C. The value of $G'G''$ represented the strength of the gels. It was found that the increase of Pluronic F127 concentration

from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [64]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which

affected the release of CO₂ [67]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [68]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [69]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure 8A and 8B, below 7.5% of Pluronics (FF1 and FF2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the compounds over 24 h.

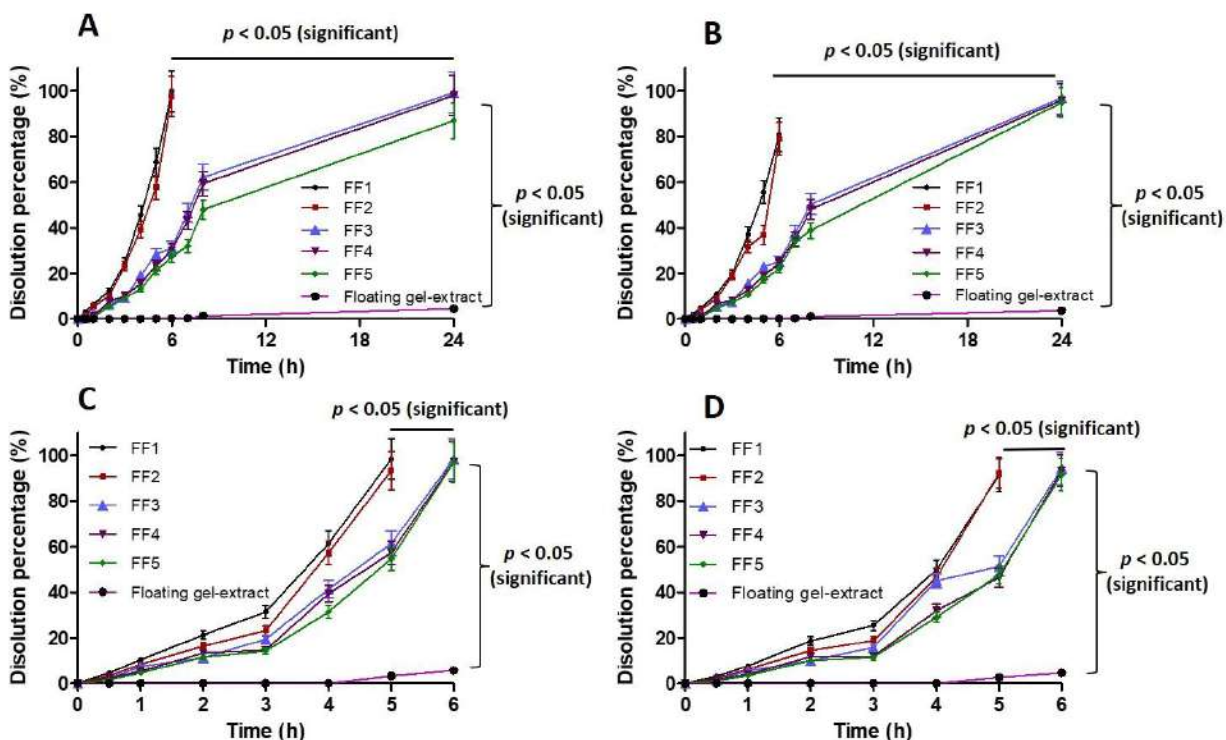


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF (mean $\pm$ SD, $n=3$).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8C and 8D, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h (Figure 9A and 9B). After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF.

Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 9C the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.

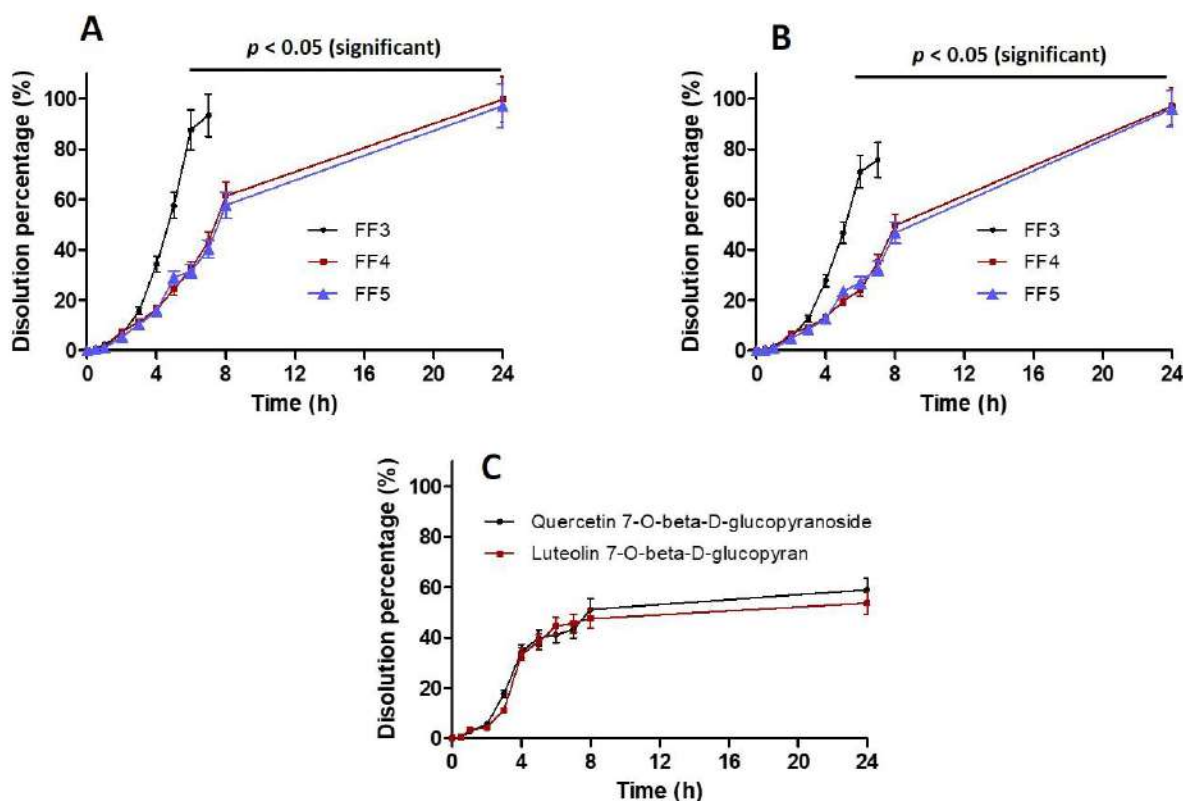


Figure 9. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (C) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, $n=3$).

Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate

its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 $\mu\text{g/mL}$ against DPPH and 48.75 ± 4.52 $\mu\text{g/mL}$ against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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Solid lipid nanoparticles cyclodextrin-decorated incorporated into gellan gum-based dry floating in situ delivery systems for controlled release of bioactive compounds of Safflower (*Carthamus tinctorius*. L): A proof of concept study in biorelevant media

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Abstract

Safflower (*Carthamus tinctorius* L.) has been explored as a source of natural antioxidant. However, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, as its bioactive compounds, possessed poor aqueous solubility, limiting its efficacy. Here, we developed solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HP β CD) incorporated into dry floating gel in situ systems to control the release of both compounds. Using Geleol® as a lipid matrix, SLNs were less than 200 nm in size with > 80% of encapsulation efficiency. Importantly, following the decoration using HP β CD, the stability of SLNs in gastric environment was significantly improved. Furthermore, the solubility of both compounds was also enhanced. The incorporation of SLNs into gellan gum-based floating gel in situ provided desired flow and floating properties, with < 30 seconds gelation time. The floating gel in situ system could control the release of bioactive compounds in FaSSGF (Fasted-State Simulated Gastric Fluid). Furthermore, to assess the effect of food intake on release behavior, we found that the formulation could show a sustained release pattern in FeSSGF (Fed-State Simulated Gastric Fluid) for 24 h after being released in FaSSGF for 2 h. This indicated that this combination approach could be a promising oral delivery for bioactive compounds in safflower.

Keywords: Safflower, solid lipid nanoparticles, floating gel in situ

1. Introduction

Recently, oxidative stress, caused by free radicals, has been reported to be the main foremost reason for numerous chronic diseases [1,2], including autoimmune diseases [3], chronic kidney disease [4], neurodegenerative diseases [5], cardiovascular diseases [6], cancer [7] and liver diseases [8]. Normally, as free radical, reactive oxygen species (ROS) are regularly generated in human cells. To neutralize the undesired effects of ROS, cells continuously produce endogen antioxidant systems consisting of enzymes and non-enzymatic compounds [9,10]. These endogen antioxidants are able to inhibit the reaction of ROS. Nevertheless, in some disease conditions, there is a decrease in the generation of endogen antioxidants, resulting in the increment of the ratio of antioxidant and ROS [11]. Despite the fact that the synthetic antioxidants have been extensively explored, the natural antioxidants from plants, vegetables, and fruits have been found to be a significant resource of antioxidants [12]. Accordingly, a plethora of studies have investigated different sources of natural plants for their potential as antioxidant agents [13–15]. The main reason for this was mainly due to several advantages of natural antioxidants over synthetic antioxidants, namely less adverse effects and fewer economic costs.

Safflower (*Carthamus tinctorius*. L), belonging to from Asteraceae family, has been widely explored for its antioxidant compounds. Safflower is mainly cultivated exclusively for its flowers, which are applied in the therapy of several diseases. The antioxidant properties of safflower are mainly due to several compounds, namely flavonoid and phenolic compounds [16–18]. Specifically, the glycosides form of the flavonoid compounds in safflower, namely quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside have been found to possess strong antioxidant properties which can potentially protect the body from stress oxidative [16,19,20]. However, although those compounds possess adequate antioxidant activities, they have poor aqueous solubility, limiting their bioavailability and efficacy as antioxidant in the human body. Accordingly, there is a necessity to develop a delivery system to improve the solubility of these compounds.

Among several delivery systems, the interest of researchers in solid lipid nanoparticles (SLNs) has increased significantly. This system has shown significant benefits, namely high entrapment efficiency, enhancement of solubility profiles and control release manner in comparison with other lipid-based delivery system [21]. SLNs are consisted of physiological lipids, making this system to be considered safe for the administration. Importantly, SLNs have

64 been extensively applied to enhance the solubility and control the release of numerous hydrophobic
65 compounds [22–24]. With respect to the administration route, the oral route has been considered
66 the most convenient and acceptable route for drug administration [25]. However, the poor stability
67 and high propensity to aggregate in acid conditions limited their application through the oral route
68 [26]. Several studies have shown that the oral administration of SLNs could results in instability
69 of the particles in the gastric environment [27,28]. Accordingly, it is crucial to investigate suitable
70 approach to protect the SLNs from the gastric environment during oral administration. Previously,
71 hydroxypropyl beta-cyclodextrin (HP β CD) has been found to improve the stability of SLNs
72 administered orally [29]. The decoration of SLNs containing amphotericin B, paramomycin and
73 paclitaxel using HP β CD could improve their efficacies for their respective pharmacological effects
74 [29,30]. Therefore, the incorporation of bioactive compounds of safflower into SLNs decorated
75 with HP β CD could be an appropriate strategy for improved solubility.

76 To facilitate the oral administration of the SLNs, it was important to develop a suitable
77 system. One of delivery systems to control and sustain the release of drugs through oral
78 administration is gastroretentive drug delivery system (GRDDS). In this formulation, 100% of
79 drugs would stay in the stomach for a long period [31–33]. As one of GRDDS, in situ forming gel
80 system has attracted the interest in drug delivery due to the transformation of the formulation from
81 solution into a floating gel when administered orally and reaching the gastric environment [34–
82 36]. Numerous studies have explored the effectiveness of this system to control the release some
83 drugs administered orally [32,37,38]. As one of macromolecules compounds, gellan gum has been
84 widely used to develop GRDDS preparations, showing the controlled release pattern of the
85 bioactive compounds [32,39–41].

86 In this study, we extracted the bioactive compounds from safflower and formulated the
87 extract into SLNs. The SLNs were decorated using HP β CD to improve its gastric stability. Several
88 characterizations were carried out to optimize the SLNs. Afterwards, the SLNs were further
89 incorporated into floating gel in situ system using gellan-gum as a main matrix molecule in the
90 dry form which would be dispersed in water prior to the oral administration. The floating gel in
91 situ was evaluated for their properties and release behavior of quercetin 7-O-beta-D-
92 glucopyranoside and luteolin 7-O-beta-D-glucopyranoside as the interest compounds. The effect
93 of food intake on the dissolution profile was further investigated as a proof of concept study in
94 biorelevant media. The main finding of this investigation showed the potential of using natural

product as antioxidant sources in sustain release formulation for potential treatment of oxidative stress induced diseases.

2. Materials and Methods

2.1. Materials

The safflower samples were collected from Bone, South Sulawesi, Indonesia. Geleol® was kindly gifted by Gattefosse Pvt. Ltd., France. Hydroxypropyl beta-cyclodextrin was kindly gifted by Cyclolab Ltd., Budapest, Hungary. Gellan gum, sodium citrate, calcium chloride, poly(vinyl alcohol) PVA (9–10 kDa), Tween80 and sodium dihydrogen phosphate were obtained from Sigma-Aldrich (Dorset, UK). Other chemicals utilized in this study were analytical grade and applied without purification process.

2.2. Extraction of Safflower

The safflower flowers were dried and grounded. Afterward, the samples (500 g) were extracted using three different solvents, namely absolute ethanol (Et100), 75% ethanol (Et75), 50% ethanol (Et50), 25% ethanol (Et25) and water (Wat). Percolation method was used to extract the bioactive compounds at room temperature [42]. The mixtures were then filtered, and the filtrate was subjected to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) and freeze drying (Christ, Osterode am Harz, Germany), obtaining a dry extract of Et100, Et75, Et50, Et25 and Wat.

2.2. Determination of Extraction Yields of Safflower

The yield of the extraction process using different solvents was calculated using the following equation [43]:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of initial samples}} \times 100\%$$

2.3. Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content (TFC) of safflower extract was measured by colorimetry utilizing AlCl₃ [43], with minor modification. Briefly, the methanolic solution of safflower extract was mixed with 10% w/v AlCl₃ (0.25 mL) and potassium acetate solution (0.5 mL). The solution was incubated for 25 min in the dark. The absorbance of the solution was detected using a

spectrophotometer (Shimadzu, Kyoto, Japan) at 415 nm. Quercetin was used as a standard compound. The amount of TFC was reported as milligrams of quercetin equivalent per gram of extract (mg Qu/g).

The determination of total phenolic content (TPC) of safflower extract was carried out utilizing the Folin-Ciocalteu method [43], with a slight modification. The extract was initially solubilized in methanol. Afterwards, 250 µL of the extract solution was mixed with 1 N Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL). The solution was incubated for 10 min at room temperature, followed by the addition of 5% w/v sodium carbonate solution (0.5 mL) and methanol (up to 10 mL) in the volumetric flask. The solution was incubated at room temperature for 25 min in dark condition. Finally, the absorbance of the solution was detected using a spectrophotometer at a wavelength of 760 nm. Gallic acid was used as the standard compound. The concentration of TPC was reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.4. Evaluation of Antioxidant Activity Assay Using DPPH Scavenging Capacity

The antioxidant properties of various extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [43]. Briefly, the mixture of methanolic solution of DPPH (2.5 mL), methanolic solution of extract in various concentrations (0.25 mL) and methanol (2.5 mL) was incubated for 25 min in the dark at room temperature. The absorbance of the solution was detected using a spectrophotometer at 520 nm. The absorbance of the DPPH solution with the absence of extract solution was used as a control. The extraction solvents were utilized as blank. The antioxidant properties were determined using the following calculation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of samples}} \times 100\%$$

Moreover, the concentration inhibiting 50% of DPPH free radical (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.5. Evaluation of Antioxidant Activity Using Lipid Peroxidation Method

The lipid peroxidation antioxidant properties of the extract were assessed using thiocyanate, with slight modification [43]. Initially, linoleic acid (0.25 g), Tween-80 (0.25 g), and 20 mM phosphate buffer pH 7.0 (20 mL) were used to prepare linoleic emulsion. All compositions were mixed using an Ultra-Turrax® homogeniser (IKA, model T25, impeller 10 G, Germany). To perform the evaluation, the emulsion (2.5 mL) was mixed with methanolic solution of extract in various concentration levels. The mixture was incubated at 37 °C for 6 h. Afterward, the mixture (0.1 mL) was mixed with 75% ethanol (5 mL), 20 mM of ferrous chloride in 100 mM HCl (0.1 mL) and 30% w/v ammonium thiocyanate (0.2 mL). Then, the mixture was incubated at room temperature for 5 min. The absorbance of the solution was detected using a spectrophotometer at 500 nm. As per control, the absorbance of the mixture without the addition of extract was measured. The lipid peroxidation inhibition percentage was determined using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Similarly, the concentration inhibiting 50% of lipid peroxidation (IC₅₀) was determined using linear regression in GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA).

2.6. HPLC Assay of Bioactive Compounds in safflower extract

The quantification of bioactive compounds of safflower extract was performed using reverse phase HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan). The gradient method (Table 1) using the mixture of 0.1% v/v trifluoro acetic acid (TFA) in water and acetonitrile was used to separate the compounds with a reversed-phase column C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Inc., CA, USA). The samples were analyzed at 255 nm with injection volume of 25 µL. All the analysis process were carried at 25°C. Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside were used as analytes of interest of the extract. The chromatogram analysis was carried out using Shimadzu LC solution software (ver. 1.21 SP1). The HPLC method applied to analyze both compounds was validated according to the International Committee on Harmonisation (ICH) 2005. The limit of detections of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were 0.15 µg/mL and 0.21 µg/mL with

limit of quantifications of 0.35 µg/mL and 0.44 µg/mL, respectively. Importantly, the method was found to be precise and selective following inter-day and intraday measurements with R values > 0.999.

Table 1. Condition of gradient analysis of HPLC method

Time	TFA in water	Acetonitrile	Flow (mL/min)
0	90	10	0.5
3	70	30	0.5
6	40	60	0.6
9	30	70	0.6
12	75	25	0.5
15	90	10	0.5

2.7. Solid lipid nanoparticles (SLNs) formulation

The SLNs were prepared using the emulsion-solvent evaporation technique [29]. The composition of the formulation is depicted in Table 2. Briefly, Geleol and extract were dissolved in 5 mL of mixture of chloroform and methanol (1:1). The organic phase was emulsified into 15 mL of stabilizer solutions under probe sonication (an amplitude of 75% with 20 s pulse on and 10 s pulse off).

To modify the surface of SLNs using HPβCD, the SLNs dispersion was mixed with the same volume of HPβ-CD solution 1% for 4 h at 100 rpm at 4°C. In order to concentrate the SLNs and remove free compounds, the dispersion was spun for 30 min at 7500 rpm at 4 °C using an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa).

212

213 Table 2. The formulation parameters of SLNs

	Geleol	Extract	Tween	PVA	Sonication time
F1	100	100	1%	-	5 minutes
F2	150	100	1%	-	5 minutes
F3	200	100	1%	-	5 minutes
F4	250	100	1%	-	5 minutes
F5	100	100	-	1%	5 minutes
F6	150	100	-	1%	5 minutes
F7	200	100	-	1%	5 minutes
F8	250	100	-	1%	5 minutes
F9	100	100	1%	-	10 minutes
F10	150	100	1%	-	10 minutes
F11	200	100	1%	-	10 minutes
F12	250	100	1%	-	10 minutes
F13	100	100	-	1%	10 minutes
F14	150	100	-	1%	10 minutes
F15	200	100	-	1%	10 minutes
F16	250	100	-	1%	10 minutes

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215

216 **2.8. Determination of Particle Size, PDI and Zeta Potential**

217 Particle size analyzer, Zetasizer (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK)
 218 was utilized to evaluate the particle size, PDI and zeta potential of all the SLN formulations [21].
 219 The measurements were carried out at a scattering angle of 90° at 25 °C. Prior to analysis, the
 220 SLNs was diluted in water accordingly to achieve a suitable count rate. In the determination of the
 221 particle size, Z-average (d.nm) with intensity (%) was used.

222 **2.9. Encapsulation Efficiency and Drug Loading Determination**

223 Indirect method was applied to determine the encapsulation efficiency of the compounds,
 224 by quantifying the free compounds [21]. Due to the low solubility of the compounds in water, the
 225 insoluble free compounds were initially collected by centrifugation at 7500 rpm for 30 minutes.
 226 The precipitate materials were dissolved in methanol and analyzed using HPLC. The free-
 227 dissolved compounds were quantified by determining the compounds from the filtrate from the
 228 purification step to concentrate the SLNs using HPLC. The encapsulation efficiency and drug
 229 loading of both compounds were calculated using the following equation:

Encapsulation efficiency (%) = $\frac{\text{Compounds in formulation} - \text{unencapsulated compound}}{\text{Compounds in formulation}} \times 100\%$

Drug loading (%) = $\frac{\text{Amount of encapsulated compound}}{\text{Total weight of final formulation}} \times 100\%$

2.10. FTIR study

To evaluate the possible intervention between the compounds in extract and excipients used to prepare SLNs, FTIR spectrophotometer (Shimadzu® FTIR-8400) was used [21]. In this study, extract, lipid, the physical mixture, and SLNs were scanned between 4000 and 600 cm⁻¹ wavenumbers. Specifically, the measurements were carried out with a mirror speed of 0.05 cm/s and a resolution of 8 cm⁻¹. The FTIR spectrum was obtained from an average of 64 scans.

2.11. DSC evaluation

The thermal properties of the compounds in extract, as well as in SLNs formulation were evaluated using differential scanning calorimetry (DSC) (DSC 2920 (TA Instruments) [21]. Extract, lipid, the physical mixture, and SLNs were weighed of around 5 mg and were closed in aluminum pan. The evaluation was performed from 25 to 300 °C with a flow rate of 10 °C/min under N₂ flow (40 mL/min).

2.12. XRD study

X-Ray diffractometer (Rigaku Corporation, England) was used to analyze the solid-state of the compounds in extract, as well as in SLNs formulation [37]. Extract, lipid, the physical mixture, and SLNs were scanned from 5° to 50° (2θ) with a step scan speed of 5°/min. All samples were measured at a sampling width of 0.02°.

2.13. SEM evaluation

In this study, the morphologies of SLNs were detected using a scanning electron microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan) [44].

2.14. Solubility Analysis

In order to investigate the solubility of both compounds in extract and SLN preparations, two different solvents, namely water and n-octanol were used [12]. Briefly, extract and SLNs were

added in an excess amount to 5 mL of solvents. The mixture was stirred for 1 h at room temperature at 500 rpm. The supernatant was collected by centrifugation at 3000 rpm for 15 min and analyzed using HPLC.

2.14. In vitro drug release and mathematic models

The release of the compounds from the lipid matrix was carried out using the dialysis method was used [45–48]. Both HP β CD-modified and non-modified SLNs were used in this study. Additionally, the release of the compounds from the pure extract was also conducted. In this study, phosphate buffer saline (PBS) (pH 7.4) containing 1% w/v of Tween 80 was used as a release medium to achieve sink condition during the release. Initially, the formulations were sealed inside the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO, Spectrum Medical Industries, CA, USA). The membrane was placed in 100 mL of release medium. The study was conducted at 37 °C at 100 rpm for 24 h. At each interval time, 1 mL of medium was withdrawn and replaced with similar volume of fresh media. The concentration of both compounds was analyzed using HPLC.

To further analyze the mathematic model kinetic, the release profiles were fitted to various models, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. The calculation was performed using DDSolver (China Pharmaceutical University, Nanjing, China).

2.15. Stability Evaluation in Gastric Environment

The stability of both HP β CD-modified and non-modified SLNs was assessed in FaSSGF media [12]. The formulations were mixed with 10 mL of FaSSGF and incubated at 37 °C. At 2 h, 4 h, 6 h and 8 h, the particle size, PDI and zeta potential of all formulations were determined.

2.16. Hemolytic Evaluation

To assess the possibility of the formulation to cause any toxicity, in vitro hemolytic assay was conducted [49,50]. A fresh red blood cells (RBC) from Wistar rats was used in this study after three times washing steps. The RBS was prepared in a concentration of 10% v/v in PBS. Briefly, 900 μ L of RBC was mixed with 100 μ L of samples. The mixture was incubated for 1 h. at 37 °C and spun for 10 min at 7000 rpm. The supernatant was collected, and the absorbance was detected using UV–Vis Spectrophotometer at 540 nm. The percentage of hemolysis was calculated using the following equation.

$$\text{Hemolysis (\%)} = \frac{\text{Abs samples} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100\%$$

In this evaluation, PBS (7.4) and water were used as positive and negative control, respectively.

2.17. Formulation of SLNs Loaded Dry Floating Gel In-Situ

Prior to the preparations, the concentrated SLNs were initially freeze dried with the addition of 2.5% w/v trehalose as a cryoprotectant. Dry floating gel in situ was prepared using the composition depicted in Table 3. All formulations contained lyophilized SLNs, gellan gum, Pluronic F127, sodium citrate, calcium carbonate and microcrystalline cellulose. All the components were mixed using dry blender and further evaluated for their characterizations.

Table 3. Formulation chart of SLNs loaded dry floating in-situ gel system (%w/w)

Composition	FF1	FF2	FF3	FF4	FF5
SLNs	25	25	25	25	25
Gellan gum	5	5	5	5	5
Pluronic F127	2.5	5	7.5	10	15
Sodium citrate	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	61	56	51	46	41

2.18. Angle of Repose Determination

The assessment of angle of repose of the dry formulation was carried out using funnel method [32]. The funnel was initially fixed on a stand. Briefly, the powder was placed in the powder funnel until all the powder passed the funnel. Afterwards, the diameter of the height of the dry formulation under the funnel were measured. The angle of repose was calculated using the following calculation:

$$\text{Angle of repose} = \tan^{-1} \frac{\text{Height}}{\text{Diameter}} \times 2$$

2.19. In Vitro Gelation Study

The gelation properties of the formulations were carried out by dispersing the dry powder into distilled water in a ratio 1:5. Following this, the dispersion (5 mL) was carefully added into

FaSSGF media (100 mL) inside the glass Beaker under the stirring at 50 rpm at 37 ± 0.5 °C. The time required by the liquid formulation to form a gel was observed and recorded as gelation time [51].

2.20. In Vitro Floating Study

The formulation was evaluated for the floating study using USP Dissolution Apparatus. Firstly, the dispersion of the formulation (10 mL) was put into opened Petri dish. Afterwards, the dish was put into FaSSGF media (100 mL) inside the dissolution vessel at 37 ± 0.5 °C. The floating lag time was recorded by observing the time required by the gel formed to float on the surface. Furthermore, the floating duration time was also recorded by observing the time required by the gel to stop floating [51].

2.21. Measurement of Viscosity and Gel Strength

Prior to the evaluations, the formulation was dispersed in 100 mL, and 10 mL of the formulation was placed in the 100 mL of FaSSGF media, forming gel structure. The viscosity of the formulation was assessed using a rheometer (AR-2000ex rheometer, TA Instruments, UK). The determination was conducted using the oscillatory method with 0.5 mm trim gap and parallel plate of 50 mm. Initially, the formulation was placed into the bottom part of the instrument and the sample shearing was decreased using the trim gap. The determination was carried out at 25 °C at 1 Hz frequency. Furthermore, to assess the gel strength, the frequency sweep was carried out using 1% strain at 37 °C. The analysis was performed between 0.05 and 10 Hz of frequency. The gel strength was indicated by the value of the ratio of the modulus of storage (G') and the modulus of loss (G'') (G'/G'' value) [52,53].

2.22. Preparation of dissolution media

Two different type of dissolution media were used, namely FaSSGF (Fasted-State Simulated Gastric Fluid) and FeSSGF (Fed-State Simulated Gastric Fluid). The preparation of FaSSGF was carried out by solubilizing 1.999 g of NaCl in 1000 mL of purified water. The pH of the solution was adjusted to 1.5 using HCl 1 M [12]. Meanwhile, the preparation of FeSSGF was carried out by mixing skimmed milk and acetate buffer pH 5.0 at a ratio of 1:1. To mimic the pH of fed state in the gastric, the pH of the mixture was adjusted to pH 5 using 1 M HCl [54].

2.23. In Vitro Drug Dissolution Study

The dissolution study of both compounds in safflower extract from the formulation was carried out using USP Dissolution Apparatus II (paddle). Initially, the dissolution vessel was filled with 900 mL of FaSSGF. Then, following the dispersion of the dry formulation in water, the formulation (10 mL) was put into dissolution media. At predetermined interval times, 5 mL of the media was taken, replaced with the fresh media and the amount of both compounds was analyzed using HPLC. The study was carried out at 50 rpm at 37 ± 0.5 °C. Additionally, the dissolution of both compounds in three different conditions was performed, as previously reported in our study, namely the FeSSGF and FeSSGF after being released in 2 h in FaSSGF [37]. The release profiles of both compounds from crude extract-loaded dry floating gel in situ and non-decorated SLNs were also investigated.

2.24. Statistical analysis

The statistical analysis of all results was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Statistically different was denoted when $p < 0.05$. Where appropriate, an independent and an unpaired *t*-test was used to compare two groups. Specifically, The Kruskal-Wallis test with post-hoc Dunn's test was applied to compare data from multiple groups.

3. Results and Discussion

3.1. Extraction Yield, TFC and TPC of Safflower Extract

Safflower has been found to show strong antioxidant properties against free radicals. This is related to the rich flavonoid and phenolic compounds in safflower [17,19,20]. It has been previously reported that both compounds and their glycoside forms can protect the damage caused by an oxidative reaction from free radicals [16–20]. In our study, initially, we extracted safflower using different solvents, mainly containing ethanol and water. The yield of the extraction, TFC, TPC, and antioxidant properties of all extract are depicted in Figure 1. It was found that the high concentration of ethanol was able to increase the extraction yield, TFC and TPC. The highest values were achieved by the use of Et100 as extraction solvent. The extraction yield, TFC and TPC of Et100 were found to be $23.12 \pm 1.87\%$ w/w, 187.37 ± 13.87 mg/g extract (equal to quercetin)

and 198.43 ± 12.87 mg/g extract (equal to gallic acid), respectively. The difference in solvent type could significantly affect the extract yield, TFC and TPC ($p < 0.05$). Accordingly, the bioactive compounds, including flavonoid and phenolic contained in safflower possessed low solubility in water which could further limit its application.

3.2. Antioxidant Activity using DPPH

Furthermore, as the main purpose of this study was related to antioxidant properties, the antioxidant activities of all extract were assessed. Antioxidant could have various benefits in medical applications, particularly the condition triggered by oxidative stress from free radical. In our study, the antioxidant activities of safflower extract were in good agreement with the results from TFC and TPC determination. Using DPPH as free radical, in comparison with other extracts, Et100 possessing the highest TFC and TPC showed the lowest IC₅₀ value, implying its strongest antioxidant behavior. This could be explicated that both flavonoid and phenolic compounds in safflower could act as antioxidant agents. The IC₅₀ values of Et100, Et75, Et50, Et25 and Wat are depicted in Figure 1D, showing the values of 36.87 ± 2.81 μ g/mL, 52.91 ± 4.69 μ g/mL, 81.98 ± 7.53 μ g/mL, 378.28 ± 21.17 μ g/mL and 698.43 ± 54.93 μ g/mL, respectively. Analyzed statistically, there was a statistical difference in IC₅₀ values of all extracts ($p < 0.05$).

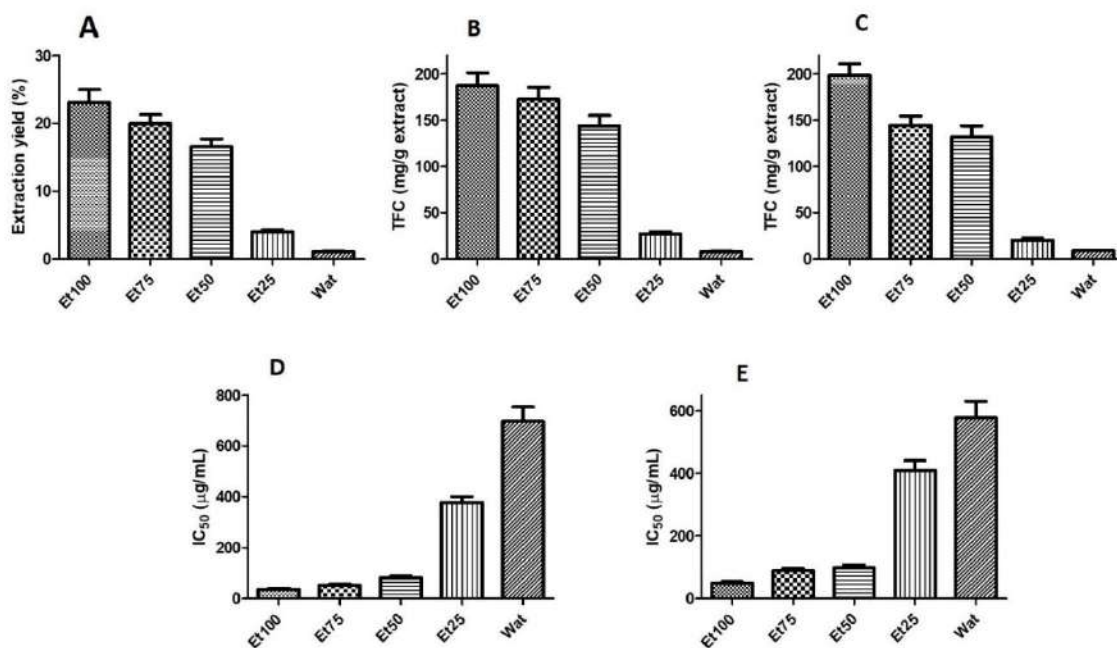


Figure 1. Extraction yield (A), TFC (B), TPC (C), IC₅₀ values against DPPH (D) and IC₅₀ values against lipid peroxidation (E) of safflower extract obtained from different solvents (mean $\pm$ SD, n=3)

3.3. Antioxidant Activity Using Lipid Peroxidation

The antioxidant properties of all extracts were also investigated using the lipid peroxidation method. As lipids are the major component of the cell membrane, this method represents the ability of the compounds to protect the damage of the cell membrane caused produced by peroxy radicals [43]. In this evaluation, it was found that the IC₅₀ of Et100, Et75, Et50, Et25 and Wat against lipid peroxidation were 48.75 ± 3.42 μ g/mL, 89.04 ± 6.56 μ g/mL, 98.97 ± 8.91 μ g/mL, 409.51 ± 31.67 μ g/mL and 577.43 ± 43.87 μ g/mL, respectively (Figure 1E). Similar to the DPPH test, the difference in solvent type significantly influenced the IC₅₀ values in lipid peroxidation method ($p < 0.05$).

3.4. HPLC Analysis of Active Compounds

The investigation was intended to encapsulate the safflower extract into SLNs delivery system for further incorporation into floating in situ system. It was important to analyze the interest compound in safflower extract. In this study, quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were used as the interest compounds, analyzed using HPLC. The chromatogram of both compounds and the concentration in all extracts are depicted in Figure 2A. The concentrations of quercetin 7-O-beta-D-glucopyranoside in safflower extract were 7.65 ± 0.63 mg/g for Et100, 4.32 ± 0.32 mg/g for Et75, 2.97 ± 0.19 mg/g for Et50, 0.74 ± 0.05 mg/g for Et25 and 0.02 ± 0.001 mg/g for Wat. Moreover, the concentrations of luteolin 7-O-beta-D-glucopyranoside of Et100, Et75, Et50, Et25 and Wat were 2.32 ± 0.21 mg/g, 1.92 ± 0.15 mg/g, 0.67 ± 0.05 mg/g, 0.29 ± 0.03 mg/g and 0.01 ± 0.001 mg/g, respectively. From these results, it was shown that the concentrations of both compounds were very low in Wat (Figure 2B), due to the non-polar properties. Importantly, Et100 produced the highest concentrations of both interest compounds. Taking into consideration of the results from extraction yield, TFC, TPC and antioxidant activity evaluations, this extract was used for further experiments.

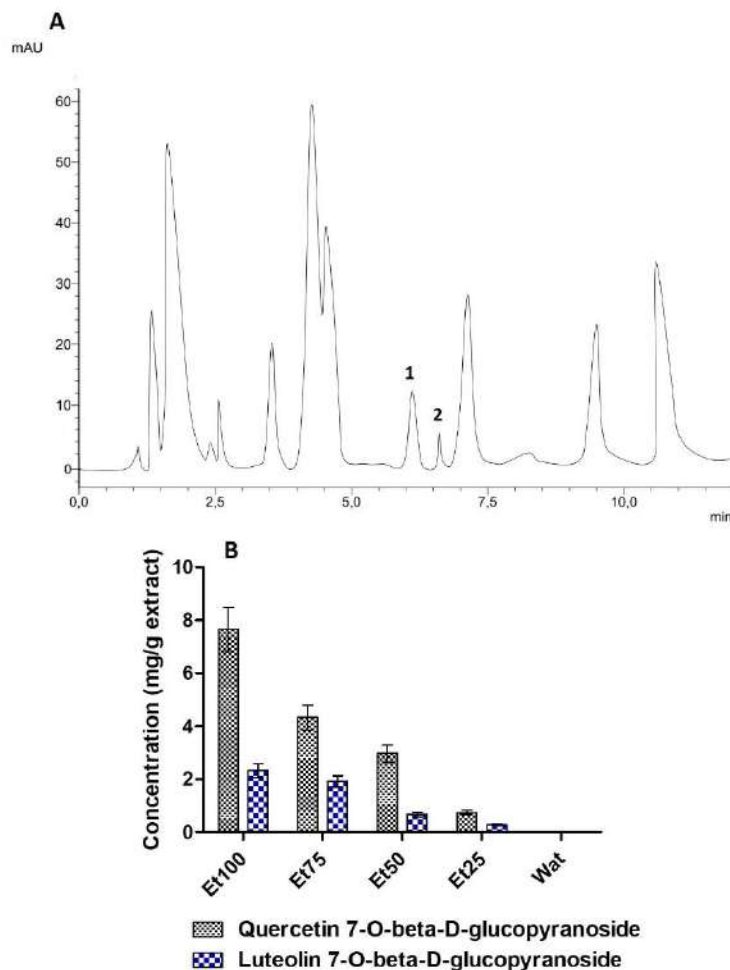


Figure 2. HPLC chromatogram of quercetin 7-O-beta-D-glucopyranoside (1) and luteolin 7-O-beta-D-glucopyranoside (2) in safflower extract (A). Concentration of both compounds safflower extract obtained from different solvents (B) (mean $\pm$ SD, n=3)

3.5. Solid Lipid Nanoparticles Preparation and Characterizations

In an attempt to enhance the solubility and bioavailability of the compounds in safflower extract, it was incorporated into SLNs formulation decorated with HP β CD. In this study, Geleol® was selected as lipid matrix. In our preliminary study (data not shown), we previously screened different type of lipids, namely, Compritol® 888 ATO, Precirol® ATO 5 and stearic acid. According to the particle size and encapsulation efficiency values, other lipids were not able to produce desire particle sizes and encapsulation efficiencies. With respect to the size, compared to other lipids, Geleol®, produce the smallest particle sizes. This might be due to the lowest melting point of Geleol®, causing the low viscosity in the solvent and when mixed with stabilizer solution.

In our preliminary study, we found that the viscosity of the dispersion of Compritol® 888 ATO, Precirol® ATO 5 and stearic acid were 198.54 mPa.s, 243.18 mPa.s and 319 mPa.s, respectively. The low viscosity could improve the sonication effectiveness, resulting in smaller particle sizes compared to other lipids [43]. Regarding the encapsulation efficiency, the higher encapsulation of SLNs prepared from Geleol® might be caused by the ability of hydroxyl group of glyceryls as hydrogen bond donor functional groups contained in Geleol® to form hydrogen bonds with hydrogen bond receiver functional groups of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside. This formation could generate a molecular complex, resulting in improved solubilization of both compounds in Geleol® [55]. Therefore, this increased the encapsulation efficiency of both compounds in lipid matrix.

In this study, several parameters were evaluated to achieve SLNs with desired characteristics. The particle size, PDI, and zeta potential values of all formulations are shown in Figure 3A-C. Moreover, the encapsulation efficiencies of quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside are depicted in Figure 3D. Initially, we evaluated the effect of lipid matrix concentration. It was found that the increment of lipid concentration could increase the particle size and encapsulation efficiency. However, the increase of lipid from 200 mg (F3) to 250 mg (F4) did not improve the encapsulation efficiency ($p > 0.05$) while increasing the particle size significantly ($p < 0.05$). The effect of different type of stabilizers, namely Tween80 and PVA, was also observed. The results showed that Tween80 could produce smaller particle size and higher encapsulation efficiency compared to PVA. It might be due to the difference in HLB values. It has been previously described that the HLB values of between 12 and 16 could form a stable oil-in-water (O/W) emulsion, leading to smaller droplet of nanoparticles formed [56]. As the HLB of Tween80 and PVA were > 24 and 15 , respectively, SLNs prepared Tween80 were smaller in size. The effect of sonication time was also studied, showing that the increase of sonication time could reduce the particle size of nanoparticles. However, this could reduce the encapsulation efficiency and drug loading of both compound in SLNs matrix. This might be caused by the increase of the small droplet number and the separation of the generation of the core-shell of the nanoparticles. This could result in escape of the encapsulated compounds during the emulsification process, decreasing the encapsulation efficiency and drug loading of both compounds in lipid matrix [43]. Additionally, it was found that all SLNs produced zeta potential of around -30 mV, indicating the high stability of SLNs. The negative charge of SLNs might be

due to the anionic nature of the lipid [21]. Following the optimization process, F3 prepared from 200 mg of Geleol® and 100 mg of extract, stabilized with 1% of Tween80 and sonicated for 10 minutes was selected as the optimum formulations. The SLN possessed particle size, PDI, and zeta potential of 178.97 ± 16.57 nm, 0.102 ± 0.01 , -31.24 ± 2.87 mV, respectively. The encapsulation efficiency values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $87.31 \pm 7.65\%$ and $91.36 \pm 8.61\%$. Moreover, the drug loading values of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside were found to be $30.65 \pm 2.19\%$ and $31.34 \pm 2.86\%$. This formulation was further decorated using HP β CD. As previously explained, was used to decorate the SLNs to improve the stability especially in the gastrointestinal tract [26,29]. Using similar preparation process, the decorated SLNs possessed particle size of 187.36 ± 12.02 nm, PDI of 0.114 ± 0.01 and zeta potential of -30.25 ± 2.14 mV. The encapsulation efficiencies were $86.98 \pm 7.12\%$ for Quercetin 7-O-beta-D-glucopyranoside and $90.78 \pm 7.83\%$ for Luteolin 7-O-beta-D-glucopyranoside. Furthermore, the drug loading capacities were $29.54 \pm 2.16\%$ for Quercetin 7-O-beta-D-glucopyranoside and $29.88 \pm 2.54\%$ for Luteolin 7-O-beta-D-glucopyranoside. It was found that following the incorporation of HP β CD, importantly, the properties of SLNs did not change significantly ($p > 0.05$).

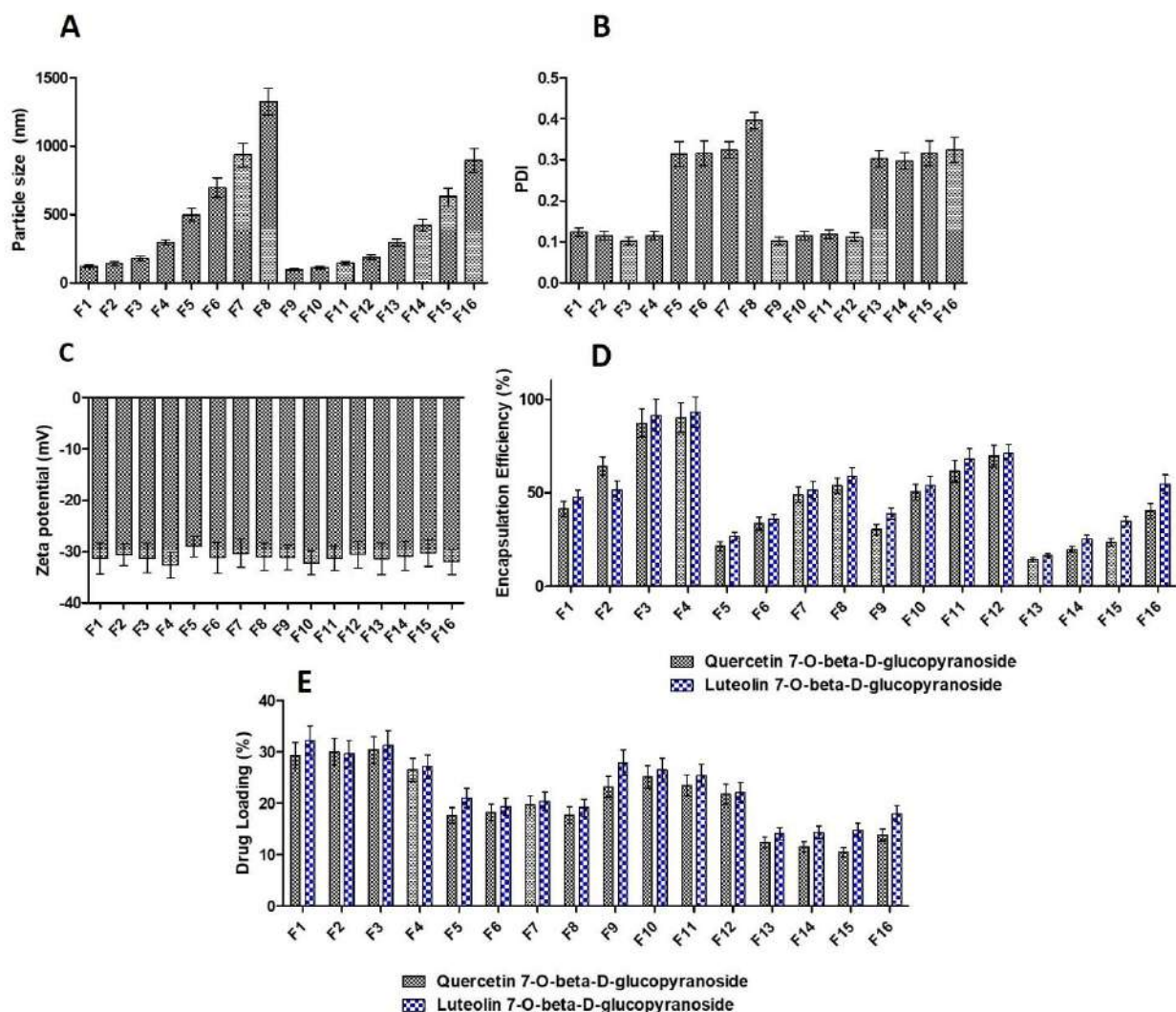


Figure 3. Particle size (A), PDI (B), zeta potential (C), encapsulation efficiency (D) and drug loading (E) results of SLNs prepared from safflower extract (mean $\pm$ SD, n=3)

3.5. FTIR study

FTIR study was conducted to investigate the possible interaction between interest compounds and all the excipients used in the formulation. As shown in Figure 4A, safflower extract showed at 3239 cm^{-1} due to the vibration of -OH functional groups of the phenolic parts of both compounds. Additionally, another peak was detected at 1684 cm^{-1} which might be due to the presence of C=O stretching of the ketone carbonyl. Distinct peak was observed at 1614 cm^{-1} due to -C-O stretching of quercetin 7-O-beta-D-glucopyranoside and central heterocyclic ring of luteolin 7-O-beta-D-glucopyranoside. Other peaks were found at 1519 cm^{-1} , 1363 cm^{-1} and 1237

cm⁻¹ due to C-C=C asymmetric stretch, C-OH stretch and C-O-C bond, respectively. All the peaks were also detected in both physical mixture and SLN formulations. Interestingly, the peak at 3239 cm⁻¹ was found to be broader, which might be due to the formation of hydrogen bonding between lipid matrix and interest compounds. It should be noted that the spectrums observed in the extract between 1100-1200 cm⁻¹ were not identified in the SLNs following the purification step. This indicated that the method could remove unencapsulated compounds, while maintaining the presence of the interest compounds inside the lipid matrix.

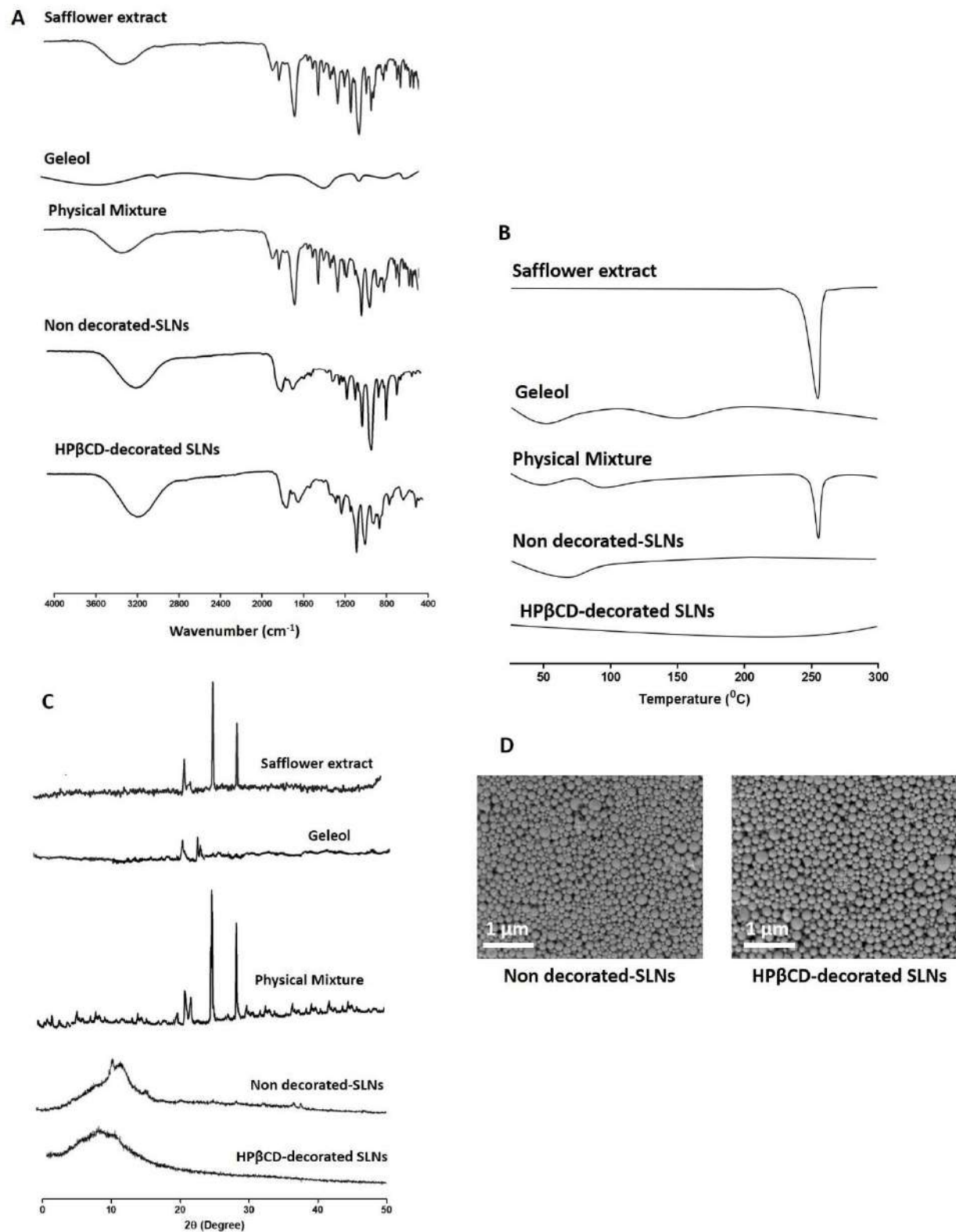


Figure 4. The results of FTIR (A), DSC (B), XRD (C) and SEM (D) evaluations

3.6. DSC analysis

Figure 4B shows the DSC thermograms of safflower extract, the physical mixture, and both SLN formulations. As shown in the thermogram, safflower extract showed a sharp peak at 252°C due to the melting point of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside which also showed the crystalline form of the compounds. In the case of Geleol, broad peak was observed at 61°C, representing the melting point of the lipid. Following the observation in the physical mixture, the peaks were still identified, showing that all compounds did not change when mixed physically. In the SLN formulations, the sharp peak was not observed, showing the formation of the crystalline compounds to amorph. Additionally, this could also show that all compounds were successfully entrapped inside the lipid matrix [21]. The transformation of the crystalline form to amorphous could be beneficial in the enhancement of the solubility of the hydrophobic compounds.

3.7. XRD analysis

In good agreement with DSC thermograms, XRD evaluations showed distinguishable peaks around 20-35 °2θ regions (Figure 4C), showing the crystallinity of the compounds. Furthermore, these peaks were not found in SLN formulations, indicating the amorphous state of the system developed in this study. In the XRD pattern of Geleol, the small peaks were observed between 20-25°, demonstrating the low crystallinity of the lipid. The XRD analysis data was in a good agreement with the DSC analysis, showing that the presence of the peaks in the physical mixture and the absence of the peaks in the SLNs, showing the formation of amorphous SLNs.

3.8. Solubility analysis

The formulation of hydrophobic compounds into SLNs has been widely reported to improve the solubility of the compounds. Table 4 showed the comparison of the inherent solubility values in water and n-octanol of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in safflower extract and SLN formulations. As depicted, due to their hydrophobicity, the water solubility values of both compounds were $18.23 \pm 1.32 \mu\text{g/mL}$ and $16.54 \pm 1.36 \mu\text{g/mL}$ for quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside, respectively. On the other hand, significant higher ($p < 0.05$) solubility values were found in n-octanol, namely $408.45 \pm 29.34 \mu\text{g/mL}$ and $434.31 \pm 39.31 \mu\text{g/mL}$. Following the

incorporation into SLNs, the water solubility increased significantly ($p < 0.05$) up to approximately 15-fold for both compounds. Furthermore, the solubilities of these compounds were also enhanced in n-octanol, despite non-significant ($p > 0.05$) increment. Furthermore, following the decoration using HP β CD, due to the solubilization enhancement ability of HP β CD [57], the solubility of both compounds in decorated SLNs was higher compared to non-decorated SLNS. However, there was no significant different ($p > 0.05$) in the solubility values.

Table 4. The solubilities of interest compounds in water and n-octanol from extract, SLNs and HP β CD-SLNs (mean $\pm$ SD, n=3)

Compound		Samples	Aqueous solubility ($\mu\text{g/mL}$)	n-octanol solubility ($\mu\text{g/mL}$)
Quercetin 7-O-beta-D-glucopyranoside		Extract	18.32 ± 1.32	408.45 ± 29.34
		SLNs	278.43 ± 20.31	411.23 ± 31.72
		HP β CD-SLNs	301.21 ± 26.32	415.87 ± 33.67
Luteolin 7-O-beta-D-glucopyranoside		Extract	16.54 ± 1.36	434.73 ± 39.42
		SLNs	201.23 ± 17.44	454.31 ± 40.38
		HP β CD-SLNs	243.12 ± 21.27	461.21 ± 38.17

This evaluation confirmed that the formulation of SLNs was able to enhance the solubility of hydrophobic compounds in safflower extract. Numerous studies have shown that the reduction of the size in nanoparticles could potentially enhance the solubility of hydrophobic drugs in many cases [58–62].

3.9. In vitro drug release and mathematic models

In this study, dynamic dialysis using dialysis membrane was applied to evaluate the release of both compounds from SLNs. Figure 5 depicts the cumulative release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs and HP β CD-decorated SLNs. Overall, it was found that the incorporation of safflower extract into SLNs could improve and sustain the release patterns of both compounds. For Quercetin 7-O-beta-D-glucopyranoside, only $0.43 \pm 0.03\%$ of compound were released from extract after 3 h. No compound was detected in the first 2 h. After 24 h, the release percentage was found to be $20.35 \pm 1.87\%$. In the SLN formulations, after 3 h, $10.69 \pm 1.19\%$ and $12.98 \pm 1.21\%$ of compound were released from non-decorated SLNs and HP β CD-decorated SLNs, respectively. The releases were

sustained over 24 h, reaching the final release percentages of $88.63 \pm 7.87\%$ and $93.76 \pm 8.18\%$, respectively. In terms of the release profile of luteolin 7-O-beta-D-glucopyranoside, the release was found after 4 h from the extract, which was only $0.65 \pm 0.05\%$. In case of SLN formulations, the release percentages were $11.36 \pm 1.27\%$ for non-decorated SLNs and $14.32 \pm 1.29\%$ for HP β CD-decorated SLNs. Similar to another compound, sustained release behavior was also observed after 24 h, showing that $81.06 \pm 7.65\%$ and $88.69 \pm 8.01\%$ of the compound were released from both SLNs, respectively. Specifically, it was observed that during the first 3 h, there was a fast release pattern of both compounds from SLNs. This might be caused by the low percentage of compounds in the outer layer of lipid matrix, leading to the quick diffusion of both compounds into the release medium [63]. Following this, the slow-release pattern was observed. In an attempt to understand the release kinetic and mechanism of both compounds from SLNs matrix, the release profiles were fitted into release kinetic models. It was found that the release kinetic of both compounds followed Higuchi model with R values of > 0.98 . Therefore, it could be explained that both compounds released from lipids due to the diffusion and the erosion process [21,49].

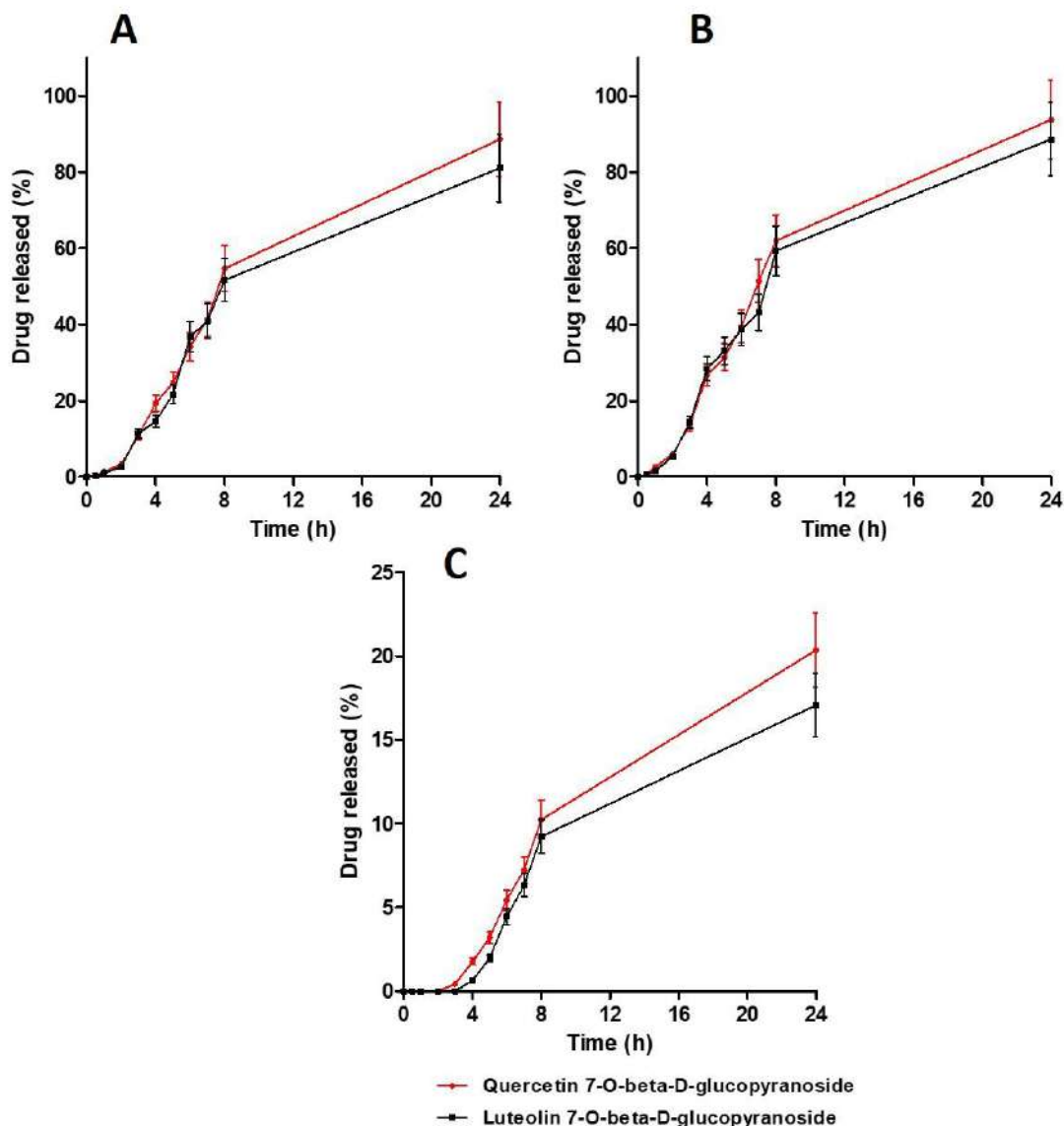


Figure 5. In vitro release profiles of of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from non-decorated SLNs (A), HPβCD-decorated SLNs (B) and extracts (C) (mean $\pm$ SD, n=3).

3.10. Stability evaluation in gastric environment

In order to investigate the stability of SLNs in the gastrointestinal tract, the characteristics of non-decorated SLNs were compared to HPβCD-decorated SLNs. The particle sizes, PDIs, zeta potentials of both formulations during the incubation in FaSGGF media are depicted in Table 5. It was shown that without the addition of HPβCD, there were significant improvements ($p < 0.05$)

of the particle size during 8 h. On the other hand, following the decoration using HP β CD, the particle size did not change significantly ($p > 0.05$), indicating the improvement of the stability of SLNs following the decoration using HP β CD.

Table 5. The particle size (nm) of non-decorated SLNs and HP β CD-decorated SLNs following several incubation times in FaSGGF (mean $\pm$ SD, n=3)

Samples	0 h	2 h	4 h	6 h	8 h
SLNs	178.97 $\pm$ 16.57	193.83 $\pm$ 12.12	234.56 $\pm$ 20.18	382.28 $\pm$ 21.82	476.12 $\pm$ 38.89
HP β CD-SLNs	187.36 $\pm$ 12.02	189.32 $\pm$ 13.71	190.31 $\pm$ 12.73	191.27 $\pm$ 17.29	204.87 $\pm$ 15.34

3.11. Hemolytic evaluation

Initial potential toxicity of nanoformulations could be assessed using in vitro hemolytic assay. This study was performed to investigate the ability of SLNs to lyse erythrocytes, indicating the toxicity. The results of this evaluation are depicted in Figure 6 As shown, the hemolysis percentage values of both SLNs were below 5%. Accordingly, it can be hypothesized that the SLNs developed in this study are considerably safe at the evaluated concentrations [47,48].

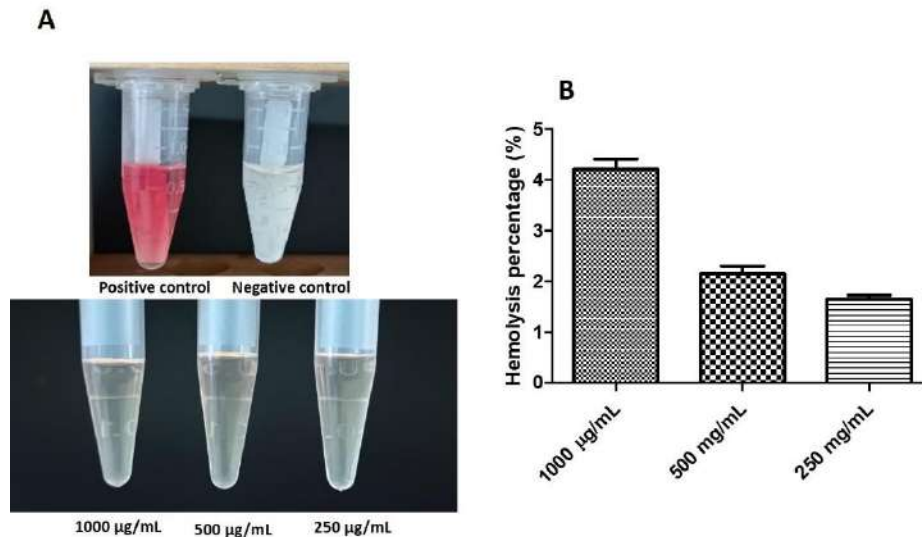


Figure 6. Representative images of in vitro hemolysis evaluation (A) and hemolysis percentages of decorated SLNs in three different concentrations (B) (mean $\pm$ SD, n=3).

3.12. Formulation of SLNs Loaded Dry Floating Gel In-Situ

In this study, we incorporated HP β CD-decorated SLNs into dry floating in situ gel using gellan gum. The dry formulation should be initially dispersed in water before the oral administration. The principle of in situ gelation formation is due to the reaction of gellan gum and divalent ions. Categorized as anionic polysaccharide, gellan gum solution forms gel in the existence of Ca²⁺ [32]. In this formulation, following contact with the acidic condition in the gastric juice, calcium carbonate would be solubilized and release Ca²⁺ which are further complexed by sodium citrate [37]. Additionally, the release of free Ca²⁺ from the complex has been found to be slow, resulting in gelation of gellan gum. Additionally, following the release of Ca²⁺ from calcium carbonate, carbon dioxide (CO₂) would be released and entrapped inside the gel network, decreasing the density of the system and causing the floating system in the gastric environment [64]. Additionally, Pluronic F127 was also used as thermogelling system which would also control the release of the compounds [65,66]. As a diluent of the dry formulation, microcrystalline cellulose was used, due to its ability to enhance the flow property of the system.

3.13. Angle of repose determination

As the main form of the system developed in this study was dry powder, it was crucial to evaluate its flowability. Here, repose angle was assessed to represent the flowability of the powder. As shown in Figure 7A, all repose angles were above 25°, indicating the excellent flowability of dry powder formulation. Specifically, the angle of repose values was 35.76 ± 2.98 , 35.09 ± 3.02 , 33.82 ± 3.21 , 33.12 ± 2.78 and 32.19 ± 3.29 for FF1, FF2, FF3, FF4 and FF5, respectively. It was important to note that there was no significant difference ($p > 0.05$) in the values of the repose angles of all formulations.

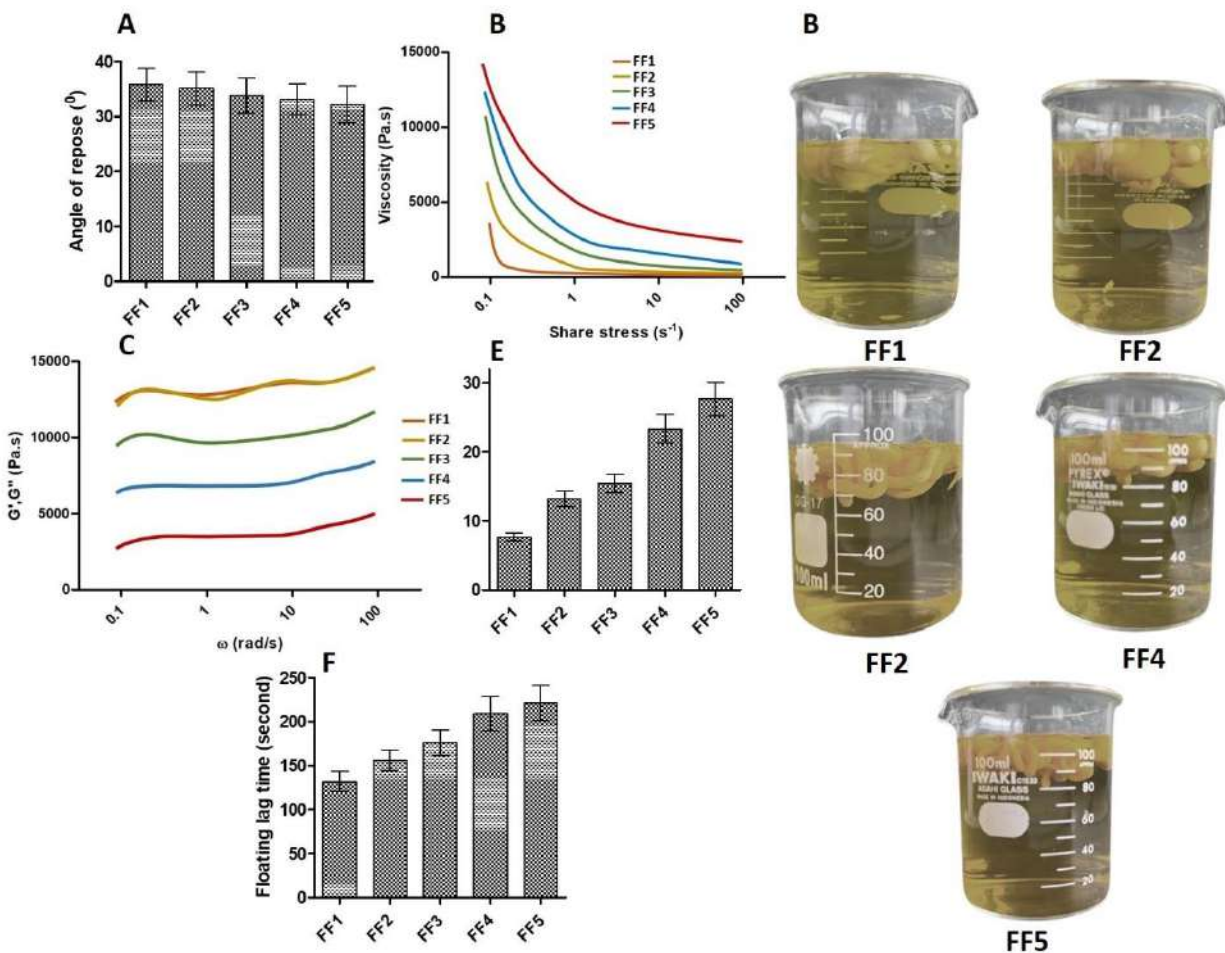


Figure 7. Angle of repose (A), viscosity (B), gel strength (C), gelation time (D) and floating lag time (E) of SLNs loaded floating gel in situ (mean $\pm$ SD, $n=3$). Representative images of floating gel in situ (F).

3.14. Viscosity and gel strength determination

Furthermore, the viscosity of the pre-gelation dispersion was determined. The result is shown in Figure 7B. The rheogram showed that all the formulations exhibited non-Newtonian behavior. Analyzed statistically, there was a significant difference ($p < 0.05$) in the viscosity value in all formulations. The difference in viscosity was caused by the use of Pluronic F127 as thermogelling agent. Several studies have explored the use of Pluronic F127 as gelling agent, causing the increase in viscosity with the increase of the concentrations [65,66].

The strength values of the gel formulation are shown in Figure 7C. The value of $G'G''$ represented the strength of the gels. It was found that the increase of Pluronic F127 concentration

from 2.5% to 10% could significantly improve ($p < 0.05$) the gel strength of the formulation. However, the increase from 10% to 15% ($p > 0.05$) did show any significant improvement in the gel strength. This was in a good agreement with the viscosity determination, showing that the higher viscosity resulted in stronger gel strength. It was necessary to maintain rigid gel assembly in floating gel formulation. The drug loaded into the gel matrix would release to gastric environment depending on the strength of the complex 3D structure of the gel network [64]. Accordingly, the gel should be strong enough to maintain its integrity. The strength values of the gel were similar to the values reported previously, showing the ability of the formulation in maintaining the gel form in the gastric environment.

3.15. Gelation time

Gelation time is one of critical parameters in in situ gel formulation. In order to control the release of drugs, the formulation should be able to form gel before the release of the drugs from the gel matrix. Figure 7D exhibits the time required by the formulation to form a gel structure, showing that all formulation were able to form a gel in FaSGGF media at body temperature less than 30 seconds. As explained in previous section, gellan gum could form gel following the presence of Ca^{2+} release from calcium carbonate. Importantly, it was found that the concentration of Pluronic F127 could affect the gelation time of the formulation. The increase in Pluronic concentration ($p < 0.05$) could significantly decrease the gelation time of the formulation. Pluronics are water soluble nonionic triblock copolymers with polar and non-polar region, namely polyethylene oxide and poly propylene oxide, respectively. This polymer can undergo sol-to-gel conversion following the change of the temperature [49].

3.16. Floating lag time and duration of floating

Following the gel formation of the in situ formulation, to further sustain the release based on its gastroretentive characteristics, the formulation should be able to float in the gastric environment. Floating lag time represent this parameter and Figure 7E depicts the floating lag times of the formulations. Following the concentration of Pluronics, the time required of the gelled formulation to float also increased. This might be associated with the increase of the viscosity of the formulation when the concentration of Pluronics increased. As a result, the penetration of the gastric fluid would decrease, retarding the reaction between the calcium carbonate with acid which

affected the release of CO₂ [67]. A previous study has shown the same phenomenon showing that the floating lag time increased following the increase of the polymeric material in the system [68]. It should be noted that all formulations remained floating after 24 h (Figure 7F).

3.17. In Vitro Drug Dissolution Study

The dissolution profile of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside from SLNs incorporated into floating in situ formulation was investigated in three different conditions. To ensure that the formulation could control the dissolution of both compounds, the burst release should be avoided [69]. In this study, initially we evaluated the dissolution profile of Quercetin 7-O-beta-D-glucopyranoside and Luteolin 7-O-beta-D-glucopyranoside in FaSSGF. The dissolution profiles of both compounds in FaSSGF for 24 h are depicted in Figure 8. The results showed that the incorporation of SLNs into floating gel in situ could potentially control the dissolution profile of active compounds from safflower extract. Without the SLNs formulation, the dissolution percentages were found to be significant lower ($p < 0.05$) in all cases. Specifically, the difference in Pluronic concentrations resulted in various control release patterns. As shown in Figure 8A and 8B, below 7.5% of Pluronics (FF1 and FF2), the sustained release patterns were only observed for 6 h. On the other hand, formulations containing 7.5% (FF3), 10% (FF4) and 15% (FF5) of Pluronics were capable of controlling the release of the compounds over 24 h.

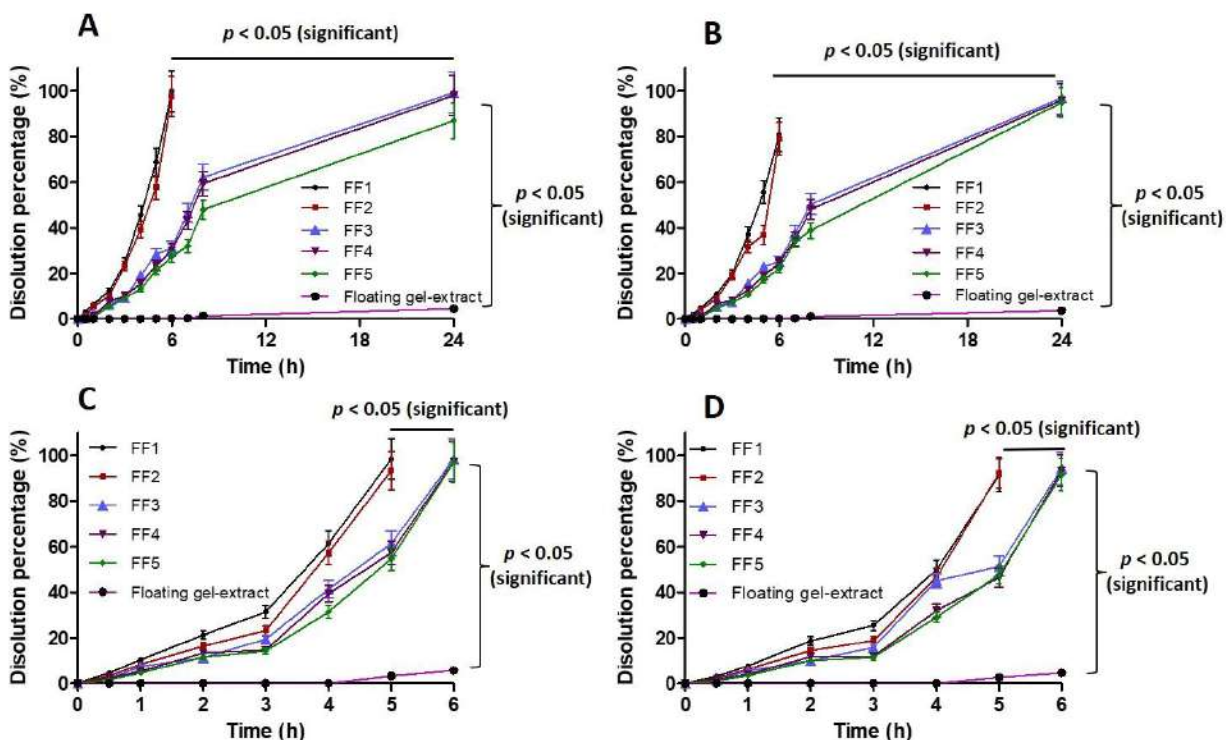


Figure 8. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF, in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (C) and luteolin 7-O-beta-D-glucopyranoside (D) from SLNs loaded floating gel in situ system in FeSGGF (mean $\pm$ SD, $n=3$).

In FeSGGF, all formulations were not able to control the release of active compounds. As shown in Figure 8C and 8D, all compounds were released less than 6 h. It was caused by the inability of gellan gum to form gel in the FeSGGF pH. Accordingly, this confirmed that the formulation should be taken in fast state. However, it was important to evaluate the effect of the release of active compounds from the developed formulations after food intake. In this investigation, only FF3, FF4 and FF5 were selected due to their ability to control the dissolution behavior for 24 h (Figure 9A and 9B). After 2 h in FaSGGF, it was found that F3 with 7.5% of Pluronic were not able to control the dissolution behaviour of both compounds. On the other hand, FF4 and FF5 could potentially maintain and control the release of the active compounds encapsulated into SLNs. This could be related the difference in the gel strengths between the formulations. As the gel strength of FF3 was significantly lower ($p < 0.05$) than FF4 and FF5, the ability to maintain its rigid gel decreased when the formulation was moved into FaSGGF.

Importantly, in this study, we also evaluated the dissolution profile of both compounds in the floating gel from the non-decorated SLNs. As shown in Figure 9C the non-decorated SLNs were not able to control the release of both compounds. From 4 h onwards, there was no significant improvement of the dissolution percentages of both compounds. This could be the aggregation of the non-decorated SLNs following 4 h contact with the gastric environment (Table 5). Accordingly, this showed that it is crucial to decorate the SLNs using HP β CD to maintain the stability during the oral administration of this approach.

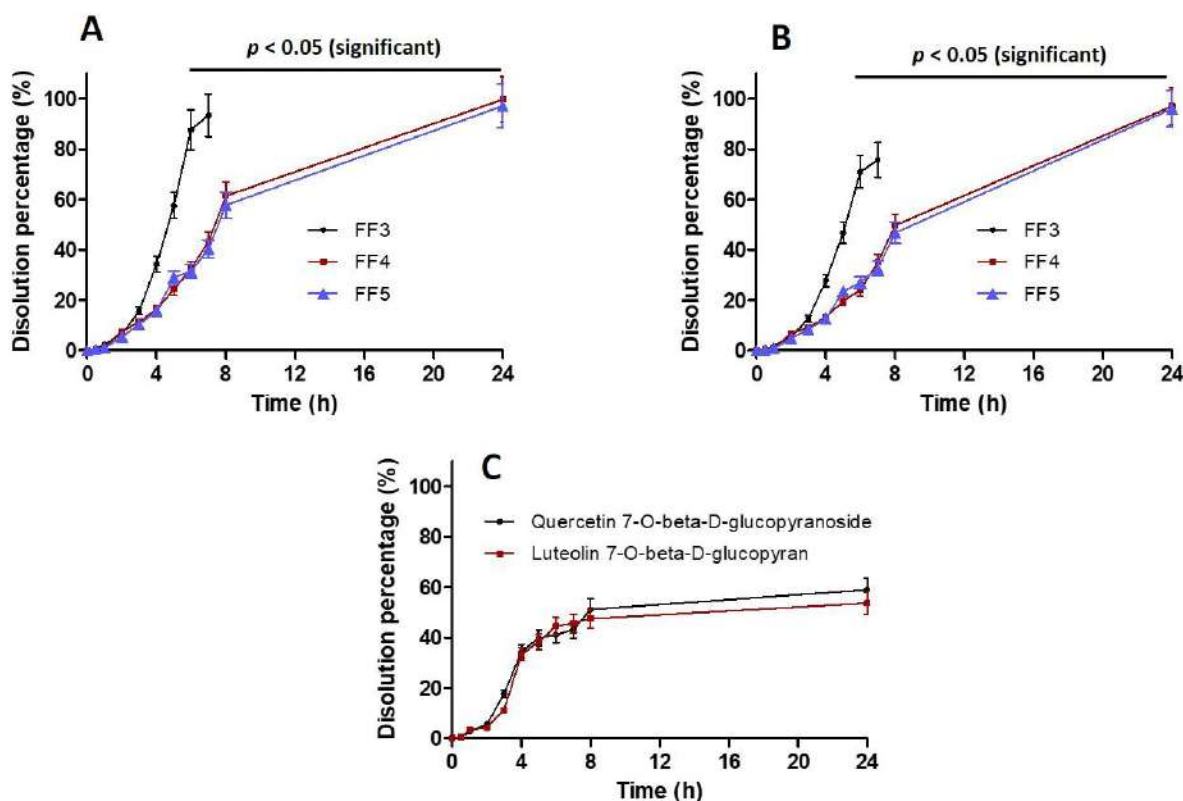


Figure 9. In vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside (A) and luteolin 7-O-beta-D-glucopyranoside (B) from SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h; in vitro dissolution profiles of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside (C) from non-decorated SLNs loaded floating gel in situ system in FaSGGF after being released in FeSGGF for 2 h (mean $\pm$ SD, n=3).

Overall, this study showed the possibility to improve the solubility of bioactive compounds in safflower using SLNs and further control the release using floating gel in situ system. As the data presented in our study is a proof of concept study in the biorelevant media, to further evaluate

its efficacy, in vivo studies should now be conducted using appropriate animal model. The pharmacokinetic study is critical to investigate the effectiveness of this approach to improve the bioavailability of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in Safflower extract.

4. Conclusions

In this study, safflower extract with antioxidant activity was obtained using ethanol as extraction solvent. The extract showed antioxidant activity, expressed as IC₅₀ values of 36.87 ± 2.81 µg/mL against DPPH and 48.75 ± 4.52 µg/mL against lipid peroxidation. Specifically, the extract contained 7.65 ± 0.63 mg/g extract of quercetin 7-O-beta-D-glucopyranoside and 2.32 ± 0.21 mg/g extract of luteolin 7-O-beta-D-glucopyranoside. To improve the solubility, the extract was formulated into solid lipid nanoparticles (SLNs) decorated with hydroxypropyl beta-cyclodextrin (HPβCD) with particle size of 178.97 ± 16.57 nm, showing the improvement of 15-fold of solubility in water. The SLNs possessed gastric solubility with no particle size improvement after 8 h. The SLNs were further formulated into floating gel in situ delivery system using gellan gum as gelling agent. With desired gelation and floating properties, the formulation could control the release of quercetin 7-O-beta-D-glucopyranoside and luteolin 7-O-beta-D-glucopyranoside in biorelevant media. Furthermore, in vivo studies should be carried to explore the effectiveness of this approach in a suitable animal model.

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