Determination of Free Radical Protective Activity from Hydrogen Peroxide, Antioxidant and Melanogenesis Stimulating Activities of *Eclipta prostrate* Linn. and *Sapindus rarak* DC. for Hair Dye Product

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Background: *Thai medicinal plant namely Eclipta prostrate Linn. (EP) and Sapindus rarak DC. (SR) are reported to improve hair growth and hair color. Theories of grey hair occurred from the gradual loss of pigmentation. Intrinsic factors are age, genetic, stress and extrinsic factor such as chemical that involved melanogenesis process and oxidative mechanism. Moreover, hydrogen peroxide (H_2O_2) is known as an oxidant which can destroy melanocytes cell. On the other hand, if the herb extracts can increase melanocytes cell and high antioxidant, they will protect melanocytes cell.*
Objective: *To investigate free radical protective activity from hydrogen peroxide, antioxidant and melanogenesis stimulating activities of Eclipta prostrate Linn. and Sapindus rarak DC.*
Material and Method: *The leaves of EP and the pericarps of SR were maceration in 95% ethanol (EPE95 and SRE95), 50% ethanol (EPE50 and SRE50), and were decoction with water (EPA and SRA). All extracts were tested antioxidant activity by DPPH radical scavenging assay and ABTS radical cation decolorization assay. The effect of extracts on melanocytes proliferation in mouse melanoma cell (B16F10) and in preventing cells from H_2O_2 by MTT assay were also investigated.*
Results: *The results showed that EPA had the highest DPPH radical ion inhibition with the EC_{50} value of 15.00 μg/ml. For ABTS assay, EPE_{50} and EPA showed the highest inhibitory effect on ABTs radical assay (EC_{50} = 54.24 and 30.28 μg/ml, respectively). For melanocytes proliferation assay, EPE95 showed the highest stimulating effect on melanocyte proliferation at the concentration of 50 μg/ml. Moreover, the SRA showed high activity on the effect of herbs in preventing cells from H_2O_2 at the concentration of 10 and 1 μg/ml.*
Conclusion: *These results can be concluded that SRA showed the effect of prevention of cell from H_2O_2 and EPE95 showed melanocytes proliferation stimulating effects and EPA showed high antioxidant activities. Thus, these extracts should be continuously developed as a hair product.*

Keywords: *Antioxidant, Hydrogen peroxide, Melanocyte proliferation, Grey hair, Eclipta prostrata Linn., Sapindus rarak DC.*

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Nowadays, Thailand is an aging society. The increasing number of hair graying results in decreasing self-confident, beauty and older than actual age. All hair has pigment cells called melanocytes. These melanocytes produce a pigment called melanin, more specifically, the chemicals eumelanin and pheomelanin. Hair with more eumelanin will be darker, and hair with more pheomelanin will have a red/orange/yellow tint. Hair with less of either will be lighter[1]. These melanocytes also pass this pigment to cells called keratinocytes (which produce hair's primary component and the protein keratin). When the keratinocyte cells die, they keep the melanin and gives hair its color. As we get older, the melanocytes get less active, thus producing of pigment is reduced and making hair lighter. Eventually, all the melanocyte cells die, and there

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are none left to produce any color[2]. However, stressors induce alterations in pigment-producing, antioxidative enzymes, cofactors, the production of endogenous antioxidants and repair enzymes as well as growth factors. This reaction results in a breakdown of the hair follicle melanocyte redox-capacity and subsequent deleterious oxidative stress damage[3].

Hydrogen peroxide is the most commonly used as an oxidizing agent. The ideal herbal product was needed in the areas of color saturation, color development without causing hair damage and reducing skin irritation. However, the oxidizing of the oxidative coloring agents which are color from herbs cannot solubilize and decolorize the colored melanin component in the hair. Then hydrogen peroxide can be mixed with herbs to bleach and penetrate the hair shaft and removes the natural pigment of the hair[4].

In the past, the use of local herbs for hair care has been continuously use for a long time such as false-daisy white head (*Eclipta prostrata* Linn.). It is a local herb in Thailand, and it can change hair color from white to black and prevent premature gray hair[5]. In Thai traditional medicine has used juice from fruits of *Sapindus rarak* DC. to inhibit the growth of fungi that cause ringworm, tinea capitis and dandruff[6]. However, there is no report about activity on melanocyte cells and prevention from H_2O_2. Thus, the objective of this research are to investigate antioxidant, melanocytes proliferation stimulating and cell protection activities of *Eclipta prostrata* Linn. (EP) and *Sapindus rarak* DC. (SR) extracts.

Material and Method

Chemicals and reagents

Dimethyl sulfoxide [$(CH_3)_2SO$)] (DMSO) was purchased from RCI Lab scan, Thailand. Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were purchased from Univar, Australia. Dulbecco's modified Eagle's medium (DMEM), Penicillin-Streptomycin (P/S), Trypan blue stain 0.4% and Trypsin-EDTA were purchased from Gibco, USA. Phosphate buffered saline (PBS) was purchased from Amresco, USA. Sodium bicarbonate ($NaHCO_3$) was purchased from BHD, England. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 5-Diphenyltetrazolium Bromide (MTT), Hydrogen peroxide 30% (H_2O_2) from Qrec, Newzealand, UV-vis spectrophotometer, microplate reader.

Plant materials

The parts of plants of *Eclipta prostrata* Linn. (EP) and *Sapindus rarak* DC. (SR) were collected from Pathumthani, Thailand in 2014, voucher specimens shown in (Table 1). The voucher specimens were verified at the herbarium of Southern Center for Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Preparation of crude extracts

Plant materials were cleaned with water, sliced into small pieces and dried in a hot air oven at temperature 50°C and its ingredients herbs (200 grams of each) were macerated with 95% ethanol and were macerated with 50% ethanol (100 grams of each) for 3 days, filtered using Whatman No.1 filter paper and concentrated to dryness by evaporator (Rotavapor R-205, Germany). The filtrate was pooled and dried using an evaporator. Each herb (100 grams of each) was boiled with water (decoction) for 15 minutes in 3 times and filtered through a Whatman No. 1 filter paper. This residue of aquous extract was repeated twice and the filtrate was dried by lyophillizer. Percentage yields of all extracts were calculated by using the following equation.

$$\% \text{ yield} = \frac{\text{Weight of all extracts (g)}}{\text{Weight of herb for extracting (g)}} \times 100$$

Antioxidant activities

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay[7]

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Yamasaki et al 1994[7]. A sample for testing was dissolved in absolute ethanol or distilled water in various concentrations. 100 µl of extracts were transferred into a 96-well microplate. Then 100 µl of $6x10^{-5}$ M DPPH (in absolute ethanol) were added to each well. A portion of the sample was mixed with an equal volume of $6x10^{-5}$ M DPPH. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 520 nm. BHT was used as a positive control. The concentration of antioxidant needed to decrease the initial DPPH concentration (EC_{50}) by 50% is a parameter widely used to measure the antioxidant activity. The scavenging activity was calculated as percentage inhibition in the formulae below: Inhibition % = [(A Control-A Sample)/ A Control]x100] Effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis of the dose-response curve of % inhibition versus concentration,

Table 1. The percentage of yield of extracts from EP and SR by 95%, 50% ethanolic and aqueous extraction, 50% effective concentration (EC_{50}) of 95%, 50% ethanolic and aqueous extracts by DPPH assay and ABTs assay (n = 3)

Botanical name	% yield			Code	DPPH assay			Code	ABTs assay		
					EC_{50} ± SEM (µg/ml)				EC_{50} ± SEM (µg/ml)		
	95% ethanol	50% ethanol	Aqueous		95% ethanol	50% ethanol	Aqueous		95% ethanol	50% ethanol	Aqueous
Eclipta prostrata Linn: Compositae	7.825%	27.70%	23.30%	EP	73.46±0.57	>100	15.93±1.79	EP	>100	54.24±3.02	30.28±3.77
Sapindus rarak DC: Sapindaceae	13.52%	31.79%	21.40%	SR	>100	>100	>100	SR	>100	>100	>100
				BHT (positive)	13.40±0.27	13.40±0.27	13.40±0.27	Trolox (positive)	5.709±0.501	5.709±0.501	5.709±0.501

and EC_{50} is calculated using Prism program. All determinations were carried out in triplicate.

ABTS radical scavenging assay[8]

Total antioxidant capacity of the extracts was tested according to $ABTS^{\bullet+}$ solution which was produced by reacting 7 mM $ABTS^{\bullet+}$ stock solution in distilled water with 2.45 mM potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 h. The $ABTS^{\bullet+}$ solution was diluted with distilled water to get the absorbance of 0.68 to 0.72 at 734 nm before use. The assay was performed in 96-well microplates by mixing $ABTS^{\bullet+}$ solution with 10 µl of the extracts or standard Trolox. The reaction was carried out for 6 min, and then the absorbance was measured at 734 nm using a microplate reader (PowerWave XS, BioTek). The scavenging activity of the extracts against $ABTS^{\bullet+}$ was expressed as EC_{50} (µg/mL) and Trolox equivalent antioxidant capacity (TEAC) (mg Trolox equivalents/g extract).

$$\% \text{ inhibition} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \text{ x100}$$

Melanin proliferation by MTT assay

Cell culture[9]

B16F10 murine melanoma cell (ATCC® CRL-6475™) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified 95% air 5% CO_2 atmosphere.

Melanin proliferation by MTT assay[10]

Melanin proliferation was determined after treatment with the tested compounds using 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide (MTT; Sigma Chemical Co., St. Louis, USA) which measures mitochondrial activity in living cells as described previously. Briefly, $B_{16}F_{10}$ cells were cultured at 3,000 cells/well in a 96-well plate. After 24 h, cells were then treated with 95% or 50% ethanolic or aqueous extracts at 100 - 1 µg/ml. 100 µl sample solution was added and mixed where 2% DMSO solution was used as control solvent. Cells in a 96-well plate were incubated in the CO_2 incubator for 24 hours. Medium was removed and added 100 µl fresh media. Then incubated with MTT solution (1 mg/mL) 15 µl for 4 h at 37°C. The medium was removed and DMSO was added to dissolve the formazan product in these cells. Absorbance was measured at 570 nm using a microplate reader and Proliferation Index were calculated from this equation.

$$P.I = \frac{\text{Mean of OD sample}}{\text{Mean OD of control}} \times 100$$

Preventing cell for hydrogen peroxide in mouse melanoma cells (B16F10) by colorimetric MTT assay[11,12]

Effect of herbs in preventing cell for hydrogen peroxide with the tested compounds using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide (MTT; Sigma Chemical Co., St. Louis, USA) which measures mitochondrial activity in living cells as described previously[11]. Briefly, B16F10 cells were cultured at 3,000 cells/well in a 96-well plate. After 24 h, cells were then treated with 95% or 50% ethanolic or aqueous extracts at 100 to 1 µg/ml. Then 50 micromolar of H_2O_2 was added (the concentration which can make B16F10 cells die in 50%). 100 µl sample solution was added and mixed where 2% DMSO solution and 50 micromolar of H_2O_2 were mixed and used as control solvent. Cells in the 96-well plate were incubated in the CO_2 incubator for 24 hours. Medium was removed from 96 well plate and 100 µl fresh media was added. Then incubated with MTT solution (1 mg/mL) 15 µl for 4 h at 37°C. The medium was removed, and DMSO was added to dissolve the formazan product in these cells. Absorbance was measured at 570 nm using a microplate reader and Proliferation index were calculated from this equation. Calculate for % proliferation index.

$$P.I = \frac{\text{Mean of OD sample}}{\text{Mean OD of control}} \times 100$$

Statistical analysis

Data are expressed as mean ± SEM (n = 3). IC_{50} and EC_{50} values were calculated using GraphPad Prism software (version 4.03). Student t-test and one-way ANOVA followed by Duncan's or Dunnett's T3 was used to compare all groups. Statistical analysis was performed using SPSS for Windows (version 16.0). Statistical significance was set at a level of $p<0.05$. For correlation analysis, Pearson's correlation coefficient (r) with *p*-value between antioxidant activities and contents was identified. The $p<0.05$ was considered statistically significant.

Results

The percentage of yields (w/w) of the 95% ethanolic extracts, 50% ethanolic extracts and aqueous extracts of EP and SR were shown in Table 1. They were tested antioxidant activity which based on chemical methods. All extracts showed the results of DPPH assay and ABTS assay with their particular EC_{50} value (Table 1). The results showed that EPA showed the highest inhibitory DPPH radical ion with the EC_{50} value of 15.00 µg/ml. For ABTS assay, EPE50 and EPA showed the highest inhibitory effect on ABTs radical assay (EC_{50} = 54.24 and 30.28 µg/ml, respectively). From melanocytes proliferation assay, the proliferation index of the 95% ethanolic extracts, 50% ethanolic extracts, and aqueous extracts were shown in Fig. 1. The EPE95 showed the highest stimulating effect on melanocyte proliferation at the concentration of 50 µg/ml. The Proliferation index in preventing cell from hydrogen peroxide of 95%, 50% ethanolic and aqueous extracts from EP and SR (Fig. 2). The SRA showed high activity on preventing cells from H_2O_2 at the concentration of 10 and 1 µg/ml, respectively.

Discussion

In the present study, EP can increase melanin proliferation and SR as a plant which can inhibit the growth of fungi that cause ringworm, tinea capitis and dandruff can protect cell melanocytes from hydrogen peroxide. The results of the EP water extract related with the previous report which also showed high antioxidant , however the antioxidant activity by DPPH assay of the EP water extract showed higher than the previous study (IC_{50} values = 15.93 and 230 µg/ml respectively)[13]. The different results depend on source of growing, age of plant which make the active compound difference. The results of antioxidant testing by DPPH and ABTS assay of EP extract relate in the same way, the water extract on both antioxidant assay showed higher effective than ethanolic extract respectively. For EP 95% ethanolic extract which was tested by DPPH, showed higher antioxidant than EP 50% ethanolic extract. In the opposite result, EP 95% ethanolic extract which was tested by ABTS, showed less antioxidant than EP 50% ethanolic extract. The results depend on polarity of extract especially ABTS assay, the polar extraction showed effective than nonpolar extract. The previous study of the EP 95% ethanolic extract exhibited high antioxidant activities, with IC_{50} = 41.8 mg/ml[14]. Our results indicated that EPE50 and EPA showed the highest inhibitory effect on ABTs radical assay (EC_{50} = 54.24 and 30.28 µg/ml, respectively) but EPE95% had no activity. Thus this result is opposite the previous study.

The proliferation index of the 95% ethanolic (EPE95) showed the highest stimulating effect on melanocyte proliferation at the concentration of 50 µg/ml which related with the previous study which reported

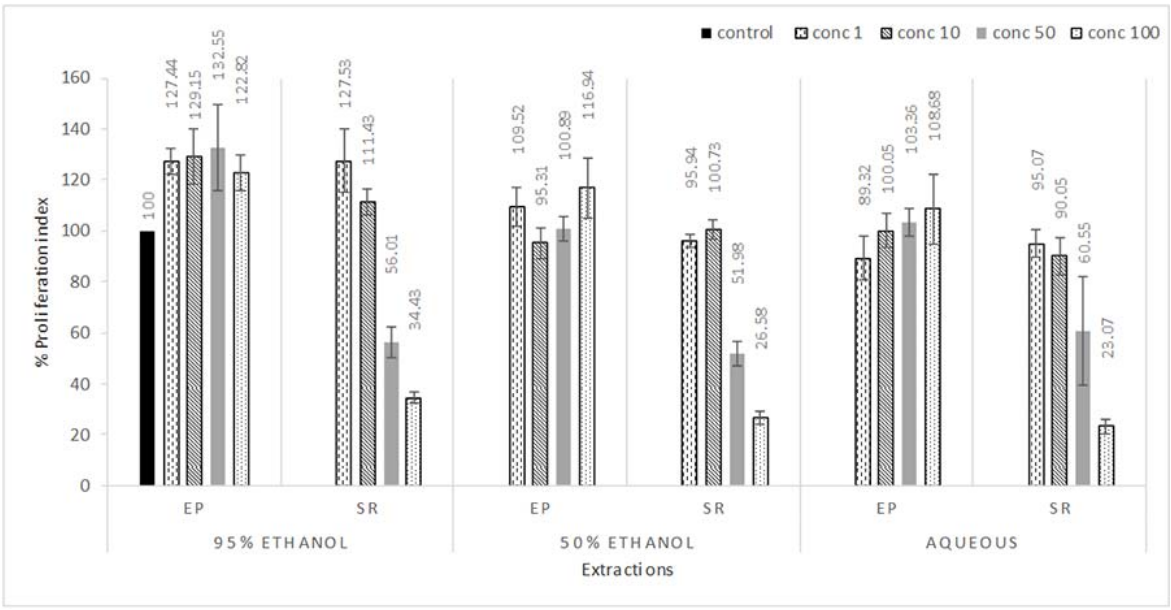

Fig. 1 Proliferation index in melanocytes proliferation assay of 95%, 50% ethanolic and aqueous extracts of EP and SR by MTT assay (n = 3).

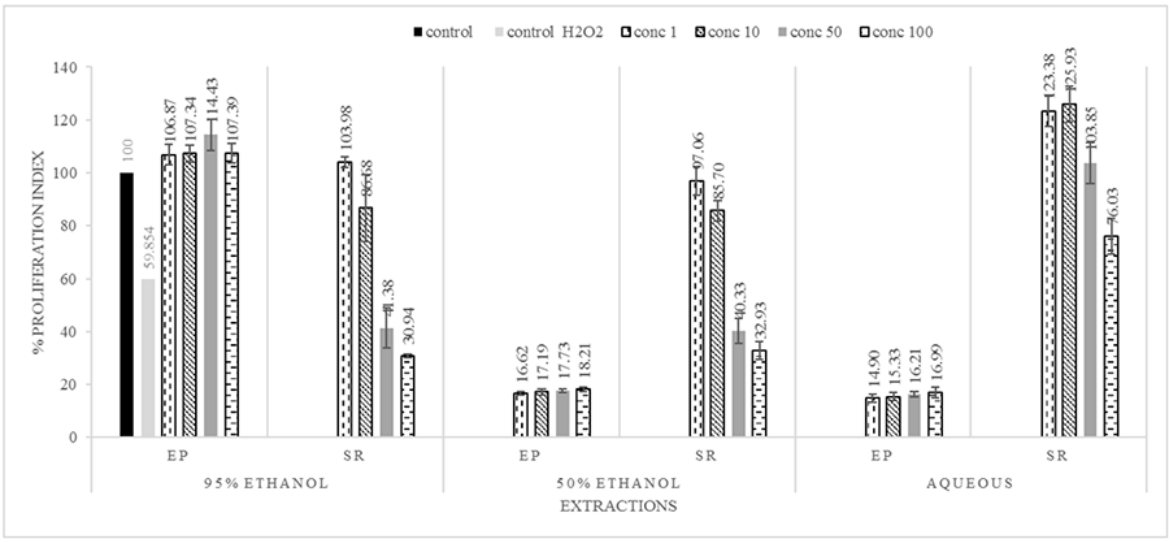

Fig. 2 Proliferation index in preventing cell from hydrogen peroxide of 95%, 50% ethanolic and aqueous extracts of EP and SR (concentration of Hydrogen peroxide to kill 50% melanocyte cells is 0.05 M or 50 micromolar) by MTT assay (n = 3).

that the methanolic extract of the EP has also efficacy in promoting hair growth. An extract at dose of 3.2 mg/15 cm^2 showed 87.5%[15]. However, this study is the first report of EP 95% ethanolic extract which was study in cells. For SR results, it was concluded that all extract of SR had no antioxidant by both antioxidant assay. The high concentration of all SR extract can kill melanocyte cells except SRA at the concentration of 10 and 1 µg/ml can stimulate cell growth and also showed high proliferate activity when added H_2O_2. The results can be concluded that the water extract of SR showed the highest protecting cells from H_2O_2 at the

concentration of 10 and 1 µg/ml, respectively.

This finding indicates that SRA protects cell from H_2O_2, EPE95 stimulates melanocytes proliferation and EPA has high antioxidant activities. Thus, these extracts should be continuously developed as the hair product. However, the effect of SRA and EPA on anti-tyrosinase activity should be the best tested for strong support data on melanin production.

Conclusion

To our knowledge, these results can be concluded that SRA protects cell from H_2O_2, EPE95 stimulates melanocytes proliferation and EPA has high antioxidant activities. Thus, these extracts should be continuously developed as the hair product.

What is already known on this topic?

Thai traditional herbs used *Eclipta prostrate* Linn. (EP) for dying hair and preventing premature gray hair. For EP, the aqueous extract of EP showed antioxidant activities and the methanol extracts of EP showed efficacy for promoting hair growth. For *Sapindus rarak* DC. (SR), the concentration 1, 10, 50, 100 and 200 ppm of SR pulp showed antioxidant activities and it could inhibit the growth of fungi which was caused ringworm, tenia capitis and dandruff. However, SR had no melanin proliferation. In the end, there had no research in EP and SR about the effect in preventing cell for hydrogen peroxide activity.

What this study adds?

The present study showed that the aqueous extract of *Eclipta prostrate* Linn. (EP) was the highest antioxidant activities but all extracts of *Sapindus rarak* DC. (SR) had no antioxidant activities. In addition, the 95% ethanolic extract of EP in 50 µg/ml concentration showed the highest melanin proliferation assay. However, the aqueous of SR in 10 µg/ml concentration showed the highest effect of herbs in preventing cell from hydrogen peroxide assay.

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Potential conflicts of interest

None.

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การศึกษาฤทธิ์ป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์กระตุ้นการสังเคราะห์เม็ดสีเมลานินของสารสกัดสมุนไพรกะเม็งตัวเมียและมะคำดีควาย

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ภูมิหลัง: จากทฤษฎีการลดลงของเม็ดสีและการทำงานที่น้อยลงของเอนไซม์ ทั้งสองกระบวนการมีความเกี่ยวข้องกับกลไก melanogenesis และกลไกการเกิดออกซิเดชัน โดยไฮโดรเจนเปอร์ออกไซด์เป็นสารอนุมูลอิสระ ซึ่งสามารถทำลายเซลล์เมลาโนไซต์ ในทางกลับกันถ้าสารสกัดจากสมุนไพรสามารถเพิ่มเซลล์เมลาโนไซต์และต้านอนุมูลอิสระได้สูง สมุนไพรนั้นก็จะสามารถปกป้องเซลล์เมลาโนไซต์ได้ ซึ่งพืชสมุนไพรที่มีรายงานว่าสามารถกระตุ้นการเจริญเติบโตของเส้นผมและย้อมสีผม ได้แก่ กะเม็งตัวเมีย (Eclipta prostrate Linn. (EP)) และมะคำดีควาย (Sapindus rarak DC. (SR))

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์กระตุ้นการสังเคราะห์เม็ดสีเมลานินของสารสกัดสมุนไพรกะเม็งตัวเมียและมะคำดีควาย

วัสดุและวิธีการ: สกัดสารด้วยวิธีการหมักเอทานอล 95% และ 50% ทำแห้งด้วยเครื่องปั่นเหวี่ยงภายใต้ระบบสุญญากาศ (evaporator) สกัดสารด้วยการต้มน้ำทำให้แห้งด้วยเครื่องทำแห้งแบบแช่แข็ง (lyophillizer) การศึกษาในขั้นตอนแรกนำสารสกัดไปทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH radical scavenging assay และ ABTS radical cation decolorization assay จากนั้นนำมาทดสอบการกระตุ้นการเพิ่มจำนวนเซลล์เมลาโนไซต์ในเซลล์มะเร็งของหนู (B16F10) และการป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ทำลายเซลล์ด้วยวิธี MTT assay

ผลการศึกษา: สารสกัดกะเม็งตัวเมียชั้นน้ำมีฤทธิ์ดีที่สุดในการยับยั้งสารอนุมูลอิสระ DPPH โดยแสดงผลในรูปแบบ EC_{50} ซึ่งมีค่าเท่ากับ 15.00 μg/ml. ในส่วนการทดสอบฤทธิ์ของ ABTS assay พบว่าสารสกัดกะเม็งตัวเมียชั้น 50% เอทานอล มีฤทธิ์ดีที่สุดในการยับยั้งสารอนุมูลอิสระ ABTs และในส่วนสารสกัดกะเม็งตัวเมียชั้นน้ำมีฤทธิ์ดีรองลงมาโดยมีค่า EC_{50} เท่ากับ 54.24 และ 30.28 μg/ml ตามลำดับ) การทดสอบฤทธิ์การกระตุ้นเซลล์เมลาโนไซต์ พบว่าสารสกัดกะเม็งตัวเมียชั้น 95% เอทานอลที่ความเข้มข้น 50 μg/ml มีฤทธิ์กระตุ้นในการเพิ่มจำนวนเซลล์ดีที่สุดโดยแสดงผลในรูปแบบ proliferation index นอกจากนั้นการทดสอบฤทธิ์การป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ พบว่าสารสกัดมะคำดีควายชั้นน้ำที่ความเข้มข้น 10 μg/ml มีฤทธิ์ในการป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ดีที่สุดรองลงมาคือ ที่ความเข้มข้น 1 μg/ml

สรุป: จากผลการศึกษาทำให้ทราบว่าสารสกัดมะคำดีควายชั้นน้ำมีฤทธิ์ในการป้องกันสารอนุมูลอิสระไฮโดรเจนเปอร์ออกไซด์ดีที่สุด ส่วนสารสกัดกะเม็งตัวเมียชั้น 95% เอทานอล มีฤทธิ์กระตุ้นเซลล์เมลาโนไซต์ดีที่สุดและสารสกัดกะเม็งตัวเมียชั้นน้ำมีฤทธิ์ต้านอนุมูลอิสระดีที่สุดด้วยเหตุนี้จึงควรมีการพัฒนาสารสกัดมะคำดีควายและกะเม็งตัวเมียในรูปแบบผลิตภัณฑ์ที่เกี่ยวกับเส้นผมต่อไปในอนาคต