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Investigation of DPPH Radical Scavenging, Antioxidant and Melanogenesis Stimulating Activities of Various Pigment Extracts from Thai Herbal Plants.

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ABSTRACT

The purpose of this study was to investigate the DPPH radical scavenging, antioxidant and melanogenesis stimulating activities of various pigment extracts from Thai herbal plants. All ethanolic extracts were determined for their antioxidant activity by DPPH assay and FRAP assay. The melanogenesis stimulating activities were studied by the stimulation of tyrosinase enzyme activity and melanocyte proliferation of these extracts at various concentrations (50, 100 and 500 µg/mL) was evaluated by MTT assay. The results indicated that high potential of DPPH radical scavenging was achieved from both extracts of *S. cumini* (151.67 µg/mL) and *G. magostana* (153.00 µg/mL). In addition, the extract of *G. magostana* showed strongest value for determination of FRAP assay. Among all tested extracts, the strongest stimulating of tyrosinase enzyme activity was obtained from the extract of *C. sappan* (95.75 %) whereas the extract of *S. cumini* exhibited lowest stimulating activity (27.11%). From the determination of stimulating activity of the melanocyte proliferation, three extracts of *C. ternatea*, *A. puncticulatum* and *O. sativa* could stimulate the melanocyte proliferation among all tested extracts, and the proliferation index (P.I.) of these extracts (500 µg/mL) were 1.5, 1.3 and 1.3, respectively. Therefore, the results of this study attributed to the high potential activity of appropriate pigment extract from Thai herbal plant for further study on grey hair prevention agent.

Keywords: Antioxidant activity; melanogenesis stimulating activities; melanocyte proliferation; pigment extract

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INTRODUCTION

Hair graying is one of the typical signs of human aging, and the maintenance of hair pigmentation is dependent on the presence and functionality of follicular melanocytes [1]. This change is based on an amount and classification of melanin, resulting from the complex genetic control. It is produced by the follicular melanocytes which derived from the neural crest cells locate in the hair follicles and produces the content of melanins by the melanogenesis pathway. Melanogenesis, a complicated pigment biosynthesis, involves the oxidative reaction of tyrosine to be either brown/black eumelanin or yellow/red pheomelanin, depending on the cysteine or glutathione existing. These produced melanins are packed into granules known as melanosomes and then transferred to the cortical keratinocytes. The onset of white hair in Thailand are at the late 30 years old and in the mid of 40 years old [2-4]. Currently, there is no treatment procedure for this condition and the masking of white hairs with hair dye is popularly used. Nevertheless, the hair masking with hair dye has been reported to have the numerous harmful effects, such as dermatitis, hair loss and cancer [5]. Many researchers have investigated the low toxic compounds which can induce the melanogenesis pathway. For the development of gray hair prevention agent, we have carried out a screening program to find a potential stimulant of melanogenesis from the natural resources by using cultured murine B_{16} melanoma cells with theophylline as a reference drug [3, 6-8]. Based on the result, theophylline could enhance the pigmentation in cultured murine B_{16} melanoma cells without any effects on cell proliferation [3, 6-8]. Somvong et al. [2] investigated the antioxidant and melanogenesis stimulating activity of Thai traditional medicinal extracts included aqueous, ethyl acetate, methanol and hexane extracts of *Tiliacora triandra*, *Centella asiatica*, *Clitoria ternatea*, *Morus alba* and *Pueraria mirifica*. The result found that ethyl acetate extract of *T. triandra* exhibited the strongest stimulating activity with percentage of stimulation about 94.34. Among all tested extracts, the stimulating activity on melanocyte proliferation of methanol extract of *C. ternatea* and aqueous extract of *T. triandra* had strong stimulating activities with the proliferation index (P.I.) of 1.7 and 1.6, respectively.

Therefore, the objective of the present study was to investigate the DPPH radical scavenging, antioxidant and melanogenesis stimulating activities of various pigment extracts from Thai herbal plants e.g. *Oryza sativa L.*, *Clitoria ternatea L.*, *Zea mays ceratina*, *Syzygium cumini L.*, *Hibiscus sabdariffa Linn.*, *Caesalpinia sappan L.*, *Antidesma puncticulatum Miq.* and *Garcinia magostana Linn.*

MATERIALS AND METHODS

Materials

2,2-diphenyl-1-picrylthydrazyl (DPPH) (Sigma Aldrich, USA), Ascorbic acid, α-tocopherol (Nam Siang Co., Ltd., Bangkok, Thailand) were used as received. All pigment plants were obtained from Agricultural Technology Department, Thailand Institute of Scientific and Technological Research. All other chemicals were of standard chemical grade.

Preparation of ethanolic extracts

All pigment plants e.g. *Oryza sativa L.*, *Clitoria ternatea L.*, *Zea mays ceratina*, *Syzygium cumini L.*, *Hibiscus sabdariffa Linn.*, *Caesalpinia sappan L.*, *Antidesma puncticulatum Miq*. and *Garcinia magostana Linn* were prepared by maceration. Initially, 500 g of herb powders was accurately weighed, mixed with 70% ethanol for 4 nights, filtered through Whatman paper No. 41 and rinsed in the same solvent. The solvent was removed under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision) at 45 ^{0}C.

Determination of total phenolic contents

The total phenolic contents of eight extracts were determined according to the method described by other studies [9 - 11] and modified the procedures of measurement by using a microplate reader. Briefly, 10 µL of each extract was transferred into a 96-well microplate containing 160 µL of distilled water. After mixing the contents, 10 µL of Folin-Ciocalteu reagent and 20 µL of a saturated sodium carbonate solution were added. The plate was mixed well and the absorbance of blue mixtures was recorded at 750 nm with microplate reader after 30 min incubation. The readings of sample and reagent blanks were subtracted from the reading of

reagent with extract. The total phenolic contents were calculated as a gallic acid equivalent (GAE) from a calibration curve of gallic acid standard solutions (ranging from 25 to 800 mg/mL), and expressed as mg of gallic acid per 100 gram of dry sample. All measurements were done in triplicate.

Determination of DPPH radical scavenging activity

The effect of pigment extracts on the DPPH radical was adapted from other reports [12, 13, 14]. 50 µg of extract was accurately weighted and dissolved in 1 mL of 20% DMSO. After that, the solution of extract (50 µg/mL) was mixed with 100 µM DPPH in absolute methanol and adjusted to the final volume at 2000 µL. The sample was incubated at 37 ^{0}C for 20 min and then evaluated the absorbance by using a microplate reader (Sunrise, Tecan Co., Austria) at 517 nm. The absorbance of sample and control were calculated on the effective concentration at 50% (EC_{50}) according to the following equation as: EC_{50}= [(Abs. control – Abs. sample)/ Abs. control] x100.

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Determination of ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP)

The effect of pigment extracts on the ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP) was adapted from another report [15]. First, a solution of ferric chloride solution (20 mM) was prepared in acetate buffer (300 mM, pH 3.6) and TPTZ (2,4,6-Tris(2-pyridyl)-1,3,5-triazine, 10 mM) solution. These solutions were vortexed thoroughly and incubated in dark. Second, 1 mg/mL of sample was dissolved in 20 % DMSO and mixed with FRAP reagent and distilled water. After that, the sample solution was adjusted to the final volume (2,000 µL) with distilled water. The absorbance value of the reaction mixture was recorded at 593 nm. The relative antioxidant activity (FRAP value) of sample was calculated using a standard curve of Ferrous sulfate ($FeSO_4$). The sample was performed in triplicate.

Determination of stimulation of tyrosinase enzyme activity

The stimulation of tyrosinase enzyme activity was tested by tyrosinase activity assay as adapted from other studies [8, 10, 16]. Each extracts was diluted with distilled water at 20 and 50 mg/mL. The dilution of extract was mixed with tyrosinase enzyme (100 µg/mL) in phosphate buffer pH 6.8 and then incubated at 37 ^{0}C for 10 min. After that, the samples were added with 2 mM tyrosine and incubated at 37 ^{0}C for 40 min. After incubation, the absorbance of solution was determined by using a microplate reader (Sunrise, Tecan Co., Austria) at 450 nm. The tyrosinase stimulation was calculated according to the following equation as: % stimulation = [(A-B)/A] x 100 where A is the absorbance of extract solution, and B is the absorbance of control. The sample was performed in triplicate.

Determination of stimulation of melanocyte proliferation

The stimulation of melanocyte proliferation of various pigment extracts was tested by MTT assay with mouse melanoma cells (B16F10) [7, 8]. Briefly, mouse melanoma cells (B16F10) were seeded at the density of 1.5 x 10^4 cells/well in 96-well plates and incubated at 37 ^{0}C for 24 h under 5% CO_2 atmosphere for cell adhesion. Then, the cell was diluted with completed medium DMEM at different concentrations (50, 100 and 500 µg/mL). After that, 100 µL of different concentrations of all pigment extracts were added in the 96 well plate system and then incubated at 37 ^{0}C for 72 h. After incubation, 50 µL of MTT in PBS at 1 mg/mL was added to the medium in each well and incubated for 4 -6 h. Medium and MTT were then removed from the well and solubilized with 100 µL of DMSO. The absorbances of all samples were assessed by using a microplate reader (Sunrise, Tecan Co., Austria) at 570 nm. The proliferation index (P.I.) was calculated according to the following equation as: proliferation index (P.I.) = [Mean absorbance of sample well x 100] /Mean absorbance of control. The sample was performed in triplicate.

Data analysis

All experimental measurements were triplicate performed. Result values were expressed as mean value ±standard deviation. Statistical significance in this study was examined using analysis of variance (ANOVA). The value of p <0.05 was considered statistical significant.

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RESULTS AND DISCUSSION

Total phenolic contents

Total phenolic contents were determined spectrophotometrically by the most common method of Kruawan and Kangsadalampai [13] using the Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalents. The total phenolic contents was calculated using the standard curve of gallic acid (R^2 = 0.9995). The results were shown in Table 1. It was found that the total phenolic contents among different extracts were as follows: *S. cumini* > *G. magostana* > *C. ternatea* >*O. sativa*> *Z. mays ceratina*> *A. puncticulatum* > *C. sappan* > *H. sabdariffa*. The result indicated that the highest significantly (p < 0.05) total phenolic contents were found in *S. cumini* (533.03 mg gallic acid equivalent/ 100 g sample) and *G. magostana*. (522.10 mg gallic acid equivalent/ 100 g sample) whereas *H. sabdariffa* (249.67 mg gallic acid equivalent/ 100 g sample) showed the lowest significantly (p < 0.05) total phenolic contents. The high total phenolic contents might be linked to high antioxidant activity.

Table 1: Total phenolic content (mg gallic acid equivalent/ g sample) of different extracts

Different pigment extracts	Part used of the studied plants	Total phenolic content (mg gallic acid equivalent/ g sample)
1. *Oryza sativa L*	Seed	508.23± 2.17
2.*Syzygium cumini L.*	Fruit	533.03 ± 2.78
3.*Hibiscus sabdariffa Linn*	Flower	249.67 ± 1.09
4.*Clitoria ternatea L.*	Wood	301.24 ± 3.09
5.*Zea mays ceratina*	Fruit	417.00 ± 2.98
6.*Caesalpinia sappan L.*	Flower	513.45 ± 3.56
7. *Antidesma puncticulatum Miq*	Fruit	415.36 ± 3.64
8. *Garcinia magostana Linn.*	Peel	522.10 ± 2.89

DPPH radical scavenging activity

The antioxidant activity was evaluated by a widely used and convenient method – the DPPH radical scavenging assay [17, 18]. This method has been used as a model system to evaluate the scavenging activity of various natural compounds such as phenolic compounds, anthocyanins or crude extracts of plants. The antioxidant activity and total phenolic contents vary considerably among all herbs. The decrease in DPPH by antioxidants in the herbal extracts was expressed in term of percentage of radical scavenging activity (from highest to lowest). Furthermore, the DPPH radical could be directly scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H•. This was indicated by the change in color from purple to yellow after reduction, and it could be quantified by the decrease in absorbance at wavelength 517 nm. From the result, the radical scavenging activity of all pigment extracts form Thai herbal plants were expressed in term of the effective concentration at 50% (EC_{50}, µg/mL). As shown in Table 2, the scavenging activity against DPPH radical was in the range of 150 – 1000 µg/mL. The DPPH radical scavenging activity of different pigment extracts were as follows: *S. cumini* > *G. magostana* >*O. sativa* > *A. puncticulatum* > *C. sappan* > *H. sabdariffa* > *C. ternatea* > *Z. mays ceratina*. The extracts of *S. cumini* and *G. magostana* had the high potential of antioxidant activity with the EC_{50} about 151.67 µg/mL and 153.00 µg/mL, respectively compared with other extracts. Nevertheless, *Z. mays ceratina* showed the significantly lowest (p < 0.05) antioxidant activity and the EC_{50} value was 973.33 µg/mL compared with other extracts. The pigment extracts with the high antioxidant activity indicated the relatively high total phenolic contents and there was a statistically significant (p < 0.05) correlation between radical scavenging activity and total phenolic contents. The antioxidant properties of herbs may be attributed to the plant pigments that are the main components of each herbal extract which may act as an antioxidant [17, 18].

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Ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP)

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a coloured ferrous tripyridyltriazine [Fe^{2+}-TPTZ] [19]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom [20]. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction [20]. For the determination of ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) among pigment extracts, *G. magostana* showed the significantly ($p < 0.05$) strongest value (2255.00 FeFug/mL) while *C. sappan* exhibited the lowest value (1351.67 FeFug/mL) compared with the other extracts as shown in Table 2. The high value of FRAP was attributed to the high antioxidant of samples that was in accordance to the ferric ions (Fe^{3+}) - ferrous ions (Fe^{2+}) transformation [22]. The results on reducing power exhibited the electron donor properties of active ingredient from extract and hence neutralizing free radicals by forming stable products was occurred. The outcome of the reducing reaction resulted in termination of radical chain reactions, and it may otherwise be very damaging [22-23]. The result of total phenolic contents was related to the values of ferric ion reduction (FRAP value) and the scavenging activity (DPPH assay). Hence, they should be able to donate electrons to free radicals stable in the actual biological and food system [24].

Table 2: DPPH Radical scavenging (EC_{50}, µg/mL) and FRAP value (FeFug/mL) of various pigment extracts from Thai herbal plants

Different pigment extracts	DPPH (EC_{50}, µg/ml)	FRAP value (FeFug/ml)
1. *Oryza sativa L*	169.67 ± 2.51	1643.89 ± 6.03
2.*Syzygium cumini L.*	151.67 ± 7.57	1736.11 ± 1.55
3.*Hibiscus sabdariffa Linn*	749.67 ± 2.26	1391.67 ± 2.07
4.*Clitoria ternatea L.*	712.00 ± 4.03	1351.67 ± 3.12
5.*Zea mays ceratina*	973.33 ± 4.19	1456.94 ± 5.64
6.*Caesalpinia sappan L.*	754.00 ± 3.05	1429.44 ± 5.55
7. *Antidesma puncticulatum Miq*	226.67 ± 7.64	1622.22 ± 9.27
8. *Garcinia magostana Linn.*	153.00 ± 6.24	2255.00 ± 246.82

Stimulating activity of tyrosinase enzyme

Tyrosinase (monophenol, 3, 4-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a single-chain glycoprotein enzyme essential to pigment formation in mammals which is a specifically localized in the melanocyte pathway. It is occurred primarily in the skin, hair bulbs, and eyes. The principal subcellular site of tyrosinase activity has been determined to be the melanosome while, tyrosinase is shown in the soluble, ribosomal, endoplasmic reticulum and Golgi apparatus fractions [25]. In this study, the stimulation of tyrosinase enzyme activity was tested by tyrosinase activity assay as described in other reports [8, 10, 16]. The stimulating activity of tyrosinase enzyme of all pigment extracts at both concentrations (20 and 50 mg/mL) is shown in Figure 1. The results demonstrated that seven extracts i.e., *G. magostana*, *O. sativa*, *A. puncticulatum* , *C. sappan*, *H. sabdariffa*, *C. ternatea* , *Z. mays ceratina* were able to stimulate the tyrosinase enzyme activity, because, the stimulating value of enzyme tyrosinase of these extracts at 50 mg /mL were more than 80%. In addition, the strongest stimulating of tyrosinase enzyme activity was obtained from *C. sappan* (95.75%) while *S. cumini* (27.11%) showed the lowest stimulating activity. The stimulating of tyrosinase activity was in accordance with Somvong and Prasitpuriprecha [2] which investigated to the antioxidant and melanogenesis stimulating activity of Thai traditional medicinal plant extracts included aqueous, ethyl acetate, methanol and hexane extracts of *Tiliacora triandra*, *Centella asiatica*, *Clitoria ternatea*, *Morus alba* and *Pueraria mirifica*. It was found that the stimulating activity of tyrosinase enzyme of ethyl acetate extract of *T. triandra* had the strongest stimulating activity with % stimulation of 94.34. The higher concentrations of all

extracts, the higher stimulating of tyrosinase activity were obtained, because, the concentration of extract had ability to increase the biological activity.

Stimulating of melanocyte proliferation using mouse melanoma cells ($B_{16}F_{10}$)

Many researchers have investigated the active compounds for inducing the melanogenesis pathway. For the development of gray hair prevention agent, we had performed a screening program to find a potential active ingredient for the stimulation of melanogenesis of different pigment extracts from Thai herbal plants e.g. *Oryza sativa L.*, *Clitoria ternatea L.*, *Zea mays ceratina*, *Syzygium cumini L.*, *Hibiscus sabdariffa Linn.*, *Caesalpinia sappan L.*, and *Antidesma puncticulatum Miq*. and *Garcinia magostana Linn*. by using cultured murine B_{16} melanoma cells compared with theophylline as a reference drug. In this study, the stimulating of melanocyte proliferation using mouse melanoma cells of all pigment extracts with various concentrations (50, 100 and 500 µg/mL) were expressed in term of proliferation index (P.I.) as shown in Figure 2. The results demonstrated that three ethanolic extracts e.g. *C. ternatea*, *A. puncticulatum* and *O. sativa* could stimulate the melanocyte proliferation and the proliferation index (P.I.) of these extracts (500 µg/mL) were 1.5, 1.3 and 1.3, respectively. Among all tested extracts, the extract of *C. ternatea* showed the strongest stimulation activity of melanocyte proliferation and the proliferation index was increased from 1.2 to 1.5 when the concentrations of this extract was enhanced from 50 to 500 µg/mL. However, the lowest stimulating activity of melanocyte proliferation was obtained from both extracts of *Z. mays ceratina* and *H. sabdariffa*, because, the proliferation index (P.I.) of both extracts were less than 1.1 of all concentrations. The method of stimulating activity of melanocyte proliferation was in accordance with other reports [2, 26-27]. Somvong and Prasitpuriprecha found that the stimulating activity of melanocyte proliferation of *C. ternatea* and *T. triandra* had strong stimulating activities with proliferation index (P.I.) of 1.7 and 1.6, respectively [2]. Park and Kwona [26] reported that the extract of *Pueraria thunbergiana* could stimulate the melanogenesis and prevented the follicular depigmentation and vitiligo by stimulating melanin synthesis. Takekoshi et al. found that quercetin could enhance the expression of tyrosinase protein, leading in turn to the promotion of melanogenesis. It is expected that quercetin will be put to practical use in the management of vitiligo and acquired pigment loss like gray hair [27].

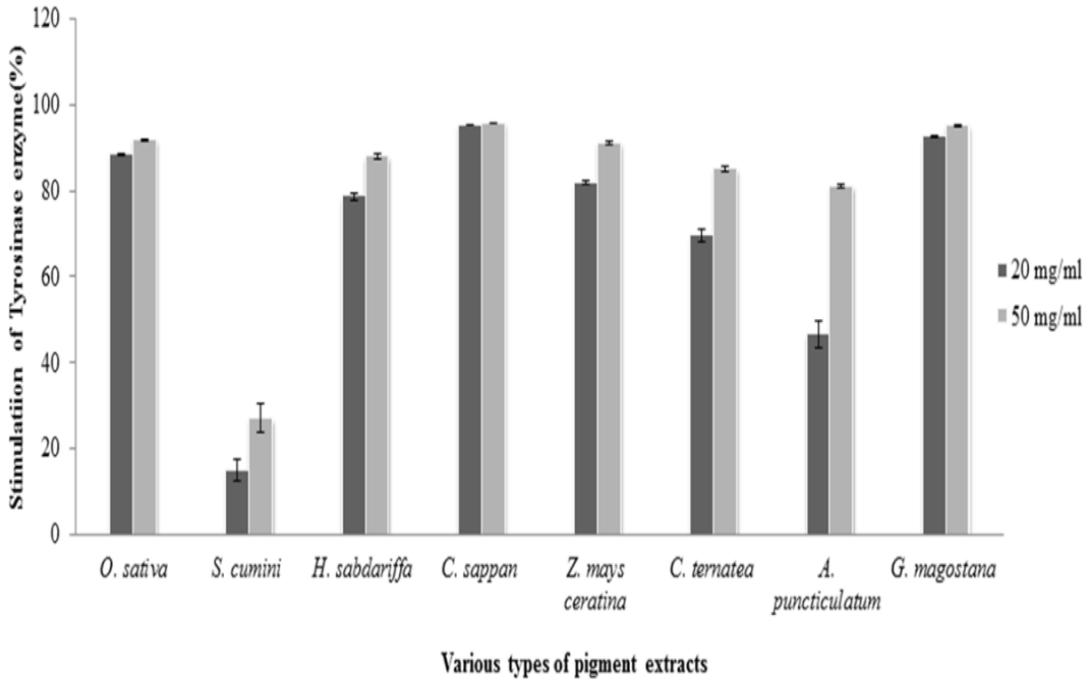

Figure 1: Stimulating activity of tyrosinase enzyme of various pigment extracts from Thai herbal plants.

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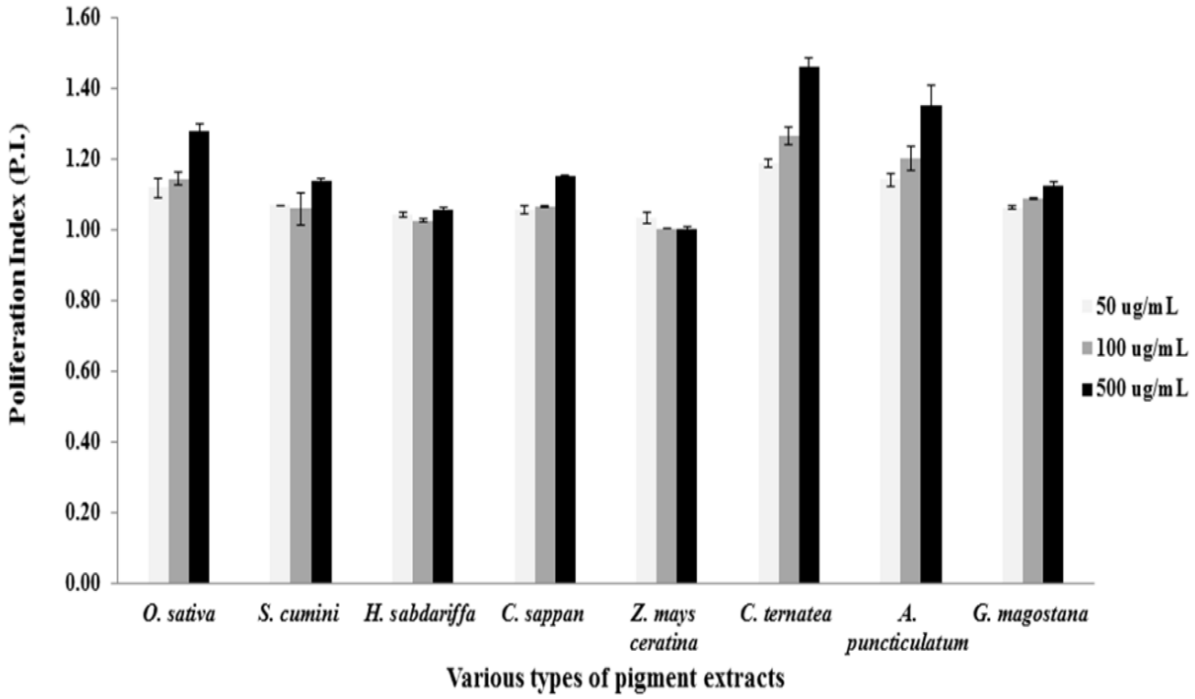

Figure 2: Stimulating of melanocyte proliferation using mouse melanoma cells ($B_{16}F_{10}$) of various pigment extracts from Thai herbal plants

CONCLUSION

From the result, it could be concluded that the extracts of *S. cumini* and *G. magostana* exhibited the high potential of antioxidant activity. In addition, extract of *G. magostana* showed strongest value for determination of ferric ions (Fe^{3+}) reducing antioxidant power. Among all tested extracts, the strongest stimulating of tyrosinase enzyme activity was obtained from extract of *C. sappan* (95.75%) whereas the extract of *S. cumini* exhibited lowest stimulating activity (27.11%). From the determination of stimulating activity of melanocyte proliferation, three extracts of *C. ternatea*, *A. puncticulatum* and *O. sativa* could stimulate the melanocyte proliferation among all tested extracts and the proliferation index (P.I.) of the extracts (500 µg/mL) were 1.5, 1.3 and 1.3, respectively. Therefore, a screening program to find a potential active ingredient for the melanogenesis stimulation of different pigment extracts from Thai herbal plants were observed. It was attributed to the high potential activity of suitable pigment extract from Thai herbal plant for further study on grey hair treatment formulation.

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