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# Molecular modeling and phenoloxidase inhibitory activity of arbutin and arbutin undecylenic acid ester

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ABSTRACT

Excessive melanin formation has been linked to various skin disorders such as hyperpigmentation and skin cancer. Tyrosinase is the most prominent target for inhibitors of melanin production. In this study, we investigated whether arbutin and its prodrug, arbutin undecylenic acid ester, might inhibit phenoloxidase (PO), a tyrosinase-like enzyme. Molecular docking simulation results suggested that arbutin and arbutin undecylenic acid ester can bind to the substrate-binding pocket of PO. Arbutin undecylenic acid ester with an $IC_{50}$ 6.34 mM was effective to inhibit PO compared to arbutin ($IC_{50}$ 29.42 mM). In addition, arbutin undecylenic acid ester showed low cytotoxicity in *Drosophila* S2 cells and the compound inhibited the melanization reaction. Therefore, the results of this study have demonstrated that arbutin undecylenic acid ester as a potential inhibitor of PO. We successfully designed a new platform utilizing *Drosophila melanogaster* and *Bombyx mori* as animal models propounding fast, cheap, and high effectiveness in method to screen tyrosinase inhibitors.



## 1. Introduction

Melanin is a pigment that plays an important role in human skin pigmentation which is produced by melanocytes through melanogenesis [1]. Skin pigmentation is a photoprotective factor mainly responsible for photo-carcinogenesis of the skin and harmful ultraviolet radiation [2,3]. However, excessive production of melanin can be a dermatologically serious problem leading to skin cancer and facial aesthetics in humans [4–6]. Apart from cosmetic considerations, treatment of skin pigmentation may also improve the quality of life.

In recent years, many substances have become available to treat hyperpigmentation disorder through various mechanisms [7] and tyrosinase, the key enzyme of the biosynthetic melanin pathway, has been the obvious molecular target for inhibiting this abnormality [8,9]. This enzyme belongs to the type 3 copper-containing metalloprotein with binuclear copper ions, CuA and CuB, coordinated by histidine residues as the conserved active site [10].

Many compounds have been identified as tyrosinase inhibitors, but only a few are currently used in topical dermatological products due to lack of clinical efficacy [11,12]. Hydroquinone is one of potent inhibitors against tyrosinase since the early 1990s [13]. However, the use of hydroquinone has been banned since January 2001 due to medium-term effects such as white patches on the skin, exogenous ochronosis, and carcinogenic for long-term effect [14]. Therefore, an increasing interest in discovery and development of tyrosinase inhibitors are observed especially in the cosmetics industry, and arbutin is the most well-studied among them [15–17].

Instead of human tyrosinase, mushroom tyrosinase isolated from *Agaricus bisporus* is more frequently used as an in vitro model for screening tyrosinase inhibitors, since this enzyme is commercially available [18]. However, previous studies have reported significant differences in inhibitor effectiveness between mushroom tyrosinase (mTyr) and human tyrosinase (hTyr) [19,20]. Moreover, structural and kinetic information of human tyrosinase still remains unclear [21].

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In line with our study, the results lead us to establish a new approach for an efficient and effective in vitro and in vivo screening of tyrosinase inhibitors by utilizing PO from fruit flies, *Drosophila melanogaster* and silkworms, *Bombyx mori*. PO has similar activity with tyrosinase in mammals that catalyzes the o-hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to quinones [22,23]. This new platform is simple preparation, easy, and low cost. We identified the effects of popular skin whitening agents that inhibit tyrosinase as well as PO, such as tropolone and kojic acid [24,25] and compared their efficacy with arbutin and its derivative, arbutin undecylenic acid ester. We found that arbutin undecylenic acid ester is effective and safe as a PO inhibitor.

## 2. Materials and methods

### 2.1. Compounds and reagents

Arbutin undecylenic acid ester was enzymatically synthesized by esterification reaction as described previously [26]. Arbutin and kojic acid were obtained from TCI Technology Co., Ltd., (Tokyo, JP). Tropolone and L-DOPA were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, JP). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

### 2.2. Cell culture

*Drosophila* S2 cell lines were maintained at 25 °C in Schneider's Drosophila medium (Thermo Fisher SCIENTIFIC) containing 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin.

### 2.3. Animal stocks and maintenance

*Drosophila* stocks were maintained in standard cornmeal-yeast agar medium at 25 °C or 18 °C. Oregon R flies were used as a wild-type. *Bombyx mori* stocks were maintained in mulberry chow silkworm food at 25 °C.

### 2.4. Molecular docking

The molecular structure of PO and tyrosinase was obtained from Protein Data Bank (PDB ID: 3HHS and 2Y9W, respectively). Preparation of proteins and rotatable bonds in the ligands were assigned by AutoDockTools4 program. Docking was performed by directing the optimized ligand molecular model on the active site of the enzymes using AutoDock Vina [27] for three replications. The protein-ligand interactions were visualized and analyzed using PyMOL. The amino acid sequences were retrieved from the UniProt database (http://www.uniprot.org/).

### 2.5. PO inhibitory activity assay

Silkworm hemolymph was collected by dissecting the legs of the 5th instar larvae of *B. mori*. 5 μL of compounds were added into a 96-well plate. Then, hemolymph solution was prepared by combining 50 μL of diluted hemolymph (5 μL of hemolymph in 45 μL of protease inhibitor cocktail) with 150 μL of a 5 mM $CaCl_2$ solution in a clean Eppendorf tube. Next, add 800 μL of L-DOPA solution (20 mM in phosphate buffer pH 6.6). Following thorough mixing, 45 μL of hemolymph solution was added into each well to a final volume 50 μL and incubated at 29 °C in the dark for 30 min. After that, the optical density (OD) at 492 nm was measured for each sample against negative control containing no compound. Each experiment was repeated three times. The intensity of the color observed will depend on the initial amount of cleaved PO in the sample. The data from this assay reflect the PO activity at the time of collection due to activation of the prophenoloxidase (PPO) was blocked by the presence of the protease inhibitor [28].

### 2.6. Cell viability assay

*Drosophila* S2 cells (1.5-8.0 × $10^5$ cells in 100 μL) were inoculated in a 96-well plate. 10 μL of compounds, arbutin, arbutin ester, tropolone, and kojic acid were added and incubated at 25 °C for 12–18 h. Cell viability was monitored by luminescence from a CellTiter-Glo Luminescent Cell Viability Assay (Promega) with a Synergy-HTX (BioTek). Cell viability is expressed as a relative value, with luminescence in cells incubated with the Elix water (negative control) being 100%.

### 2.7. Melanization assessment

To observe PO inhibitory activity during melanization, we performed pinching of 3rd instar larvae (unless otherwise noted) and pricking the adult flies of wild-type, Oregon R, after feeding with compounds for five days. For imaging of the melanization reaction upon pricking, the thorax of the animal was pricked using a sterilized needle. Pictures were taken 16 h post-pricking. Third-instar larvae were pinched dorsally near the posterior end. Pictures of melanized larvae were taken 3 h post-injury [29]. Pictures were captured with an Olympus SZX16 microscope, an Olympus DP74 camera and the Olympus Application Suite. For publication purposes, contrast and brightness were increased on some images.

### 2.8. Statistical analysis

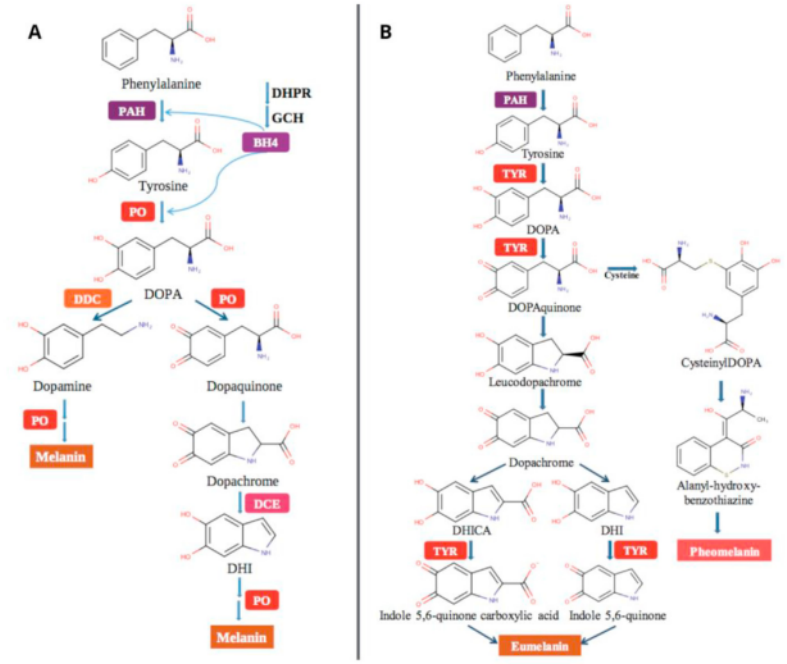
Statistical significance of PO inhibitory activity and cytotoxicity data was calculated with 2-way ANOVA with Tukey's multiple comparisons test. Statistical significance of survival data was calculated with a log-rank test compared to the water group. Blackening strength (strong, weak, or none) of the larvae and adult flies were analyzed using Chi-square test. P values are indicated in figure legends.

## 3. Results

### 3.1. Molecular modeling

In insects, PO refers to an enzyme with tyrosinase-like activity. The main role of PO as a key component in melanogenesis is to convert phenols to quinones, which subsequently polymerize to form melanin (Fig. 1A and B). The *Drosophila* genome encodes three isoforms of PPO, two of them, PPO1 and PPO2 contribute to melanization [30]. We checked the amino acid sequences of PPO1 and PPO2 of *D. melanogaster* and *B. mori*. The result showed homology when its amino acid sequences were aligned with that of human tyrosinase and mushroom tyrosinase isoforms (PPO3 and PPO4, the main components of commercially available mTyr) (Fig. 1C).

To evaluate the interaction of compounds with PO and tyrosinase, we performed molecular docking. With the docking strategies, the orientation of the ligand when it is bound to an enzyme or protein can be predicted [31]. In PO, arbutin has low binding energy compared to other compounds (Table 1). On the other hand, arbutin ester has similar binding energy with tropolone and kojic acid as positive controls (Fig. 2). The results suggest that arbutin ester could bind closer to the active site of PO. The same interaction was also shown in the arbutin ester against the tyrosinase.

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| | | | |
|---|---|---|---|
| *D. melanogaster* | PPO1 | 365 369 405 | YGNLHNEGHNII ..... FGVMGDVTTVMRDPIFYRWHGFIDTVF |
| *D. melanogaster* | PPO2 | 367 371 407 | YGDLHNNGHTFI ..... FGVMGDVSTAMRDPVFYKWH SYIDRIF |
| *B. mori* | PPO1 | 366 370 406 | YGDLHNMGHVFI ..... FGVMGDSATAMRDPVFYRWHAYIDDIF |
| *B. mori* | PPO2 | 366 370 406 | YGSIHNNGHSFT ..... FGVIADEATTMRDPFFYRWHAYIDDVF |
| *A. bisporus* | mTyr PPO3 | 259 263 296 | LEMVHNTVHFLI ..... PGHMGSVPHAAFDPIFWMHHCNVDRLL |
| *A. bisporus* | mTyr PPO4 | 251 255 283 | LEAVHDDIHGFV.... RGHMTHALFAAFDPIFWLHHSNVDRHL |
| *H. sapiens* | hTyr | 363 367 390 | QSSMHNALHIYM..... NGTMSQVQGSANDPIFLLHHAFVDSIF |

**Fig. 1.** Melanin biosynthesis pathway and sequences alignments of PO and tyrosinase. (A) and (B) melanin biosynthesis pathway in *Drosophila* and in mammals, respectively. (C) Amino acid sequences of *Drosophila melanogaster* and *Bombyx mori* isoforms PPO1 and PPO2 for comparison with human tyrosinase (hTyr) and mushroom tyrosinase (mTyr) isoforms PPO3 and PPO4 in the Cu−B site. Cu-coordinating Histidine (H) residues are shown in red. PAH, phenylalanine hydroxylase; BH4, tetrahydrobiopterin, DHPR, dihydropteridine reductase; GCH, GTP cyclohydrolase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme, TYR, tyrosinase, DHI, 5,6-dihydroxyindole, DHICA, 5,6-dihydroxyindole-2-carboxylic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**
Interaction energy of ligand with phenoloxidase and tyrosinase.

| No | Ligand | Interaction energy (kcal/mol) | |
|---|---|---|---|
| | | PO | mTyr |
| 1 | Arbutin | −6.2 | −5.8 |
| 2 | Arbutin Ester | −5.3 | −6.5 |
| 3 | Tropolone | −5.7 | −5.1 |
| 4 | Kojic Acid | −5.1 | −4.2 |

### 3.2. Inhibition of phenoloxidase

To assess the potential inhibitory effects of these compounds, we performed the PO inhibitory activity assay using silkworm hemolymph. We show that arbutin ester was able to inhibit PO with half-maximal inhibitory concentration ($IC_{50}$) of 6.36 mM (Fig. 3A B). Kojic acid and tropolone as well-known inhibitor of PO had $IC_{50}$ values of 0.83 mM and 3.44 mM, respectively. With an $IC_{50}$ of about 29.4 mM, arbutin was 4 times less potent than arbutin ester.

### 3.3. Cytotoxicity activity

We checked the cytotoxicity of compounds in *Drosophila* S2 cells (Fig. 3C and D). We observed significant cytotoxicity of tropolone with a $LC_{50}$ value of 4.14 mM. Arbutin ester and kojic acid have $LC_{50}$ values of 22.5 mM and 54.7 mM, respectively. Arbutin was by far the most safe compound with $LC_{50}$ values of 72.8 mM.

### 3.4. Melanization in Drosophila melanogaster

To further understand anti-melanogenic effects of compounds, we then tested the blackening reaction at the wound site upon pinching the 3rd instar larvae and pricking the adult flies after compound treatment. We categorized the blackening into three levels: strong, weak, and none (Fig. 4A). After pinching, 63% of the water group blacken strongly, 35% blacken weakly, and 2% no blacken. This ratio shifts for arbutin (33% strong, 52% weak, 15%

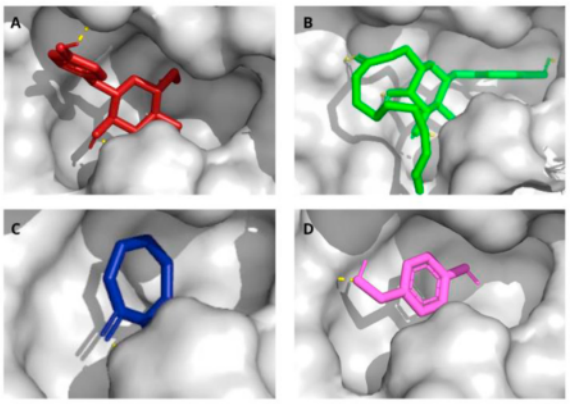


**Fig. 2.** Docking poses of phenoloxidase inhibitors. (A) The binding mode of phenoloxidase (blue sticks) with arbutin (red sticks), (B) arbutin ester (green sticks), (C) tropolone (blue sticks), and kojic acid (magenta sticks). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

none). Interestingly, as we expected, blackening reaction of arbutin ester group (15% strong, 47% weak, 38% none) was not significantly different with kojic acid as positive control group (13% strong, 42% weak, 45% none), whereas tropolone group were almost deficient for blackening (3% strong, 15% weak, 82% none) (Fig. 4B).

Concordant with blackening reaction in larvae, after pricking, the water group 56% blacken strongly and 44% blacken weakly. Blackening reactions of arbutin and arbutin ester group were 11% strong, 44% weak, 39% none and 17% strong, 66% weak, 17% none, respectively (Fig. 4C).

We also reveal that the melanization responses were contributed with survival in adult flies post-injury. A synergistic effect can be observed in arbutin ester group, we noticed that they have a significantly lower survival rate than the arbutin, tropolone, and water group (Fig. 4D). A similar trend towards reduced survival rate was evaluated in kojic acid group. However, as shown in Supplemental Fig. 1, we examined the effects of the compounds on survival rates without pricking and we confirmed that the kojic acid being strongly lower survival rates than other compounds. This indicates that arbutin ester was potent and safe as an inhibitor of PO with a remarkable efficacy not only in vitro but also in vivo over other compounds.

## 4. Discussion

Tyrosinase inhibitors are highly warranted by pharmaceutical and cosmetic industries for skin whitening agents. Most tyrosinase inhibitors have been evaluated with mushroom tyrosinase. However, recent research has reported that the commercially available mushroom tyrosinase is a mixture of several tyrosinase isoenzymes and additional enzyme activity that may influence inhibition studies [32]. Thus, it is important to explore an alternative model for screening the tyrosinase inhibitor. The main objective of this study was to investigate the effects of derivative of arbutin, arbutin undecylenic acid ester using PO from *D. melanogaster* and *B. mori* as a new model.

Although arbutin had already been identified as a tyrosinase inhibitor, the published $IC_{50}$ values of arbutin range from 0.04 mM to more 30 mM for mTyr [33]. Mann [34] reported that arbutin only weakly inhibited human tyrosinase. On the other hand, penetration of arbutin through the skin to reach the melanocytes is rather difficult due to low hydrophobicity (log P value = −1.49) [35]. Therefore, new derivatives of arbutin are being sought out.

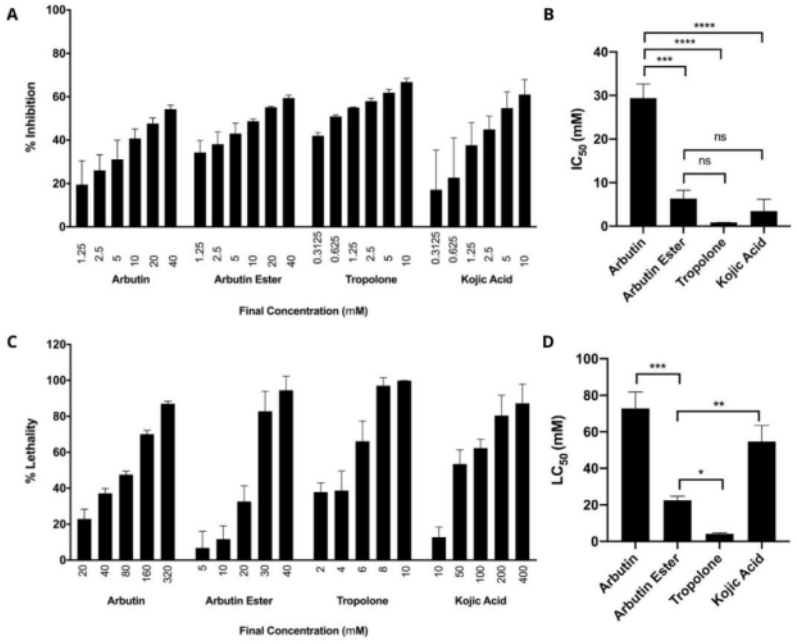


**Fig. 3.** Inhibition of phenoloxidase and the cytotoxicity effect of compounds. (A) % Inhibitory and (B) $IC_{50}$ of arbutin, arbutin ester, tropolone, and kojic acid (***$P = 0.0001$; ****$P < 0.0001$; ns $P > 0.05$). (C) % Lethality and (D) $LC_{50}$ of arbutin, arbutin ester, tropolone, and kojic acid ($P = 0.0326$; **$P = 0.0021$; ***$P = 0.0002$). Data represent the mean ± SEM of experiments performed in triplicate (A–D).

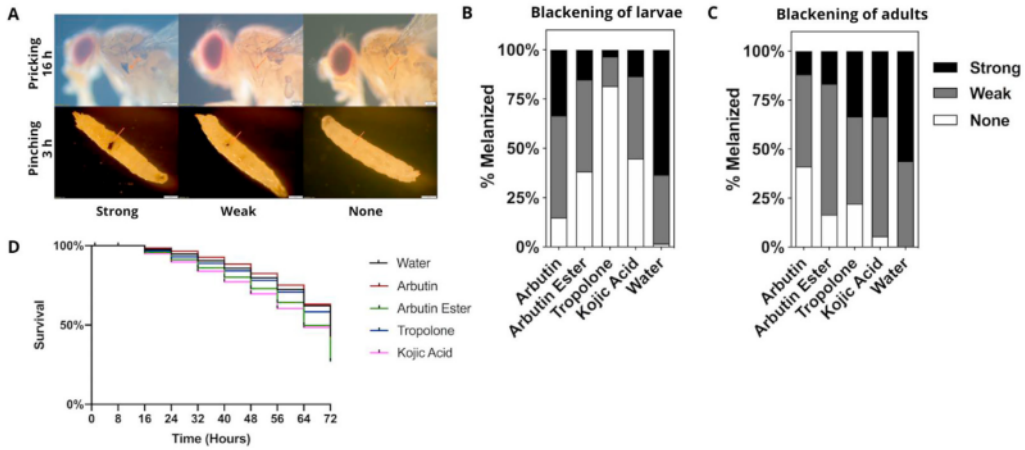


**Fig. 4.** Melanization reaction in *D. melanogaster*. (A) Category of blackening reaction: strong, weak, and none. (B) Blackening reaction of larvae (arbutin vs arbutin ester $P = 0.0002$, tropolone vs arbutin ester $P = <0.0001$, kojic acid vs arbutin ester $P = 0.6023$, and water vs arbutin ester $P = <0.0001$). (C) Blackening reaction of adult flies of *Drosophila melanogaster* after treatment with compounds (arbutin vs arbutin ester $P = 0.0008$, tropolone vs arbutin ester $P = 0.0060$, kojic acid vs arbutin ester $P = 0.0031$, and water vs arbutin ester $P = <0.0001$). (D) Survival rate of flies post-injury (water vs arbutin $P = 0.5176$, water vs arbutin ester $P = 0.0010$, water vs tropolone $P = 0.2790$, water vs kojic acid $P = 0.0007$).

Arbutin undecylenic acid ester is an arbutin prodrug from enzymatic esterification of arbutin and undecylenic acid which is considered to maintain the stability of the physical and chemical properties of arbutin and as a strategy for enhancing the absorption in human skin because of the acylation with chain fatty acid may increase the hydrophobicity (prediction of log P value = 3.37). Ester prodrugs have been targets of a number of studies in recent decades, especially for applications in cosmetic products [36].

Our docking data reveal that the arbutin undecylenic acid ester can bind to the substrate-binding pocket of PO (Fig. 2). The hydroxyl group of the compound has been proposed as essential structural requirements to display inhibitory activities against PO due to their chelating properties [37]. The results are in agreement with previous studies which in tropolone, kojic acid, and arbutin, take part in chelation inhibiting tyrosinase [38].

Tropolone and kojic acid have been known as strong inhibitors of tyrosinase [39]. Nevertheless, tropolone was never seriously considered for clinical developments in humans due to its highly toxic substances [40]. Our results exhibit tropolone has $IC_{50}$ values about 0.38 mM and significantly cytotoxic in *Drosophila* S2 cells (Fig. 3). Additionally, our study found that the growth of larvae has delayed after feeding with the tropolone and kojic acid. Regarding the safety of kojic acid for cosmetic products, the European Scientific Committee on Consumer Safety [41] considers kojic acid concentrations up to 1.0% to be safe. However, due to cytotoxic and instability on storage, its use has been limited [42].

In our in vitro experiments, we used hemolymph (insect blood) from the silkworm, *B. mori*. PO exists in hemolymph, which is primarily produced as the inactive zymogen, PPO [43]. We found that arbutin ester with an $IC_{50}$ 6.34 mM was effective to inhibit PO compared to arbutin (Fig. 3A and B). Further experiments exhibited that arbutin and arbutin ester was low cytotoxic activity (Fig. 3C and D). However, the European Union Scientific Committee Consumer Products [44] published a critical opinion regarding the use of arbutin to be unsafe in cosmetics products because it can release hydroquinone from the molecule.

In melanization assessment, we performed injury in the larvae and adult flies of *D. melanogaster*. Upon injury, they release PPOs into the hemolymph, where they are activated by a cascade of patterns-recognition proteins, serine proteases (SPs) [29]. These experiments confirmed that arbutin ester can inhibit the melanization reaction (Fig. 4). Binggeli [45] have analyzed the association of melanization to survival of flies. They showed that $PPO1^{\Delta}$, $PPO2^{\Delta}$ double mutants have a significantly shorter lifespan than the wild-type flies. Therefore, our results suggested that the arbutin undecylenic acid ester might provide important information for the development of pigmentation-related skin diseases and serve as a useful skin whitening agent in future cosmeceutical applications.

Furthermore, the results of this study have demonstrated that *D. melanogaster* and *B. mori* provide new insight and alternatives into novel melanogenesis inhibitor discovery. There are many advantages of using these animal models, they are inexpensive and easy to culture in laboratory conditions [46]. This new platform is simple, economic, and high-throughput screening for the rapid determination of tyrosinase inhibitors. Better yet, the low-cost of this platform enables researchers to conduct large-scale drug screening at keen prices. In comparison, commercial tyrosinase inhibitor screening kit demand an exorbitant price and only available for in vitro assay. In fact, the most striking feature of this new platform is that it can enable researchers to screen potential tyrosinase inhibitors efficiently not only in vitro but also in vivo assays, as well as it can be detected the toxicity effect of compounds.

## 5. Conclusions

Arbutin undecylenic acid ester was potent and safe as PO inhibitor as well as tyrosinase inhibitor. Indeed, *D. melanogaster* and *B. mori* could be used as a new efficient and effective platform for screening tyrosinase inhibitors. The full potential of arbutin undecylenic acid ester to reduce hyperpigmentation of human skin and the powerful *D. melanogaster* and *B. mori* as an alternative model need to be explored in future studies.

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## Declaration of competing interest

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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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.02.006.

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