Tyrosinase inhibitory activity of Sargassum plagyophyllum and Eucheuma cottonii methanol extracts

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Abstract. Tyrosinase is an enzyme that plays a role in the formation of melanin on two reactions, namely monophenolase and diphenolase. The process of melanin formation can be reduced through the inhibition mechanism of the tyrosinase enzyme. Seaweeds are natural organisms that have recognized lightening agents. Sargassum plagyophyllum contains secondary metabolite compounds such as alkaloids, steroids, flavonoids, saponins, and tannins while Eucheuma cottonii contains alkaloids and terpenoids. The aim of this research was to determine the tyrosinase inhibitory activity of seaweeds. The yield of methanol extract amounted to 1.342% in S. plagyophyllum and 0.2599% in E. cottonii. The inhibitory activity of tyrosine is performed by measuring enzymatic reaction using L-tyrosine (monophenolase) and L-DOPA (diphenolase) substrates and presented as IC50 value. The IC50 values of kojic acid as a positive control were 15.566 μg/mL for monophenolase and 29.156 μg/mL for diphenolase. The IC50 values of S. plagyophyllum and E. cottonii methanol extracts for monophenolase were 2195.206 μg/mL and 2691.478 μg/mL, respectively. The IC50 value of methanol extract was 1769.336 μg/mL for S. plagyophyllum and 2631.648 μg/mL for E. cottonii on diphenolase. Methanol extracts from these two species of seaweed have the potential to be developed as bioactive compounds in skin lightening cosmetics.

Keywords: Eucheuma cottonii, Sargassum plagyophyllum, tyrosinase

1. Introduction

Melanin is a major defense mechanism against ultraviolet light and plays an important role in the prevention of sun-induced skin damages [1]. Melanin pigments are produced in melanocyte cells. Melanin formation (through browning reactions) in human skin occurs in the presence of a catalyst (a tyrosinase enzyme form) and UV light, which can lead on hyperpigmentation [2]. The skin naturally stimulates tyrosinase enzyme for melanin production to protect skin damage by ultraviolet A (UVA) and ultraviolet B (UVB) [3]. Indonesia is a tropical country with stinging and disturbing solar radiation, but melanin production can be disrupted in skin. Tyrosinase (EC 1.14.18.1) is a polyphenol oxidase (PPO) copper-containing enzyme that catalyzes two different reactions, both utilizing molecular oxygen: hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by monophenolase reaction and oxidation of L-DOPA to DOPAquinone by diphenolase reaction [4].
The process of melanin formation can be reduced by a tyrosinase enzyme inhibitor. Tyrosinase inhibitors are compounds that can inhibit the formation of melanin. These compounds are the latest approach used for skin lightening. Many synthetic compounds have been demonstrated to show inhibitory effect against tyrosinase enzyme and melanocyte on melanogenesis, such as kojic acid, mercury, hydroquinone, and arbutin. However, they have dangerous side effects in long-term use. Therefore, bioactive and harmless compounds are needed from natural sources with tyrosinase inhibitory activity. Natural raw materials from marine organisms that can be used as tyrosinase inhibiting agents include the ones that can be obtained from *Sargassum* (Phaeophyta) and *E*. *cottonii* (Rhodophyta).

*Sargassum plagyophyllum* has a main stem and cylindrical branching. Nevertheless, the stem and its branches are slippery (not thorny), and grow on rock substrate in the flat reef area. Fucoxanthin and xanthophyll pigments give a brown color to brown seaweed [5]. The IC\textsubscript{50} values of *S. plagyophyllum* fresh and dried powder are generally around 4,970 μg/mL and 11,350 μg/mL on monophenolase reactions [6]. Tyrosinase inhibitory activity in *Sargassum* sp. (CP 01) methanol extract is generally 27.50 μg/mL on L-Tyrosine substrate (monophenolase) and 209.06 μg/mL on L-DOPA substrate (diphenolase). Bioactive compounds consist of flavonoids, saponins, phenols, steroids and terpenoids [7]. *Sargassum* sp. (CP 02) methanol extract generally has an IC\textsubscript{50} value of 13.43 μg/mL on L-Tyrosine substrate (monophenolase) and 11.60 μg/mL on L-DOPA substrate (diphenolase) [8]. Some brown seaweeds have an inhibitory percentage against tyrosinase, such as *Ecklonia stolonifera* (79%), *E*. *cava* (72.1%), *S*. *miyabei* (48.5%) and *S*. *thunbergii* (46.7%) [9]. *S. silquastrum* extract can be used as a raw material in making lightening cosmetics because it has a tyrosinase inhibitory activity of 50% [10].

Ethanol extract of *S. polycystum* is known to contain a total phenolic compounds of 8287.18 mg GAE/g, flavonoids, steroids and triterpenoids [11]. The presence of phlorotannin compounds plays a role in protecting skin from being damaged by free radicals caused by UV exposure, which can inhibit the formation of melanin [12].

*E. cottonii* has green, yellow gray, or red color, and if dry it will be brownish yellow, with a cylindrical thallus, slippery surface and rubbery appearance [13]. It is mostly obtained from cultivation. *E. cottonii* cultivation is carried out in tidal areas up to 15 m in depth, and the species only takes about 45-50 days to grow. *E. cottonii* requires an environment consisting mostly of sand and coral, moderate water movement, and salinity ranging from 29-34 ppt [14]. *E. cottonii* is known to contain a lot of chlorophyll with around 74.920%, followed by xanthophyll 7.715% and carotene 0.947% [15]. *Sargassum* sp. and *E. cottonii* generally have a vitamin E content of 165.19 μg/mL and 160.01 μg/mL, respectively [16]. *E. cottonii* methanol extract is known to have a IC\textsubscript{50} value of 234.33 μg/mL on diphenolase reactions [17]. *E. cottonii* has a vitamin E content of 158.07 μg/mL, moisture 76.15%, ashes 5.62%, proteins 2.32%, fat 0.11%, carbohydrates (by difference) 15.8% and consists of flavonoids, phenol hydroquinone and triterpenoids [18]. It also consists of alkaloid [19], total phenol 141.00 mg GAE/g in methanol extract and flavonoids 17.78 mg QE/g in methanol extract [20]. The aim of this research was to obtain the tyrosinase inhibition value (IC\textsubscript{50} value) on monophenolase and diphenolase from raw natural materials such as these seaweed species.

2. Materials and Methods

2.1. Obtaining seaweeds

*S. plagyophyllum* specimens were obtained from Pasauran coastal waters and *E. cottonii* specimens were obtained from the results of community cultivation in Lontar village, Serang, Banten. *E. cottonii* samples were dehydrated back at the Laboratory of Aquatic Raw Materials, Bogor Agricultural University, under the sunlight so that they could be preserved and stored for a long time. *S. plagyophyllum* samples were fresh and immediately transported into the Laboratory of Aquatic Raw Materials using cool boxes and washed with saline solution. This washing was to minimize sand, mud, and mineral salts. The purpose of washing was to eliminate the fishy smell and stench found on brown algae [21]. The next process involved air-drying the seaweed material for 5-6 days [22]. This process was intended to maintain the seaweed’s chemical contents. Identification of brown seaweed species was carried out by the Center for Oceanographic Research, Indonesian Institute of Sciences (LIPI) Ancol.
2.2. Manufacturing seaweed coarse powder
Dried-seaweed raw materials of *E. cottonii* and *S. plagyophyllum* were reduced in size by crumbling the seaweed using a dish meal tool. The next step was to sift using a 60 mesh sieve to obtain the coarse powder. Size reduction was aimed to minimize the seaweed surface area so as to maximize the extraction process and obtain the finer fibers by grinding the thallus of dried seaweed. Low moisture content and high fiber caused the thallus to be very clayey and difficult to crumble. Calculation of seaweed coarse powder yield was obtained by comparing the weight after and prior to size reduction and expressed in percentage form.

2.3. Extracting seaweed
Extraction of bioactive compounds from the seaweeds *E. cottonii* and *S. plagyophyllum* was carried out by the maceration modification method performed previously [23]. The maceration method was done by immersing the samples in solvent for a certain time. Each seaweed coarse powder was weighed, and as much as 100 grams were put into a 1,000-mL beaker glass and soaked with methanol grade pro analyzer (PA) solvent with sample and solvent ratio 1:5 (g/mL). The beaker glass was covered with aluminum foil (alufo). Samples maceration was carried out for three days with solvent replacement daily (24 hours) at 37°C. The purpose of soaking and replacing solvent was to maximize the extraction process so that more bioactive compounds were obtained. The maceration solution was filtered with a white cloth (calico) and filter paper to obtain the filtrate and residue. Filtrates from each extraction were gathered. Methanol solvents that coined in filtrates were separated by an evaporation method using a rotary vacuum evaporator at 40-50°C. Crude methanol extracts of *E. cottonii* and *S. plagyophyllum* obtained were then scraped off and stored into black glass bottles. The extract yields were calculated by using the comparison between end weight of evaporation results and sample weight expressed in percentage form. Tyrosinase inhibitory testing was carried out on each of the crude extracts.

2.4. Tyrosinase inhibitory activity test
Tyrosinase inhibitory activity testing was carried out to determine whether the presence of inhibitory power of bioactive compounds was contained in seaweed extracts against the enzyme tyrosinase from fungi. Inhibitory tyrosinase activities of *E. cottonii* and *S. plagyophyllum* crude methanol extracts were determined by a spectrophotometric method using a microplate reader ELISA as outlined in [24]. Crude extracts were dissolved in DMSO to a final concentration of 50,000 µg/mL. Extract stock solutions were prepared by dissolving the concentrated extracts into 50 mM phosphate buffer (pH 6.5) up to 10,000 µg/mL concentration. Crude extracts were tested at concentrations ranging from 2,000 to 10,000 µg/mL. Kojic acid, as a positive control, was tested at concentrations of 31.25 to 250 µg/mL in a 96-well plate. Also in a 96-well plate, 70 µL of extract dilution was combined with 30 µL tyrosinase enzyme (Sigma, 333 unit/mL in phosphate buffer pH 6.5) and incubated for five minutes. One hundred and ten microliters of substrate (2 mM L-Tyrosine and 2 mM L-DOPA) was added and incubated for 30 minutes at room temperature. Each well absorbance was measured by using a microplate reader at a 475 nm wavelength to determine the percentage inhibition and 50% inhibitory concentration (IC50). Inhibition percentage was calculated by comparing the sample absorbance without extract addition (A) and with extract addition (B).

3. Results and Discussion

3.1. Coarse powder seaweed yield
Brown seaweed identification done by the Center for Oceanographic Research, Indonesian Institute of Sciences (LIPI) Ancol, confirmed the identification of *S. plagyophyllum* (Mertens) J.G. Agardh. Coarse powder yields of *E. cottonii* and *S. plagyophyllum* were 53.394% and 52.158%, respectively. These results were higher than results from similar research reporting the *E. cottonii* and *S. plagyophyllum* yields from the manufacturing of coarse powder using the same modification tool, followed by dry blender and sieved with 48 mesh size as being 6.79% and 4.77%, respectively [25]. We believe the manufacturing of coarse powder using dry blender did not get high yield due to the fact that not all dried-seaweed can be crumbled and this can damage the chemical contents yield.
3.2. Seaweed extract yields
Bioactive compounds were obtained by extracting using solvent [26]. Methanol solvent was chosen due to its being a polar solvent that can extract non-polar, semi-polar and polar compounds which were abundant in seaweed. Yield was an important parameter to determine the economic value and effectiveness of material or product. The higher the material yield value, the higher the economic value as well as its utilization [8]. Methanol extract yield of *E. cottonii* was 0.2599% and of *S. plagyophyllum* was 1.342%. This result was different from previously reported research where *Sargassum* sp. (CP 01) yield was 6.02% [8] and *Sargassum* sp. (CP 02) was 2.37% [9], *E. cottonii* yield were 6.6% [18] and 9.33% [19]. Differences in yield can occur due to different preparation materials and are affected by harvest time (age), environmental factors, extraction method used, and stirring for maceration held. Extract yield variation is also known to depend on the natural conditions of samples, extraction method, sample particle size, conditions and extraction time, as well as the comparison of sample with solvent [27].

3.3. Tyrosinase inhibitory activity
Tyrosinase inhibition results were expressed with IC$_{50}$ value at the end concentration of test solution. IC$_{50}$ value of kojic acid (as positive control), *E. cottonii* and *S. plagyophyllum* methanol extract on L-Tyrosine substrate were 15.566 $\mu$g/mL; 2,691.478 $\mu$g/mL and 2,195.206 $\mu$g/mL, respectively. IC$_{50}$ value of kojic acid (as positive control), *E. cottonii* and *S. plagyophyllum* methanol extract on L-DOPA substrate were 29.156 $\mu$g/mL, 2,631.648 $\mu$g/mL and 1,769.336 $\mu$g/mL, respectively. This indicates that *S. plagyophyllum* and *E. cottonii* extract act more effectively on diphenolase reaction and kojic acid acts more effectively on the monophenolase one. Generally, tyrosinase has tyrosine hydroxylation activity, L-DOPA oxidation, and hydroxyindole oxidation. Tyrosinase can be inhibited by using active compounds such as flavonoids. *Sargassum plagyophyllum* contained bioactive compounds such as alkaloids, steroids, flavonoids, saponins, and tannins, whereas *E. cottonii* contained alkaloids and terpenoids [6]. Flavonoids ability as skin depigmentation was directly inhibiting tyrosinase activity in melanogenesis process. A bond flavonoid with copper and antioxidant effect plays a role in inhibiting the tyrosinase enzyme action [28].

This is supported by similar research conducted by [9]. Tyrosinase inhibitory activity of *Sargassum* sp. (CP 02) methanol extract originating from Cipatujah Coastal Waters, Tasikmalaya, expressed in IC$_{50}$ value were 13.43 $\mu$g/mL on L-Tyrosine substrate and 11.60 $\mu$g/mL on L-DOPA substrate with IC$_{50}$ value of kojic acid on L-Tyrosine and L-DOPA substrates 3.25 $\mu$g/mL and 14.27 $\mu$g/mL, respectively [9]. IC$_{50}$ value of *Sargassum* sp. (CP 01) methanol extract originating from Cipatujah Coastal Waters, Tasikmalaya on L-Tyrosine and L-DOPA substrates were 27.50 $\mu$g/mL and 209.06 $\mu$g/mL, respectively [8]. This indicates that the extract acted more effective on monophenolase reaction. *Sargassum polycystum* originating from Malaysian Island extracted using polar solvents such as ethanol and water had an inhibitory percentage of tyrosinase enzyme, respectively 97.78% and 99.49% at 100 $\mu$g/mL concentration [23]. IC$_{50}$ value of *E. cottonii* methanol extract originating from Semporna Coastal Area of Sabah, Malaysia was 234.44 $\mu$g/mL on diphenolase reactions [3]. The differences in extract ability in inhibiting tyrosinase enzyme were caused by differences in bioactive compounds contained in extracts originating from different species, extraction method, and the purity of extract. Active compounds in a substance are influenced by various factors including age, environmental conditions, place of growth and the presence of nutrients and minerals in its different places of origin [24]. Specific enzyme activity is expressed in unit/mL or unit/mg. Several factors that influence enzyme activity are enzyme concentration, substrates, product, inhibitors and activator compounds, pH, solvent type, ionic strength and temperature [29].

Kojic acid has tyrosinase activity and acts more active on monophenolase reaction. IC$_{50}$ value of kojic acid was differently affected by period (time) and storage conditions prior to testing. IC$_{50}$ value of kojic acid was lower than sample extracts. This was affected by the extract conditions, which was a crude extract and which had not been purified, so it was suspected there were other compounds that did not act as tyrosinase inhibitors. Kojic acid has an inhibitory effect (inhibition of tyrosinase enzyme) and the greatest stability in cosmetic products [30], but it is carcinogenic and its use in high concentration can damage the skin [31].
4. Conclusion

The yield of methanol extract amounted to 1.342% for *S. plagyophyllum* and 0.2599% for *E. cottonii*. The IC50 values of kojic acid as a positive control were 15.566 μg/mL for monophenolase and 29.156 μg/mL for diphenolase. The IC50 values of *S. plagyophyllum* and *E. cottonii* methanol extracts for monophenolase were 2,195.206 μg/mL and 2,691.478 μg/mL, respectively. IC50 value of methanol extract was 1,769.336 μg/mL for *S. plagyophyllum* and 2,631.648 μg/mL for *E. cottonii* on diphenolase. *S. plagyophyllum* and *E. cottonii* extracts acted more effectively on diphenolase reaction by inhibiting the oxidation of L-DOPA to DOPAquinone, because this seaweed could inhibit the reaction of monophenolase and diphenolase, *S. plagyophyllum* and *E. cottonii* extracts can be used as active skin lightening ingredients and formulated into cosmetic preparations.

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