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Antioxidant activity and hair growth promoting activity of flavonoid extracts from *Phyllodium pulchellum* and *Uvaria rufa* Blume on cultured mouse vibrissa hair follicles

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Abstract: This study aims to determine antioxidant activity and hair growth promoting activity of flavonoid extracts from the aerial parts of *Phyllodium pulchellum* and the stems of *Uvaria rufa* Blume on cultured mouse vibrissa hair follicles. Flavonoid extracts of both plants were tested for their antioxidant activities using total antioxidant assay, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide radical scavenging assays. To investigate hair growth promotion, vibrissa follicles of mouse were isolated and cultured in 95 % CO_2, 5 % O_2 and 37ºC. Cultured vibrissa follicles were treated with flavonoid extracts of *P. pulchellum* (PPE) and *U. rufa* (URE) at concentrations of 0.01-20 µg/ml for three days. The length of hair follicles was measured every 24 hours for three days. Total flavonoids contents of PPE and URE were 10.14 ± 0.20, 10.28 ± 0.60 mg quercetin per extract, respectively. We found that PPE and URE had total antioxidant activities at 10.53 ± 0.26 and 11.29 ± 0.04 mg ascorbic acid equivalent per gram extract, respectively. Moreover, PPE and URE markedly exhibited antioxidant activities with the IC_{50} values at 1.67 mg/ml and 2.44 mg/ml for ABTS assay, and 1.34 mg/mL and 2.45 mg/ml for DPPH assay, and 0.44 mg/ml and 0.34 mg/ml for nitric oxide radical scavenging assay. PPE at 1 µg/ml significantly increased the length of hair follicles at 24 and 48 hours while URE at 0.01-1 µg/ml significantly elongated hair follicles at 48 and 72 hours when compared to those of controls. The results suggest that the flavonoid extracts from *P. pulchellum* and *U. rufa* are the new sources of antioxidants and the potential promoting hair growth agents.

Keywords: Antioxidant, hair growth, *Phyllodium pulchellum*, *Uvaria rufa* Blume

Introduction

Hair loss is a concern problem which can occur in both men and women. This problem brings loss of self-esteem and mental health problems (Williamson et al., 2001). Although there are effective drugs for treating hair loss which are finasteride and minoxidil, they contribute several adverse side effects. Finasteride caused male infertility and gynecomastia while minoxidil can lead to skin irritation, itching and dryness and hypotension (Chilba et al., 2011; Ramot et al., 2009; Rossi et al., 2012; Mehta et al., 1975). Due to the limited usage of synthetic drugs, natural product has become attractive. Hair growth promoting activities of various plants; *Centella asiatica Linn*., *Panax ginseng*, *Avicennia marina* and *Thujae occidentalis* were previously reported (Saansoomchai et al., 2018; Matsuda et al., 2003; Jain et al., 2015; Park et al., 2003). The major phytochemical constituent in these plants is flavonoids, which is well-known for its antioxidant properties. Since oxidative stress is related to hair loss, consumption of antioxidants can prevent the miniaturization of hair. Over-production of free radicals in scalp skin can trigger dermal papilla cell apoptosis, eventually leads to hair shedding (Trüeb, 2015). Therefore, natural antioxidant treatment for maintaining healthy scalp is necessary.

Phyllodium pulchellum and *Uvaria rufa* Blume are distributed in Thailand and used in traditional medicine as remedy for hair loss. *P. pulchellum* was reported to have hepatoprotective effect while *U. rufa* was reported as effective treatment against benign prostatic hyperplasia in experimental animals (Fan et al., 2018; Buncharoen et al., 2016). However, there is no evidence for hair growth promoting activity of these two plants. Thus, this study aims to investigate hair growth promoting activity as well as antioxidant of extracts from *P. Pulchellum* and *U. rufa* to confirm their traditional usage.

Materials and Methods

Reagents

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), 2,4-dinitrophenylhydrazine (DNPH), basal medium, bovine calf serum, bovine pituitary extract, fibroblast growth factor, gallic acid and insulin were purchased from Sigma Aldrich (MO, USA). Ammonium molybdate was obtained from Unilab (Sydney, Australia). Ascorbic acid was purchased from Prolabo (Leuven, Belgium). Penicillin/streptomycin was obtained from Merck (Darmstadt, Germany).

Plant materials

The aerial parts of *P. pulchellum* were collected in Chiang Mai Province and the stems of *U. rufa* were obtained from Buriram Province, Thailand.

Extraction

All plant materials were chopped, cleaned and dried. The dried sample was defatted with petroleum ether using a Soxhlet extractor at 40-60ºC. Then, the residue was extracted with ethyl acetate. The ethyl acetate extracts were evaporated by a rotary evaporator at 50ºC (Peleg et al., 1991) to obtain the flavonoid extracts of *P. Pulchellum* (PPE) and *U. rufa* (URE). Total flavonoid contents were determined by using aluminium chloride solution at 420 nm (Ordonez et al., 2006). The flavonoids in PPE and URE were expressed as mg quercetin equivalent per gram extract (mgQE/g extract).

Measurement of antioxidant capacity

ABTS radical scavenging activity

The scavenging of ABTS radical was tested according to the method described by Re et al (1998). Briefly, the working ABTS solution was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was allowed to react in the dark at room temperature for 12 h. The $ABTS^{\bullet+}$ solution was then diluted with deionized water to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Different concentrations of each sample were mixed with 1 ml of the $ABTS^{+}$. The absorbance was measured at 734 nm at 0 (initial absorbance) and 1 (final absorbance) minute of incubation. Gallic acid was used as the standard control. The inhibition of $ABTS^{\bullet+}$ was calculated by using the following equation:

$$\% \text{ inhibition of } ABTS^{\bullet+} = [Abs_{(initial)} - Abs_{(final)} / Abs_{(initial)}] \times 100$$

The results were expressed as median inhibitory concentration (IC_{50}) value.

DPPH radical scavenging activity

The DPPH assay was done according to the method of Susanti et al. (2007). 100 µl of each sample was added to 2 ml of 0.1 mM DPPH·solution. The mixture was vigorously shaken and the absorbance (initial absorbance) of the mixture was recorded immediately at 517 nm. Then, it was incubated in the dark for 30 min at room temperature and the final absorbance at 517 nm of the mixture was measured. Gallic acid was used as the standard control. The DPPH·scavenging ability was calculated as:

$$\% \text{ inhibition of DPPH}\cdot = [Abs_{(initial)} - Abs_{(final)} / Abs_{(initial)}] \times 100$$

The IC_{50} value of each sample as well as antioxidant standard were calculated. Each sample was performed in triplicate.

Nitric oxide scavenging activity

The nitric oxide scavenging activity assay was conducted according to the procedure described by Marcocci et al. (1994). Sodium nitroprusside (10 mM) in phosphate buffer saline was prepared to produce nitric oxide radicals, which was measured by using Griess reagent. 500 µl of sodium nitroprusside solution was then mixed with 1 ml of various concentrations of each extract. The mixture was incubated at room temperature for 180 min. After incubation, 500 µl of Griess reagent was added. Gallic acid was used as the standard positive control. The absorbance of the mixture was taken at 546 nm.

Total antioxidant capacity

The phosphomolybdenum method was used to investigate the total antioxidant capacity in PPE and URE extracts (Oyaizu, 1986. Phosphomolybdenum reagent was prepared by mixing 0.6 M sulfuric acid, 28 mM sodium phosphate and 1% of ammonium molybdate. The reagent was then mixed with 500 µl of each sample. The mixtures were incubated at 95ºC for 10 min. The absorbance of the mixture was subsequently measured at 695 mm. The results were expressed as mg ascorbic acid equivalent per gram extract (mgAAE/g extract)

Isolation and culture of mouse vibrissa follicles

For isolation experiment, five-week-old ICR mice were isolated as previously described (Jindo et al., 1994). Briefly, the mystacial pads were removed from upper lip region. Intact vibrissa follicles were then carefully disaggregated under a stereomicroscope using a scalpel and tweezers. Isolated vibrissa follicles were placed in 24-well plates containing 1 ml of basal medium supplemented with 0.002 ml/ml bovine pituitary extract, 0.5µg/ml bovine insulin, 0.05 ml/ml calf serum, 0.5 ng/ml fibroblast growth factor, 50 U/ml penicillin and 50 µg/ml streptomycin (Junlatat and Sripanidkulchai, 2014). The follicles were cultured in an incubator with 5% CO_2 at 37ºC. For each assay, PPE and URE were diluted with DMSO and added to the medium to yield various concentrations (0.001 - 10 µg/ml). DMSO (0.1%) was used as a control. The medium was discarded and replaced every 24 h. During the period of culture (72 h), the cells were photographed at 24, 48 and 72 h using a stereo microscope. The difference of the length of vibrissa hair follicles were calculated using ImageJ software and expressed as mean ± S.D.

Statistical analysis

All values were expressed as mean ± S.D. of at least three samples. The significant differences among experimental groups were determined by one-way analysis of variance (ANOVA), followed by Duncan's test using SPSS version 16. P-values less than 0.05 were regarded as significant.

Result & Discussion

Antioxidant activity of PPE and URE

Oxidative stress is the disturbance which leads to damaging of cellular structures and may be a factor of hair loss (Trüeb, 2015). The association between oxidative stress and hair loss has been reported by Bahta et al. (2008) that the dermal papilla cells were susceptible to oxidative stress and resulting in miniaturization of hair follicles. In addition, Beoy et al. (2010) have suggested that consuming the supplement containing antioxidants can decrease oxidative stress in the hair scalp as well as increase the numbers of hairs.

The low IC_{50} values of all parameters obtained from our results indicated that PPE and URE extracts exhibited the ability to scavenge free radicals. PPE and URE markedly performed antioxidant activities with the IC_{50} values at 1.67 mg/ml and 2.44 mg/ml for ABTS assay, and 1.34 mg/mL and 2.45 mg/ml for DPPH assay, respectively. Moreover, URE also exhibited excellent nitric oxide scavenging activity, especially URE which IC_{50} value was not much higher than that of gallic acid (0.34 mg/ml for URE and 0.23 mg/ml for gallic acid). PPE, however, exhibited lower capacity to scavenge nitric oxide than URE by showing IC_{50} value at 0.44 mg/ml (Table 1). Determination of total antioxidant capacity of PPE and URE expressed values with 10.53 ± 0.26 and 11.29 ± 0.04 mg AAE/g extract, respectively. Total flavonoid contents of PPE and URE were 10.14 ± 0.20, 10.28 ± 0.60 mgQE/g extract. The potent antioxidant activities of PPE and URE found in our study might contribute to their flavonoid compounds.

As previous study reported that flavonoids are powerful antioxidants which can reduce oxidative stress in the hair scalp (Pietta, 2000; Beoy et al., 2010). Thus, flavonoids in *P. pulchellum* and *U. rufa* may be the agents that reduce the free radicals in the hair scalp. To ensure hair growth promoting activity of PPE and URE via decreasing oxidative stress, determination of hair growth promoting activity *in vivo* needs to be further evaluated.

Elongation of vibrissa hair follicles

Flavonoids could enhance hair quality not only by decreasing free radicals in the hair scalp, but also promoting elongation of hair follicles. The previous study reported that green tea extract containing flavonoid promoted hair growth by inducing elongation of hair follicles and inhibiting apoptosis of dermal papilla cells (Kwon et al., 2007). In addition, flavonoids contained in *Panax ginseng* performed significant stimulation on hair growth of vibrissa follicles after 48 h of culture (Matsuda et al., 2003).

In this study, the vibrissa follicles treated with PPE at 1 µg/ml significantly increased the elongation of hair follicles after 24 and 48 h of culture (128.02 ± 21.97 and 116.81 ± 28.05 µm, respectively) when compared with the control (Figures 1, 3). In the URE group, the follicles were significantly longer than those treated with vehicle control after 48 and 72 h of culture (Figure 3). The follicles treated with URE at 0.01, 0.1 and 1 µg/ml for 24 h expressed elongation with values 123.76 ± 69.58, 136.47 ± 12.46 and 115.04 ± 26.20 µm, respectively. For 72 h of culture, the URE treatment at 0.01, 0.1 and 1 µg/ml enhanced the hair growth by 111.38 ± 17.82, 113.47 ± 2.83 and 149.83 ± 25.59 µm, respectively (Figure 2). Hence, these results suggested that PPE and URE have potential hair growth promoting activity *in vitro* resulting from the presence of flavonoids as the major constituents.

Conclusion

In conclusion, the flavonoid extracts from *P. pulchellum* and *U. rufa* are potent antioxidants and able to promote hair growth by inducing hair elongation. The antioxidant capacity of these two extracts is recommended as the mechanism underlying their hair growth promoting activity.

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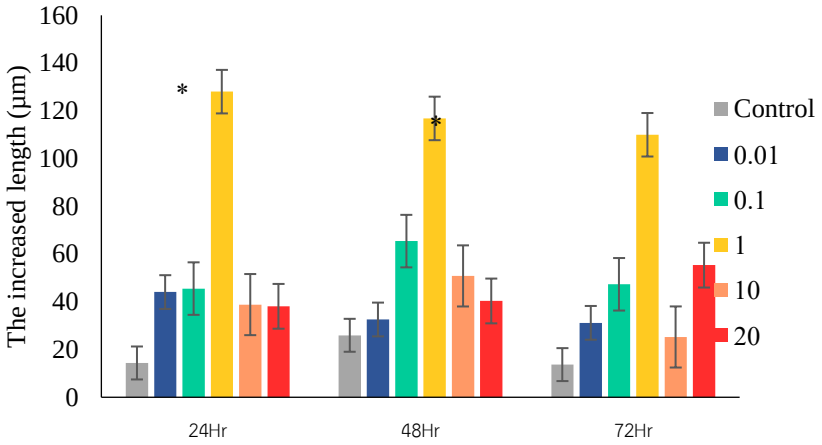

Figure 1. Hair growth promoting effect of PPE on cultured mouse vibrissa hair follicles for 24, 48 and 72 h. Follicles were treated with 0.01, 0.1, 1, 10 and 20 µg/ml of PPE. Data are exhibited as mean ± S.D. (n = 3), *p < 0.05 compared to control.

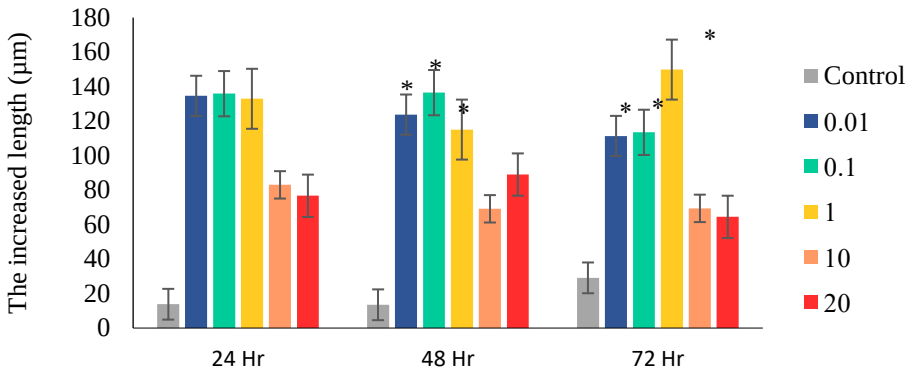

Figure 2. Hair growth promoting effect of URE on cultured mouse vibrissa hair follicles for 24, 48 and 72 h. Follicles were treated with 0.01, 0.1, 1, 10 and 20 µg/ml of URE. Data are exhibited as mean ± S.D. (n = 3), *p < 0.05 compared to control.

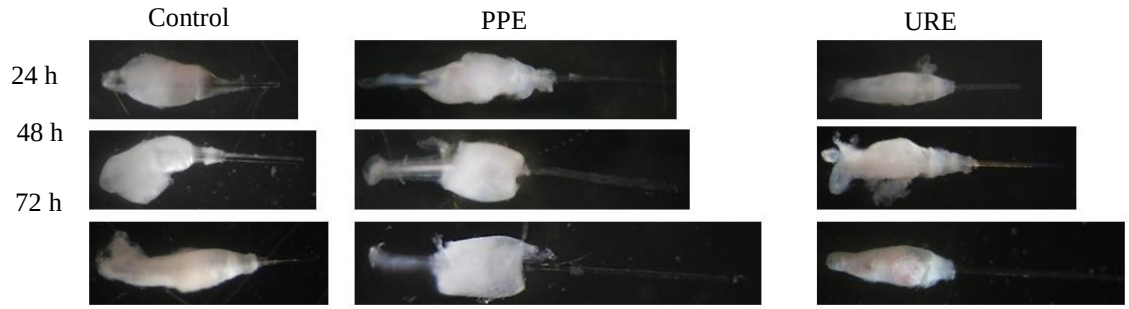

Figure 3. Elongation of cultured mouse vibrissa hair follicles treated with PPE or URE demonstrated a significant increase in length of hair shaft when compared to the control at 24, 48 and 72 h.

Table 1. Antioxidant activity of PPE and URE and gallic acid.

Treatment	IC_{50} values (mg/ml)		
	ABTS method	DPPH method	Nitric oxide inhibition method
PPE	1.67 ± 0.20	1.34 ± 0.16	0.44 ± 1.20
URE	2.44 ± 0.29	2.45 ± 0.30	0.34 ± 0.48
Gallic acid	0.08 ± 0.18	0.002 ± 0.00	0.23 ± 0.76

Each value is expressed as mean ± S.D. from triplicate.