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Submission date: 21-Jul-2022 08:08AM (UTC+0700)
Submission ID: 1873196819
File name: 43_RJPT_15_1_2022.pdf (212.47K)
Word count: 5444
Character count: 30558

ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Qualitative Phytochemical Screening and Effectiveness Analysis of *Batissa violacea celebensis* Martens 1897 Crude extract against Antioxidant and Cytotoxic Activity

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ABSTRACT:

The aim of this study was to conduct phytochemical tests aimed at identifying the bioactive components present in the powder crude extract of pokea shell can be applied in analyzing antioxidant activity and testing cytotoxic activity. The study consists of phytochemical tests, including alkaloids, steroids, triterpenoids, saponins, and flavonoids. The antioxidant activity measured by the DPPH method from the crude extract of the pokea shell is still relatively weak but the crude extract of methanol of the boiled and dried pokea shells in toxic category so potentially as a cytotoxic compound. The result of phytochemical test of crude extract of pokea shell meat, is extract of fresh and boiled pokea shell contain flavonoid, saponin, steroid and triterpenoid while extract of dried pokea shell only contain saponin, steroid and triterpenoid. The result of the antioxidant activity test of crude extract of pokea shell shows that the crude extract of dried pokea shell has the highest value of antioxidant activity. The result of the toxicity test of crude extract pokea shell boiled and dried pokea shell have an LC50 value of less than 1000 ppm. These values indicate that the extract of the boiled and dried Pokea shell is showed potential for development as an alternative anticancer agents in the future.

KEYWORDS: Phytochemical, Antioxidant, Cytotoxicity, Pokea shell, Crude extract.

INTRODUCTION:

The pokea (*Batissa violacea celebensis* Martens 1897) is a endemic bivalve species of the Corbiculidae Family found in the Pohara River of Konawe Regency, Southeast Sulawesi[1]. *Batissa violacea* is a freshwater mollusk spread in Southeast Asia and Northern Australia. In Indonesia, the animals are spread in Sumatra, Java, West Papua, and Sulawesi. Particularly in Southeast Sulawesi, the pokea shell B. violacea celebensis martens 1897 is found in the Pohara River at a depth of about 1-9 m at the edge and middle of the river with sand sediments and gravel. Pohara River is a

Received on 18.09.2020 Modified on 01.02.2021
Accepted on 09.06.2021
Research J. Pharm. and Tech 2022; 15(1):263-269.
DOI: 10.52711/0974-360X.2022.00043

watershed of Konaweha which is the source of raw water belonging to PDAM (Local water company) of Kendari City Southeast Sulawesi Province Indonesia. Pokea shells are found in strong current conditions with sandy substrate textures, pokea are commonly found with the clustered spreading type[2].

Batissa violacea celebensis is one type of clam that lives in freshwater. In general, bivalves or better known as shellfish is a group of animals with no vertebrae. Pokea shell is classified by Kingdom: Animalia, Phylum: Molusca, Class: Bivalvia, Order: Eulamellibranchia, Family: Corbiculidae, Genus: Batissa, Species: *Batissa violacea celebensis* (Martens, 1897), and area name: Pokea Shells. Pokea shell is a type of bivalves that lives on the bottom of the water and has two shells that can open and close. The shell on the dorsal part is thick, and the ventral part is thin. The top of the eggplant is called the umbo and is the oldest part of the shell. A circular line around the umbo shows the growth of the shell. The mantle in the pelecypoda is shaped as a thin, wide tissue, covering the whole body located under the shell[3].

Pokea shells are empirically believed to treat various diseases such as jaundice, malaria, asthma, lowering blood pressure and fever. The disease occurs due to infection by foreign materials and microorganisms[1].

When the body is infected with microorganisms, the body responds with macrophage and neutrophil activity mechanisms. In this case, the oxidase and oxygenase enzymes will form various free radical compounds and reactive oxygen compounds, including hypochlorous acid (HOCl) that will attack and destroy viruses and bacteria. Radical compounds are also very dangerous because of the potential to attack the body's cells. If this is not controlled, it will trigger the emergence of various chronic diseases[1].

Natural products isolated from bivalves and gastropods have been used, among others, as antioxidants, antitumors, antivirus, antibacterial, antifungal, anticancer, cytotoxic, and enzyme inhibitors[4,5,6]. Some secondary metabolites of aquatic organisms exhibit pharmacological activity[7]. Marine organisms are potential in terms of their ability to produce secondary metabolites which can be utilized as lead component in drug discovery . The search for marine derived products started 50 years ago and over the past few decades, about 3000 new compounds from various marine sources have been tested and determined so far. Metabolites from ascidians, sponges, mollusks, and sea hare, have been described as potent anti-cancer agent. Among the marine organisms, Ascidians rank second with most promising source of drugs. Ascidians are being utilized as food in various countries like Japan, China. Many anticancer compounds entered clinical trail from ascidian (ET-743, Apilidine, Kahalalide F). It is possible to isolate new molecules from marine organisms that could contribute to a better health, serve as food ingredient and also a new source of new medicine[8]. Research on bioactive components in mollusks, especially bivalves and gastropods that have the potential to be nutraceutical or pharmaceutical has been widely practiced.

In modern life we are constantly exposed free radicals, which can damage any cell in the body with which they come into contact and cause hazardous effects.The free radicals cause a state of increased oxidative stress which is mainly responsible for all the damage done to the body by free radicals. Though the body has its own mechanism to combat oxidative stress, when exposure is more than body capacity, the problem aggravates. The harmful effects of free radicals can be prevented by use of substances called antioxidants. Antioxidants are a group of compounds that neutralise or prevent free radicals or reactive species and avoid cell or tissue damage caused by oxidative potential of free radicals. Many natural substances have been explored for their antioxidant activities. There is still need to evaluate new substances for their antioxidant potential to find better alternative sources for treatment of conditions related to oxidative stress caused by free radicals. DPPH and Hydrogen Peroxide free radical scavenging methods were used for evaluating antioxidant activities. Ascorbic acid was used as standard antioxidant substance for comparative evaluation[9]

The antioxidant compounds in the diet plays an important role as a health protective factor[10]. Scientific evidence suggests that antioxidants reduce the risk of chronic diseases including cancer and heart disease. The main feature of antioxidants is the ability to trap free radicals. Free radicals are molecules having an unpaired electron in the outer orbit. They are not stable molecules and ready to react. Free radicals play an important role in living systems. The beneficial effects of free radicals occur at low or moderate concentrations and those levels are balanced by an efficient antioxidant network in the body[11].

Many synthetic compounds like butylated hydroxytoluene (BHT) and butylated hydroxy anisole (BHA) are commercially obtainable as antioxidants. However, they are not suggested for usage due to the toxicity associated with them. Therefore, many research groups have driven efforts to evaluate the antioxidant properties of natural products[12]. The same was reported that Antioxidant compounds may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation.

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have restricted use in foods as they are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants has greatly increased in the recent years and researchers focus on natural antioxidants[13]. Antioxidant compounds, phenolic acids, polyphenols and flavonoids capture free radicals that inhibit oxidative mechanisms that are the cause of degenerative diseases.

MATERIAL AND METHODS:

Sampling and sample preparation:

This research phase starts from sampling and sample preparation with materials and tools preparation for nutrient content testing and active compound extraction, The extraction method that used in this study is the stratified extraction or maceration method[14] was performed using the methods previously described[1]

Mineral analysis (SNI 01-2891-1992):

Mineral analysis was carried out using the Atomic Absorption Spectrophotometer (SAA) to determine the levels of minerals present in food, was performed using the methods previously described[1].

Phytochemical analysis[15]

Phytochemical analysis was performed to determine the bioactive components found on pokea shells crude extract powder.

Alkaloid:

Several examples were dissolved in a few drops of 2 N sulfuric acid then tested with three alkaloid reagents, that is dragendrof, meyer, and wagner reagents. The test results are positive if the meyer reagent formed yellowish white sediment, with a wagner reagent forming a brown sediment, and with a dragendrof reagent forming a red to orange sediment. The meyer reagent was prepared by adding 1.36HgCl with 0.5g of potassium iodide then dissolved and diluted with distilled water to 100mL in a measure's flask. This reagent is colorless. The wagner reagent was prepared using 10mL distilled aquades and then added 2.5g of iodine and 2g of potassium iodide, then dissolved and diluted with aquades to 200mL in a flask. This reagent is brown. Dragendrof reagents are prepared by 0.8g of bismuth subnitrate added with 10mL acetic acid and 40 mL of water. This solution is mixed with a solution made from 8g of potassium iodide in 20mL water. Prior to use, 1mL of this mixed volume was diluted with 2.3 mixed volumes of 20mL glacial acetic acid and 100mL of water. The reagent is orange.

Steroid/Triterpenoid:

Several samples were dissolved in 2ml of chloroform in the test tube. 10 drops Acetic anhydride and 3 drops concentrated sulfuric acid were added to the mixture. The positive test results of samples containing steroid and triterpenoid are the formation of red, blue, and green solutions.

Saponin:

Saponins can be detected by foam test in hot water. A stable foam for 30 minutes and not lost on the addition of 1 drop of HCl 2 N shows a sample containing saponins.

Flavonoid:

Several samples plus 0.1mg magnesium powder and 0.4 mL of amyl alcohol (37% hydrochloric acid mixture and 95% ethanol with equal volume) and 4mL of alcohol, then mixed were shaken. The result of sample test positive contains flavonoids, which is the formation of red, yellow or orange on amyl alcohol layer.

Analysis of antioxidant activity[16]

Extract of Fresh Pokea Shell, extract of boiled Pokea shell and extract of dried Pokea Shell, extraction using methanol solvent p.a. (polar) is dissolved in methanol p.a. with concentrations of 0, 250, 500, 1000, 2000 and 4000 ppm. BHT Synthetic antioxidants used as positive control, made by BHT dissolved in methanol solvent p.a. with concentrations of 0, 0,3125, 0,625, 1,25, 2,5, 5, and 20ppm. The DPPH solution to be used was prepared by dissolving the DPPH crystals in a methanol solvent with a concentration of 1mM. The process of making a 1mM DPPH solution is carried out under low temperature conditions and protected from sunlight. The extract solution and the BHT comparative antioxidant solution were each taken 4.5mL and treated with 500µL of 1mM DPPH solution in different and labeled reaction tubes. The mixture was then incubated at 37°C for 30 minutes and measured its absorbance using a spectrophotometer at 517nm wavelength. The absorbance of the blank solution was also measured to perform percent inhibition calculations. The blank solution was prepared by reacting 4.5mL of a methanol solvent with 500µL of 1mM DPPH solution in the test tube. After that, the antioxidant activity of each sample and antioxidant comparative BHT/Vitamin C.

Cytotoxicity assay[17]

The BSLT method is usually performed in a preliminary test for pharmacological screening activity on natural products. This test used *Artemia salina naupli* (Golden West Supreme Plus Great Salt Lake, USA) as an animal test. At first A. salina eggs dripped in seawater under 40 watt TL lamps for 48 hours.

A total of 10 larvae A. salina were inserted into the test tube, then incorporated an extract of the sample solution with varying concentrations and added artificial seawater to the 5 mL volume. Artificial seawater without gi[illegible] extract (0 ppm) is used as a control. All test tubes were incubated at room temperature for 24 hours under a 40 watt TL lamp. Observations were made after 24 hours by counting the amount of *A. salina* that died at each concentration. The pricing of LC_{50} (ppm) is performed using probit analysis and regression equation.

RESULTS AND DISCUSSION:

The results of the analysis of mine[illegible] content in fresh, boiled, and dried Pokea clam meat can be seen in Table 1.

Table 1. Mineral content (ppm dry weight) fresh, boiled, and dried Pokea clam meat

Parameter	Boil	Fresh	dried
[illegible]neral Makro :			
Fosfor (P)	-	2.336,6289	3.217,5233
Kalsium (Ca)	2.321,1247	-	1.456,5214
Magnesium (Mg)	688,256	-	661,8654
Kalium (K)	910,5868	-	1.712,5860
Mineral Mikro :			
Besi (Fe)	-	1.703,1455	4.622,6855
Seng (Zn)	-	62,521	142,1247
Selenium (Se)	-	-	-

Macro minerals in fresh, boiled, and dried Pokea shells are dominated by the minerals phosphorus, calcium, and potassium. Micro minerals are dominated by iron minerals. The content of heavy metals in dried pokea shell meat has been reported[18]. The concentration of heavy metal in dried pokea shell was still below the limit of AAS detection device used was Pb <0.01 ppm; Cd <0.001ppm; and Hg<0.0002 ppm. According to BPOM RI (2009) and SNI 7387 (2009), that maximum Pb in food is 1.5ppm; Cd = 1.0ppm[1].

Phytochemical tests were performed to detect the bioactive components found in pokea shell extract from different solvents of polarity Tests with this phytochemical method can detect bioactive components not limited to secondary metabolites itself, but also to primary metabolites that provide functional biological activity, namely proteins and peptides[19]. The result of phytochemical test of crude extract of pokea shell meat can be seen in Table 2.

Various biological effects of saponins are antibacterial, antiprotozoal and anticancer activities. Saponins are secondary metabolites of glycosidic nature widely [illegible]tributed in higher plants. Saponins occur [illegible]stitutively in a great many plants species, in both wild plants and cultivated crops. In cultivated crops the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties[20].

Table 2. Phytochemical test results of crude extract of pokea shell meat

Phytochemical Test	Extract of Fresh Pokea Shell	Extract of Boiled Pokea Shell	Extract of Dried Pokea Shell
Flavanoid	+	+	-
Alkaloid	-	-	-
Tanin	-	-	-
Saponin	+	+	+
Quinon	-	-	-
Steroid	+	+	+
Triterpenoid	+	+	+

Legume contain 5-13% triterpenoid saponins. Many researchers have shown the relationship between legume consumption and health benefits, such as protection from cardiovascular disease, breast cancer, colon cancer, other cancers, diabetes, anti-inflammatory, antiarthritis, anti-oxidant and laxatives effect[20].

The different types of bioactive components extracted are, presumably due to differences in polarity of each solvent, materials and chem[illegible]al compounds will dissolve readily on relatively[21].The bioactive components present in a crude extract, such as alkaloids, steroids, saponins, phenols, free amino acids, and carbohydrates are thought to act as antioxidants[22].

Flavonoid compounds are antioxidants and can inhibit the action of xanthine oxidase enzymes and superoxide reactions[23]. The extracts which have higher content of phenol also indicated that they have better antioxidant activities. So, antioxidative activity may be correlated with total phenolic contents of the extracts. Flavonoids are one of the most important natural phenols. They also possess several biological and chemical activities and are having free radical scavenging properties too. Its antioxidant property depends primarily on thier hydroxyl group position in th molecule and their ability as electron donor to a free radical[24].

Flavonoid compounds from stereospermum personatum in addition to antioxidants, these compounds can also inhibit the action of xanthine oxidase enzyme.

Flavonoids and triterpenoids are known to have important roles in cancer chemoprevention and chemotherapy[25]. These compounds have antioxidant activity, with the capacity to capture reactive oxygen species (ROS), inhibit enzymes involved in the formation of ROS, and block the oxidation of cellular and extracellular compounds[26,27,28]. In addition, flavonoids play a role in the modulation pathway of cancer proliferation, cell cycle, inducing apoptosis, and inhibiting angiogenesis[29].

Several flavonoid compounds which have bonds with sugar groups so that flavon[24] are easily dissolved in water or polar-containing solvents such as ethanol, methanol, butanol, acetone, dimethylsulfoxide, dimethylformamide, and air[30].

Flavonoids also have anticancer potential with the mechanism of inhibition of the abnormal[37]osine kinase enzyme. The tyrosine kinase enzyme is an enzyme that plays a role in cell transduction signals, namely by regulating the cell's continued cycle, transcription regulation, cell transformation, proliferation, differentiation, and apoptosis. Oncogenes fes will alter protein kinase activity and result in the formation of [25]kemia tumor types[31,32]. The same was reported that Antioxidant compounds include vitamins, carotenoids, flavonoids and phenolics. Among them, phenolics and flavonoids are the most important and exhibit substantial antioxidant activity[33]

Triterpenoids are carbon skeletons composed of 6 isoprene units and made biosynthetic from squalene (C30 acyclic hydrocarbons). Steroids are triterpene components found in the form of glycosides. Natural triterpenoids have antitumor activity. Terpenoids have antioxidant capacity functions but are not followed by cancer cell antiproliferation functions[34].

Steroid is a class of triterpenoid compounds that can be classified into steroids with no more than 21 carbon atoms namely sterols, sapogenins, cardiac[2]glycosides, and vitamin D. Natural steroids are derived from various chemical transformations of two triterpenes namely lanosterol and cycloartenol. Steroid compounds can be used as the basic ingredient[2] for the manufacture of drugs[15]. There are several steroids such as fukosterol that are isolated from non toxic marine biological resources and have the characteristic of lowering cholesterol in the blood and promoting antidiabetic activity[35].

Based on the standard curve of absorbance spectrum, DPPH solution showed maximum absorption at 517 nm wavelengths using UV-VIS spectrophotometer Hitachi U-2800, so that sample absorbance measurements are performed at those wavelengths. The antioxidant activity of crude extracts from pokea shells can be seen in Table 3. Antioxidant[27]e the molecules that can effectively neutralize the free radicals. They safely interact with free radicals and terminate the [30]ain reaction before vital molecules get damaged. Phenolics, flavonoids, vitamins (E and C), numerous minerals (Cu, Mn, Zn, Se and Fe), glutathione [19]some of the most widely recognised antioxidants. Antioxidants in blood, cells, and tissue fluids play an important role in neutralizing the normal level of oxidative damage caused by these free radicals[36].

Table 3. Result of antioxidant activity test of crude extract of pokea shell

Parameter	Extract of Fresh Pokea Shell	Extract of Boiled Pokea Shell	Extract of Dried Pokea Shell
Antioxidant IC_{50}	2483.74 ppm	3338.61 ppm	>4000 ppm

[2]

Table 3 shows that the cru[40]extract of dried pokea shell has the highest value of antioxidant activity. The IC_{50} value of the crude extract of the pokea shell is still higher than the IC_{50} value of [8]tamin C used as the standard of 6.02ppm. IC_{50} value is one of the commonly used parameters to interpret the results of DPPH testing. This IC_{50} value can be defined as a substrate concentration which can lead to a 50% reduction in DPPH activity. The smaller IC_{50} value means the higher antioxidant activity[16]. The antioxidant activity of crude extract of pokea shells measured by DPPH method is still very weak. This is presumably because the DPPH method only measures antioxidant compounds with mechanisms capable of donating their hydrogen ato[7] to radical compounds. DPPH is a constant free radical at room temperature and accepts an electron or hydrogen radical to develop into a stable diamagnetic molecule. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical, progresses, which consequences in the [13]venging of the radical by hydrogen donation[37]. Lipid peroxidation has gained more importance nowadays because of its involvement in the pathogenesis of many diseases like atherosclerosis, cancer, diabetes mellitus, myocardial infarction, immunological incompetence, neuro degenerative disorders and also in aging[37]

The antioxidant mechanism is not limited to the ability of a compound to provide a hydrogen atom, but also in its ability to inhibit the formation of reactive oxygen compounds by metal chelating, bypassing the chain-oxidation reaction of free radicals or by capturing them[38].

Antioxidant activity measurements can be performed with several methods and all methods provide a picture of different mechanisms, so to obtain an adequate assessment of the antioxidant capability in a biological system a sample is required the use of more than one method[39].

[28]

Toxicity test by Brine Shrimp Lethality Test (BSLT) method is pre scree[14]g against active compounds contained in a sample. Extract is considered highly toxic when having an LC_{50} value below 30 ppm, considered

toxic when it has a LC_{50} value of 30-1000 ppm and is considered non-toxic when the LC_{50} value is above 1000 ppm[40].

Table 4. Result of toxicity test of crude extract of pokea shell

Parameter	Extract of Fresh Pokea Shell	Extract of Boiled Pokea Shell	Extract of Dried Pokea Shell
Toxicity LC_{50}	>1000 ppm	962.93 ppm	780.81 ppm

The LC_{50} value is the concentration that can cause death of 50% of population A. salina used. The value of LC_{50} can be seen from the table above shows that extract of boiled and dried pokea shell have LC_{50} value less than 1000 ppm. These values indicate that the extract of the boiled and dried Pokea shell is included in the toxic category. This is suspected because in the crude extract type of methanol solvent there is a cytotoxic bioactive component. This Extract dried Pokea showed the potential for development as an alternative anticancer agent.

The types of cytotoxic substances commonly present in crude extracts include tannins, flavonoids, triterpenoids and coumarins. This is consistent with the phytochemical results of the crude extract of ethyl acetate pokea shell containing flavonoids and steroids/triterpenoids. The presence of flavonoid and steroid compounds in rough extract of pokea from all solvents is allegedly different, both the number and the type of cytotoxic compounds so as to have a different impact on shrimp larvae[41].

Fraction is potentially studied as a candidate compound with cytotoxic activity if it has a relatively small LC_{50} value or less than 1000ppm. The LC_{50} value of ethyl acetate extract of pokea shell is smaller than the macroalgae extract of Turbunaria decurrens type[42].

The difference in the level of secondary metabolites in extracts is proportional to the level of cytotoxicity. The study supports the results of this study that the cytotoxic activity of extract of pokea depends on their phytochemical content[43].

The death of shrimp larvae was ascertained due to administration of extracts caused by the absence of shrimp larvae death in the negative control group. Ethyl acetate fraction and water fraction have LC_{50} values greater than 1000µg/ml. Air extract, ethanol extract, and n-hexane fraction have LC_{50} values smaller than 1000µg/ml[44].

CONCLUSION:

This study shows the results of phytochemical tests which include steroids, triterpenoids, saponins, and flavonoids. The antioxidant activity of the crude pokea shell extract is still relatively weak, and the crude methanol extract of boiled and dry pokea shell is in the toxic category so that it can be used as a cytotoxic compound. Extract purification studies should be carried out. In addition, further work is needed to resolve the specific agent anticancer activity of cancer cell lines and imunostimulant.

ACKNOWLEDGEMENT:

The authors are grateful to the Ministry of Research, Technology and Higher Education, and we thank our colleagues from IPB University who provided insight and expertise that greatly assisted in this research. We are grateful to all of those with whom we have had the pleasure to work during this and other related projects.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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