Anti-melanogenesis properties of quercetin- and its derivative-rich extract from Allium cepa

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Anti-melanogenesis properties of quercetin- and its derivative-rich extract from *Allium cepa*

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Abstract

In an effort to find a new whitening agent, we have found that the methanol extract of the dried skin of *Allium cepa* showed inhibition of melanin formation. Bioassay-guided fractionation led to the isolation of quercetin (**1**) and quercetin 4'-*O*-β-glucoside (**3**) from *A. cepa* as the inhibitors of melanin formation in B16 melanoma cells with an IC_{50} of 26.5 and 130.6 μM, respectively. In addition, we evaluated the effect of some quercetin derivatives, such as isoquercitrin (**2**); quercetin 3, 4'-*O*-diglucoside (**4)**; rutin (**5**) and hyperin (**6**) on B16 melanoma cells. These quercetin derivatives did not show any inhibition of melanin formation. Furthermore, the ORAC values of compounds **1**-**6** were 7.64, 8.65, 4.82, 4.32, 8.17 and 9.34 μmol trolox equivalents/μmol, respectively. Dried skin of red onion showed inhibitory activity of melanin formation in B16 melanoma cells as well as antioxidant properties.

Keywords : *Allium cepa*, red onion, quercetin derivatives, anti melanogenesis, antioxidant

1. Introduction

Melanin pigments are formed in specialized pigment-producing cells known as

38 melanocytes, which originate in the neural crest during embryogenesis and are
39 distributed throughout the embryo during its development (Sánchez-Ferrer,
40 Rodrígez-López, & García-Carmona, 1995).

41 Melanin biosynthesis occurs in a cascade of enzymatic and spontaneous reactions
42 that convert tyrosine to melanin pigments. The initial and rate-limiting step in melanin
43 synthesis is the hydroxylation of tyrosine to dihydroxyphenylalanine or DOPA (Yoon et
44 al., 2007). DOPA-oxidation produces a highly reactive intermediate that is further
45 oxidized to form melanin by a free radical-coupling pathway. If free radicals are
46 inappropriately processed in melanin synthesis, hydrogen peroxide (H_2O_2) is generated,
47 leading to the production of hydroxyl radicals (HO•) and other reactive oxygen species
48 (ROS) (Perluigi et al., 2003).

49 The onion is a versatile vegetable that is consumed fresh as well as in the form of
50 processed products. The regular consumption of onions in food is associated with a
51 reduced risk of neurodegenerative disorders, cancer, cataract, ulcer, osteoporosis,
52 vascular disease and heart disease (Kaneko & Baba, 1999; Kawaii, Tomono, Katase,
53 Ogawa, & Yano, 1999; Sanderson, Mclauchlin, & Williamson, 1999; Shutenko et al.,
54 1999). Onion is one of the major sources of various biologically active phytomolecules
55 e.g., phenolic acids, flavonoids, cepaenes, thiosulfinates and anthocyanins (Singh et al.,

2009). The major flavonoids found in the dry peel of the onion, which has usually been considered to be waste, contain large amounts of quercetin, quercetin glycoside and their oxidative product which are effective antioxidants against the lethal effect of oxidative stress (Gulsen, Makris, & Kefalas, 2007; Prakash, Upadhyay, Singh, & Singh, 2007).

Onion (*Allium* species) plays an important role in traditional medicine in Indonesia; it is used as a diuretic, febrifuge, and poultice to cure wounds and to remove scars from the skin, and it suppresses the blood sugar level and platelet aggregation, (de Padua, Bunyapraphatsara, & Lemmens, 1999).

Based on our preliminary screening data (not shown), the methanol extract of the dried skin of the red onion (*Allium cepa)* from Indonesia showed a potent melanin biosynthesis inhibitory activity on B16 melanoma cells. These findings led us to focus on the active compounds in the dried skin of the red onion.

2. Materials and Methods

2.1. Chemicals

NaOH, DMSO, L-tyrosine, L-DOPA, and rutin (**5**) purchased from Wako (Osaka, Japan). Mushroom tyrosinase, Fluorescein sodium salt (FL), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), and

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT were obtained from Sigma (St. Louis, MO, USA). Quercetin-3, 4'-*O*-diglucoside (**4**) and hyperin (**6**) were from Tokiwa Phytochemical (Tokiwa, Japan). EMEM was from Nissui Chemical Co (Osaka, Japan). The ethylenediaminetetraacetic acid or EDTA was from Dojindo (Kumamoto, Japan). The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was from TCI (Tokyo, Japan) and isoquercitrin (**2**) was from Fluka (Steinheim, Germany). Other chemicals are of the highest grade commercially available.

2.2. Plant materials

Red onion (*A. cepa)* was purchased from traditional market in Jakarta, Indonesia, on September in 2008. Voucher specimen (ETA-CW-6) was deposited in Wood Chemistry Laboratory, Department of Forest Product Technology, Faculty of Forestry, Mulawarman University, Indonesia.

2.3. Preparation of plant extracts

Plant materials (dried skin or flesh part of *A. cepa*) were dried at room temperature and powdered. The dried materials (17.38 g) were extracted with methanol (650 mL) at

92 room temperature with shaker at 150 rpm during 48 h. The extract solutions were
93 filtered and concentrated *in vacuum*, to obtain the crude methanol extracts. The crude
94 extracts were 1.75 g.
95
96 *2.4. Isolation of Quercetin (**1**) and Quercetin 4'-O-β-glucoside (**3**)*
97 The dried skin of methanol extract of *A. cepa* (1.4 g) was applied to silica gel
98 column chromatography (71 g of Wakogel C-200, 3.5 x 50 cm). The chromatography
99 was eluted with *n*-hexane/EtOAc [10:0 (100 mL), 9:1 (50 mL), 7:3 (50 mL), 5:5 (200
100 mL), 3:7 (200 mL), 1:9 (100 mL)] and EtOAc/MeOH [9:1 (100 mL), 8:2 (100 mL), 7:3
101 (250 mL), 6:4 (50 mL), 5:5 (100 mL), 4:6 (50 mL), 3:7 (50 mL), 2:8 (50 mL), 1:9 (100
102 mL), 0:10 (100)] to give thirty three fractions (Fr 1 to Fr 33). The fractions Fr. 3 (66.9
103 mg) and Fr. 15 (84.7 mg) was analyzed by TLC (Thin Layer Chromatography) and
104 HPLC (High Performance Liquid Chromatography). Based on NMR (Nuclear Magnetic
105 Resonance) analysis, Fr. 3 was identified as quercetin (**1**) and Fr. 15 was
106 quercetin-4'-*O*-glucoside (**3**) by comparison of previous NMR data (Alfonso &
107 Kapetanidis, 1994; Tanabe, Ogawa, Tesaki & Watanabe, 1997), respectively (Figure 1).
108 The NMR spectra of compounds were recorded at 400 MHz on JNM-AL400 FT NMR
109 spectrometer (Jeo1). All compounds were dissolved in DMSO-d_6, or methanol-d_4, and

chemical shifts were referred to deuterated solvents. The compounds were assigned for ^{1}H, ^{13}C, HMQC, and HMBC.

2.5. HPLC analysis

All the crude extracts of dried skin and flesh of onion were dissolved in HPLC grade methanol, filtered through sterile 0.22 μm millipore filter and subjected to qualitative analysis by using Waters 600 HPLC instrument. The instrument is equipped with a photodiode array (detector Waters 996), controller (Waters 600s), pump (Water 626), and auto sampler injector Model 231 (Gilson). A ODS Inertsil C18 (4.6 mm i.d. x 250 mm) was used as a column. Data were integrated by Empower Build 1154-J series software (Waters). Separation was achieved by flow rate of 1 mL/min with methanol (60%)/water (40%) containing 1% triflouroacetic acid in isocratic program by monitoring the absorbance at 250 nm.

2.6. Oxygen radical absorbance capacity (ORAC) assay

Samples were directly dissolved in acetone/water/acetic acid (70:29.5:0.5, v/v/v), and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Trolox, FL and AAPH solutions were prepared with 75 mM phosphate buffer (pH 7.4). The ORAC

128 assay was performed as described by Ou, Hampsch-Woodill, & Prior (2001) with some
129 modification as follows: 300 µL of the standard (Trolox) or sample solution is mixed
130 with 1.8 mL of 48 nM FL solution, and then they were incubated independently at 37˚C
131 for 15 min. AAPH solution (900 µL; 12.9 mM, final concentration) was added to the
132 mixture and vortexed for 10s. Then, it immediately placed in a fluorescence
133 spectrophotometer (Model FP-6500, JASCO Co., Ltd., Tokyo, Japan), and measured
134 every 5s for 60 min at 37˚C (Ex: 485 nm, Em: 520 nm). A blank (FL + AAPH) using
135 phosphate buffer, standard solutions (6.25-50 µM Trolox), and sample solutions were
136 measured at same conditions. Three independent assays were performed for each
137 sample. The area under the fluorescence decay curve (AUC) was calculated as

138 $$\mathrm{AUC} = 1 + \sum \frac{f_i}{f_0}$$

139 where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at
140 time i.

141 The ORAC values were calculated as Prior et al. (2003) by using a equation (Y) a +
142 b(X) between Trolox concentration (Y)(µM) and the net area under the FL decay curve
143 (X). Linear regression was used in the range of 6.25-50 µM Trolox. Data are expressed
144 as micromoles of Trolox equivalents (TE) per micromole of sample (µmol TE/µmol).
145

146 *2.7. Cell culture*

147 A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank. The cells
148 were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and
149 0.09 mg/mL theophylline. The cells were incubated at 37^{o}C in a humidified atmosphere
150 of 5% CO_2.

151

152 *2.8. Inhibitory effect of melanin biosynthesis using cultured B16 melanoma cells*

153 This assay was determined as described by Arung, Shimizu, & Kondo (2007).
154 Briefly, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered
155 saline (PBS) and removed from the plastic using 0.25 % trypsin/EDTA. The cells were
156 placed in two plates of 24-well plastic culture plates (1 plate is for determining of
157 melanin and other is for cell viability) at a density of 1×10^5 cells/well and incubated for
158 24 h in media prior to being treated with the samples. After 24 h, the media were
159 replaced with 998 μL of fresh media and 2 μL of DMSO was added with or without
160 (control) the test sample at various concentrations (n=3). Arbutin was used as a positive
161 control. The cells were incubated for an additional 48 h, and then the medium was
162 replaced with fresh medium containing each sample. After 24 h, the remaining adherent
163 cells were assayed (see below).

2.9. Determination of melanin content in B16 melanoma cells

The melanin content of the cells after treatment was determined as follows. After removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1*N* NaOH. The crude cell extracts were assayed using a micro plate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture.

2.10. Cell viability

Cell viability was determined by use of the micro culture tetrazolium technique (MTT). The MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. Culture was initiated in 24-well plates at 1×10^5 cells per well. After incubation, 50 μL of MTT reagent [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/mL)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37^{o}C for 4 h. After the medium was removed, 1.0 mL isopropyl alcohol (containing 0.04 N HCl) was added into the plate, and the absorbance was measured at 570 nm relative to 630 nm.

3. Results and discussion

3.1. The effect of the methanol extract of the dried skin and flesh of A. cepa on B16 melanoma.

The diameter of Indonesian red onion (*A. cepa*) is approximately 1.5 – 2.0 cm. The thickness of the dried skin is approximately 0.5 - 1.0 mm. Figures 2 and 3 show the effect of the methanol extract of the dried skin and flesh of *A. cepa* on B16 melanoma cells, respectively. The dried skin extract dose dependently inhibited melanin formation in B16 melanoma cells. The inhibition of 40-50% of melanin formation was evident at concentrations of 50 and 100 μg/mL without any cytotoxicity. Arbutin, which is used in skin-whitening cosmetics, was used as a positive control (Virador, Kobayashi, Matsunaga & Hearing, 1999). In contrast, the extract of the flesh of the onion did not lead to melanin inhibition even at concentrations up to 250 and 500 μg/mL. According to these data, the dried skin extract is more potent than the flesh extract. This result may be related to the amount of active compound in the dried skin, as shown in Figures 4 and 5. Figure 4 depicts HPLC chromatograms obtained by the injection of 20 μL of solution which contained methanol extract of the dried skin of *A. cepa* at the concentration of 1 mg/mL. Based on the methanol extract of the dried skin, the content of quercetin, isoquercitrin; quercetin 4'-*O*-glucoside; and quercetin 3,4'-*O*-diglucoside

were 13.8, 10.3, 6.4 and 8.3%, respectively. We have tried to analyze the flesh extract at a concentration of 1 mg/mL but each peak of the compounds was too small to be detected; therefore, we increased the concentration for HPLC analysis. Figure 5 shows the HPLC chromatogram obtained by the injection of 20 μL of solution which contained methanol extract of the flesh of *A. cepa* at the concentration of 11 mg/mL. Based on the methanol extract of the flesh, the content of quercetin; isoquercitrin; quercetin 4'-*O*-glucoside, and quercetin 3,4'-*O*-diglucoside were 0.01, 0.59, 0.35 and 0.50%, respectively. As seen on the Y axis of both figures, the content of quercetin derivatives in the methanol extract of the dried skin of *A. cepa* was apparently much higher than that in the extract of the flesh. Also, it was shown that the extract of dried skin contained quercetin (**1**) and isoquercitrin (**2**) dominantly, but in the flesh extract, isoquercitrin (**2**) and quercetin 3,4'-*O*-diglucoside (**4**) were contained abundantly. It was reported that the content of quercetin, quercetin 4'-*O*-glucoside and quercetin 3,4'-*O*-diglucoside in different parts of the onion bulb (*Allium cepa* L.) found difference as well as the various cultivars (Beesk, Perner, Schwarz, George, Kroh, & Rohn, 2010). They found the outer layer of *A. cepa* content approximately 2.2, 2.7, 1.7% of quercetin, quercetin 4'-*O*-glucoside, and quercetin 3,4'-*O*-diglucoside in the dry weight of outer layer, respectively. In the inner parts, they reported that the content of quercetin, quercetin

218 4'-*O*-glucoside, and quercetin 3,4'-*O*-diglucoside was 0, 0.53 and 0.72% in the dry
219 weight of inner parts, respectively. In our opinion, the difference of quecertin and its
220 derivatves content should affect the difference in their melanin inhibitory activity as
221 depicted in Figures 2 and 3.

222 *3.2. Isolated compounds*

223 The biologically-guided fractionation of methanol extracts of *A. cepa* on B16
224 melanoma cells led us to focus on the active fraction of the dried skin extract. As a
225 result of fractionation, Fr. 3 was identified as one of the active compounds as well as Fr.
226 15. Thus, NMR assignment was performed to elucidate the structure of these fractions
227 by comparison with previous data (Alfonso & Kapetanidis, 1994; Tanabe, Ogawa,
228 Tesaki & Watanabe, 1997). It was revealed that Fr. 3 and Fr. 15 were **1** (69.9 mg, 4.9 %
229 yield in the extract) and **3** (84.7 mg, 6.1 % yield in the extract), respectively.

230 *3.3. Anti melanogenesis properties*

231 The anti melanogenesis effects of isolated compounds **1** and **3** (Figure 1) from the
232 dried skin of *A. cepa* were determined by using B16 melanoma cells. Table 1 depicted
233 the IC_{50} values for the melanin biosynthesis inhibition of quercetin and its derivates.
234 The activities of the tested compounds, in terms of IC_{50} values, decreased in the
235 following order: **1** (<50 μM), **3** (100–150 μM), **4**, **5**, **2** and **6** (>150 μM). Notably,

236 isolated compounds **1** and **3** from *A. cepa*, were found to be more-potent inhibitors of
237 melanin formation in B16 melanoma cells than the positive control, arbutin (198 μM). It
238 should be noted that the opposite results were reported previously, namely that **1**
239 enhanced melanogenesis in human melanoma cells and normal epidermal melanocyte
240 (Nagata, Takekoshi, Takeyama, Homma, & Osamura, 2004). Kubo, Nitoda, & Nihei
241 (2007) also reported that **1** enhanced the total melanin content in B16 melanoma cells.
242 Recently, it was reported that **1** suppressed melanin formation in B16 melanoma cells
243 with decreased intracellular tyrosinase activity and its protein expression (Fujii & Saito,
244 2009). The reason for the difference of the effect of **1** on melanin production in cells
245 remains unclear.

246 Based on our analysis, the IC_{50} of **1** to inhibit melanin formation was 26.5 μM with
247 88% cell viability, while that of **3** was 130.6 μM with 82% cell viability. The attachment
248 of a glucoside moiety in quercetin tended to reduce the ability of quercetin to inhibit
249 melanin formation in B16 melanoma cells. These results led us to evaluate other
250 quercetin derivatives such as **2**, **4**, **5**, and **6** (Figure 1) in B16 melanoma cells.
251 Compounds **2**, **5** and **6,** which represent different glycosyl moieties (glucoside,
252 rhamnoside, and galactoside) at C-3, displayed no inhibition of melanin formation.
253 Concerning this result, a previous report showed that quercetin 3-*O*-L-arabinofuranoside

254 did not suppress melanin formation (Fujii & Saito, 2009). These results revealed that the
255 attachment of a glycosyl moiety at the C-3 position may decrease the ability of
256 quercetin to reduce the melanin formation. We concluded that the hydroxyl group at the
257 C-3 position is essential to the inhibition of melanin formation in B16 melanoma cells.
258 To our knowledge, this is the first report showing that the dried skin of *A. cepa* and its
259 isolated compound, **3,** showed the ability to act as a potent skin-whitening agent by
260 inhibiting the melanin formation in B16 melanoma cells similarly to arbutin, which was
261 used as a positive control. Therefore, further experiments are needed to determine the
262 exact mechanism of this compound.

263 *3.4. Antioxidant properties*

264 Skin is a major candidate for and target of oxidative stress caused by reactive
265 species (RS), including reactive oxygen species and reactive nitrogen species. RS are
266 major and significant contributors to skin hyperpigmentation and skin aging (Kim,
267 Kang, & Yokozawa, 2008). It has been generally believed that agents having
268 antioxidant activity show anti-aging, whitening, and anti-inflammatory activities (Choi,
269 Song, Hur & Sim, 2008). Since compounds **1** and **3** have shown promising results as
270 whitening agents using B16 melanoma cells, we conducted an antioxidant assay
271 (ORAC) in order to determine their ability to counteract oxidative stress from UV

272 radiation. Table 2 shows the ORAC value results of compounds **1** – **6**, which were 7.64,
273 8.65, 4.82, 4.32, 8.17 and 9.34 μmol TE/μmol, respectively. Compounds **1**, **2**, **5** and **6**
274 showed more potent antioxidant activity than **3** and **4**. In the ORAC assay, quercetin
275 showed an ORAC value similar to that reported by Kohri et al., (2009). However, we
276 did not find any previous report of an ORAC assay for **3**. Based on the results in Table 1,
277 it was revealed that 4'-glycosylation of quercetin decreased its ability as an antioxidant
278 as represented by ORAC values.

279 The potency of **1** and **3** as antioxidants might be related to the presence of hydroxyl
280 groups in the form of a catechol moiety as reported by Cao, Soffic, & Price (1997).
281 Moreover, the relationship between flavonoid structure and antioxidant activity has been
282 studied, and it has been found that 3',4'-dihydroxy substitution in the B ring, as in
283 quercetin, increases the antioxidant activity substantially compared with a
284 mono-hydroxy substituent, as in kaempferol (Rice-Evans, Miller, Bolwell, BramLey, &
285 Pridham, 1995; Cotelle, Bernier, Catteau, Pommery, Wallet, & Gaydou, 1996).

286

287 **4. Conclusion**

288 Our study demonstrated that the melanin inhibition ability of quercetin derivatives
289 with glycosyl moieties at C-3 is reduced. Also, our results showed that quercetin

derivatives with a glycosyl moiety at C-4' reduced its antioxidant activity. In addition, compounds **1** and **3** from the dried skin of *A. cepa,* are promising compounds that could be useful for treating hyperpigmentation as skin-whitening agents and as antioxidants. However, it should be noted that safety is a primary consideration for its practical use in humans.

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Legend of Figures

Figure 1. The structure of quercetin and its derivatives.

Figure 2. Effect of the methanol extracts of the dried skin of *A. cepa* on B16 melanoma cells [Arbutin : 100 μg/mL]. Each column represents the mean ± SD of three independents test (Student's *t*-test). Significantly different from the control value : $P<0.05$ (*), $P<0.01$ (**).

Figure 3. Effect of the methanol extracts of the flesh of *A. cepa* on B16 melanoma cells. Each column represents the mean ± SD of three independents test (Student's *t*-test). Significantly different from the control value : $P<0.05$ (*), $P<0.01$ (**).

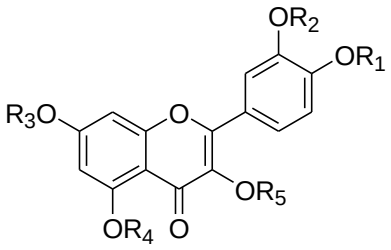
Figure 4. HPLC analysis of the methanol extracts of dried skin of *A. cepa* [1. Quercetin, 2. Isoquercitrin, 3. Quercetin 4'-*O*-glucoside, 4. Quercetin 3,4'-*O*-diglucoside, Flow rate : 1 mL/min of MeOH (60): Water (40); Sample concentration : 1 mg/mL, Wave length 250 nm, Injection volume: 20 μL]

Figure 5. HPLC analysis of the methanol extract of flesh of *A. cepa* [1. Quercetin, 2. Isoquercitrin, 3. Quercetin 4'-*O*-glucoside, 4. Quercetin 3,4'-*O*-diglucoside Flow rate : 1 mL/min of MeOH (60): Water (40); Sample concentration : 11 mg/mL, Wave length 250 nm, Injection volume: 20μL]

Legend of Tables

Table 1. Effect of quercetin and its derivatives on B16 melanoma cells (n = 3)

Table 2. Effect of quercetin and its derivatives on ORAC assay (n = 3)



1: $R_1 = R_2 = R_3 = R_4 = R_5 = H$
2: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = glc
3: R_1 = glc, $R_2 = R_3 = R_4 = R_5 = H$
4: R_1 = glc, $R_2 = R_3 = R_4 = H$, R_5 = glc
5: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = rha
6: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = gal

Figure 1.

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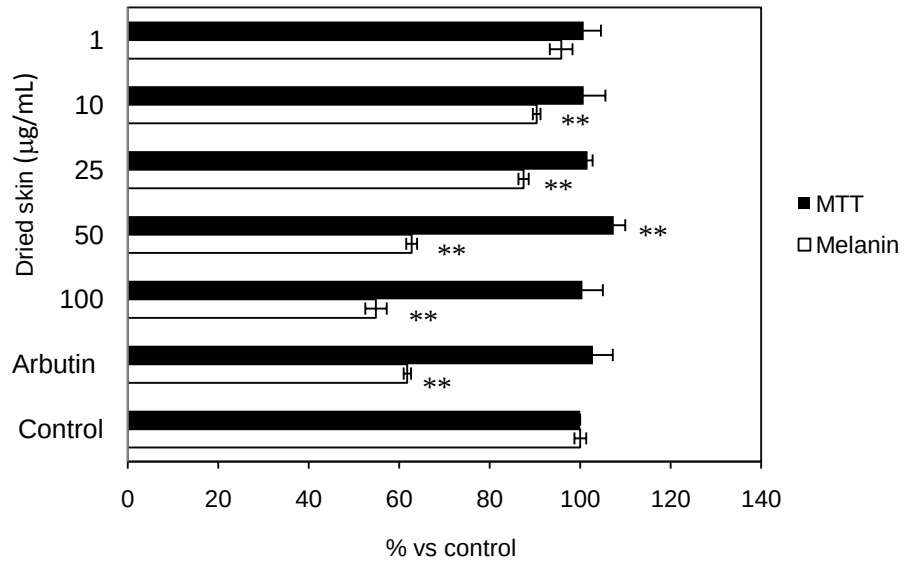

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420 **Figure 2.**

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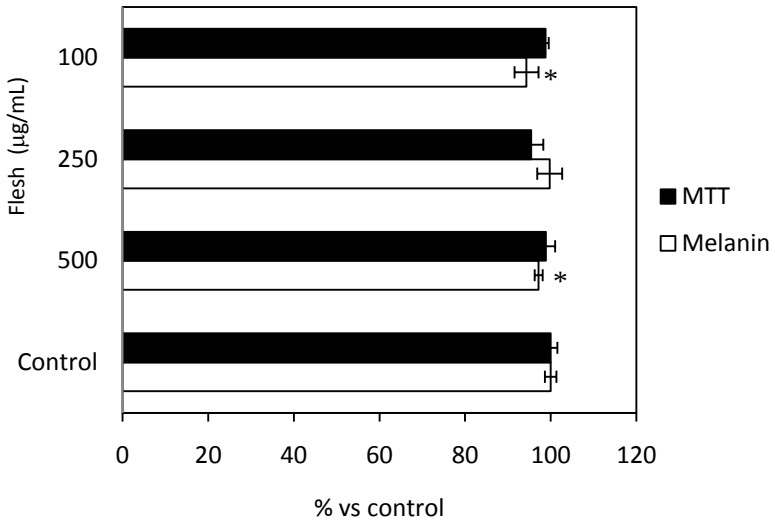

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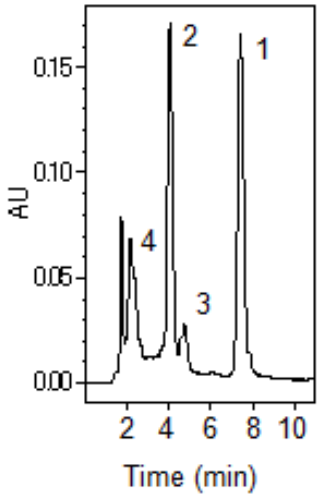
424 **Figure 3**.



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426 **Figure 4**.

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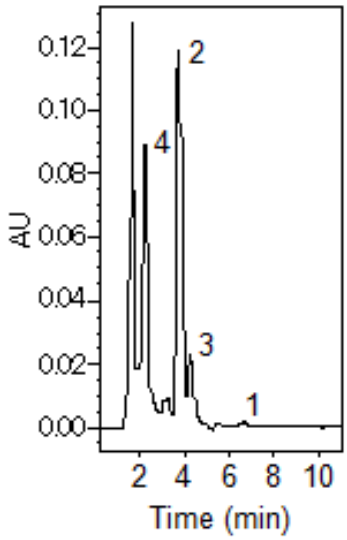

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430 **Figure 5**.

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