RESEARCH ARTICLE

In vitro and *in vivo* skin whitening and anti-aging potentials of hydroglycolic extract from inflorescence of *Etlingera elatior*

Saruth Nithitanakool, Veerawat Teeranachaideekul, Lakkhana Ponpanich, Nisa Nopporn, Tipwimol Junhunkit, Pimphaka Wanasawas and Malyn Chulasiri

Research & Development Division, S&J International Enterprises PCL, Bangkok, Thailand

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Correspondence
Saruth Nithitanakool
S&J International Enterprises PCL
115 Naradhiwas Rajanagarindra Road (Soi 10)
Bangkok 10120
Thailand

E-mail
beauty_health@snjinter.com

Abstract

Hydroglycolic extract from inflorescence of *Etlingera elatior* (Zingiberaceae) (EE) was determined for its whitening and anti-aging potentials by studying its *in vitro* antioxidant capacity using two different assays, by investigating its *in vitro* anti-tyroisnase activity against tyrosinase, by demonstrating its *in vitro* anti-aging effect against collagenase, and by evaluating its *in vivo* skin whitening and anti-aging potentials on human volunteers. EE extract possessed potent antioxidant and anti-tyrosinase activities compared with positive references. The results of *in vivo* study imply that lotion containing EE extract demonstrated significant skin whitening effects and skin wrinkles reducing capability on human volunteers. The results obtained suggest the use of EE inflorescence hydroglycolic extract as a potential source of skin whitening and anti-aging for cosmetic applications.

Introduction

Nowadays, much attention has been focused on the use of natural substances for the development of skin care products, since most of them have been proven to possess remarkable antioxidant (Saravi, 2010), emollient (Maria Elena et al, 2011) and ultraviolet (UV) protection (Perona et al, 2006) which are benefits for using in the skin care products. Moreover, natural ingredients can lower skin allergy

problems because they are easily absorbed by the superficial layers of the skin. The sources of natural antioxidants are primarily plant polyphenols which may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks. Polyphenols were re-ported as one of the potent antioxidant and have been found to exhibit several pharmacolo-gical properties such as antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-radical and anti-aging (Allaith, 2008, Amin et al, 2004, Dreosti, 2000).

Etlingera elatior (EE), also known as 'torch ginger' or 'red ginger lily' belongs to Zingiberaceae family and is a herbaceous perennial plant native to South East Asia. It has been traditionally and commercially used as food, condiment, medicine, and ornamentals. The indigenous communities use the young shoots, flower buds or fruits for consuming as a condiment, or cooked. Inflores-cence of EE is used in food as flavoring and also ornamentals. The flowers and flower buds are commonly used in Malaysian dishes such as, Penang laksa, nasi kerabu and nasi ulam (Chan et al, 2007, Khaw, 2001, Larsen et al, 1999, Noweg et al, 2003). It is well known that EE has also been used in the traditional medicine among indigenous communities in Malaysia. The fruits and leaves of EE prepared by decoction have been used to treat earache and to assist in wound healing, respectively. The young flower shoot of EE has been found to possess antimicrobial, cytotoxic and anti-tumor promoting properties (Habsah et al, 2005). Recently, it was reported that the ethanolic extract of EE inflorescence exhibited potent antioxidant properties and have a relatively high phenolic content (Haleagrahara et al, 2010; Haleagrahara et al, 2010). However, literature is scanty regarding the phytochemical studies conducted on the inflorescence of EE. Moreover, most of the previous researches of EE on the antioxidant activities were limited to rhizomes and leaves. In this study, therefore, the EE inflores-cence was selected for investigation. The hydroglycolic extract of EE was firstly characterized and evaluated for its *in vitro* antioxidant, anti-tyrosinase and anti-collagenase activities. Finally, this extract was demonstrated for its potential in skin whitening and anti-aging effects on human volunteers.

Materials and Methods

Chemicals and Reagents: Folin-Ciocalteu reagent was purchased from Merck KGaA (Darmstadt, Germany). Gallic acid (GA), kojic acid (KA) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Fluka (Buchs, Switzerland). L-3,4-dihydroxyphenyl-alanine (L-DOPA), epigallocatechin gallate (EGCG), mushroom tyrosinase (EC. 1.14.18.1) and collagenase from *Clostridium histolyticum* (ChC – EC. 3.4.23.3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vitamin E acetate was purchased from BASF (Germany). Co-Enzyme Q10 (CoQ10) was obtained from OMYA PERALTA GMBH (Germany). Propylene glycol (PG) was purchased from SKC Co., Ltd. (Korea).

Plant extraction: Inflorescences of torch ginger were purchased from local market. Excised inflorescence part was washed, dried in an oven at 50°C and powdered sample by using electric blender. The powder sample was extracted with 50% (v/v) PG. After the sample was filtered

through two layers of cheesecloth, the filtrate was centrifuged at 10000 rpm at room temperature and further filtered by using a sheet of Whatman No.6 filter paper. The filtrate or designated as the hydroglycolic (HG) extract was stored in a closed container until used.

Determination of total phenolic content: Determination of total phenolic content in EE inflorescence HG extract was carried out by using Folin-Ciocalteu reagent as previously described (Singleton *et al*, 1999). The total phenolic content of the extract was expressed as mg of gallic acid equivalence (GAE)/g crude extract.

Determination of *in vitro* antioxidant activities: The free radical scavenging potential of EE inflorescence HG extract was determined by means of DPPH method (Nithitanakool et al, 2009). The antiradical activity was calculated as the percentage of DPPH decoloration versus a control. Vitamin E acetate and Co Q10 were used as the positive references. The results were expressed as the concentration of test samples that scavenged 50% of the free radicals from the reaction mixture (SC_{50}). All experiments were performed in duplicate.

The effect of the extract on ABTS radical was estimated by using the method of Re *et al* (1999). Antioxidant capacity of the extract was determined based on the reduction of ABTS absorbance by calculating percentage of antioxidant activity. Trolox was used as positive reference. The results were expressed as the concentration of test samples that scavenged 50% of the free radicals from the reaction mixture (SC_{50}). All experiments were done in duplicate.

Determination of Anti-tyrosinase assay

The tyrosinase inhibitory activity of plant extract was investigated by using a 96-well microplate reader (Tecan, InfiniteM200 PRO model, Grodig, Austria) (Kubo *et al*, 2000). KA was used as a positive reference tyrosinase inhibitor control. The used substrate and enzyme in this test were L-DOPA and mushroom tyrosinase, respectively. The extent of inhibition by the test samples was expressed as the percentage inhibition necessary to achieve 50% inhibition (IC_{50}). All experiments were measured in duplicate.

Determination of anti-collagenase assay: The anti-collagenase assay of the extract was determined based on spectrophotometric methods as described previously by Weingarten (1985), with some modification for use in a 96-well microplate reader. EGCG was used as positive control. The *C. histolyticum* collagenase was the enzyme used in the test. Experiments were done in duplicate and anti-collagenase activity was expressed as the percentage inhibition necessary to achieve 50% inhibition (IC_{50}).

Effect of lotion containing EE inflorescence HG extract on skin whitening: The *in vivo* study was performed as a double blind test by volunteers. The formulas of the lotion are shown in Table 1. Lotion containing EE inflorescence extract (2% w/w) was applied twice a day at the inner side of the forearm using 10 female volunteers (30-55 years old) for 4 weeks. The lotion base without the extract (blank lotion) was used as the self-control within each volunteer. One tenth ml of the test or blank lotion was always used for each application. Skin color was

evaluated during treatment on week-2 and 4 after application by using Chromameter CR 400 (Konica Minolta Optics Inc, Japan) in a controlled room at 25±2°C and relative humidity between 40-60%.

The individual typology angle (ITA°) was calculated as previously described (Teeranachaideekul et al, 2013). An increased in ITA° and/or lightness (L*) indicates skin whitening or lightening effects of test samples. Measurements are in accordance with the guidelines of the European Society of Contact Dermatitis (Fullerton *et al*, 1996). Probability values (*p*) less than 0.05 were considered to be significant.

Effect of lotion containing EE inflorescence HG extract on skin wrinkles reducing capability: The *in vivo* anti-wrinkle effects of lotion containing EE inflorescence extract (2% w/w) was performed as a double blind test by human volunteers. The formulas of the lotion are listed in Table 1. Ten healthy human volunteers aged between 30-55 years were enrolled in the experiment. All the volunteers had no sign of skin disease and were not using any topical agents on the test area for 4 weeks prior to the study. The test samples were applied to the eye area twice daily at home for 4 weeks. The area without the treatment was used as a negative control. The areas were measured for anti-aging activity before sample and after sample applications at 4 weeks. Before each measurement, the volunteers were accommodated in a controlled room at 25±2°C and relative humidity between 40-60% for 20 min. All volunteers finished the study without any drop-outs. The replica images (black and white images) were recorded with a CCD camera. The images were analyzed by Skin-Visiometer VL 650 software (Courage & Khazaka, Cologne, Germany) to obtain the parameters of average depth and average area of wrinkles which were used to assess the skin wrinkles. A decreased in average depth and average area of wrinkles indicates skin wrinkles reducing capacity of test samples. The percentage reduction of wrinkle depth and wrinkle area of all formulations was calculated by the following equation:

$$\%\text{Reduction} = [(V_0 - V_m)/V_0] \times 100$$

V_0 is the value at initial point (day 0), and V_m is the value at measuring point (4 weeks). Probability values (*p*-value) less than 0.05 were regarded as indicating significant differences.

Results and Discussion

Total phenolic content, antioxidant, anti-tyrosinase and anti-collagenase activities of EE inflorescence HG extract: Polyphenolic compounds are very important plant constituents which have been reported to possess several potent pharmacological activities including free radical scavenging, anti-tyrosinase and anti-collagenase activities (Nithitanakool et al, 2009, Wahab et al, 2014). In the present study, the total phenolic content of EE inflorescence HG extract was firstly evaluated and consequently the antioxidant, anti-tyrosinase and anti-collagenase activities. It was found that the total phenolic content of EE inflorescence HG extract was 13.85 mg GAE/g crude extract (data not shown).

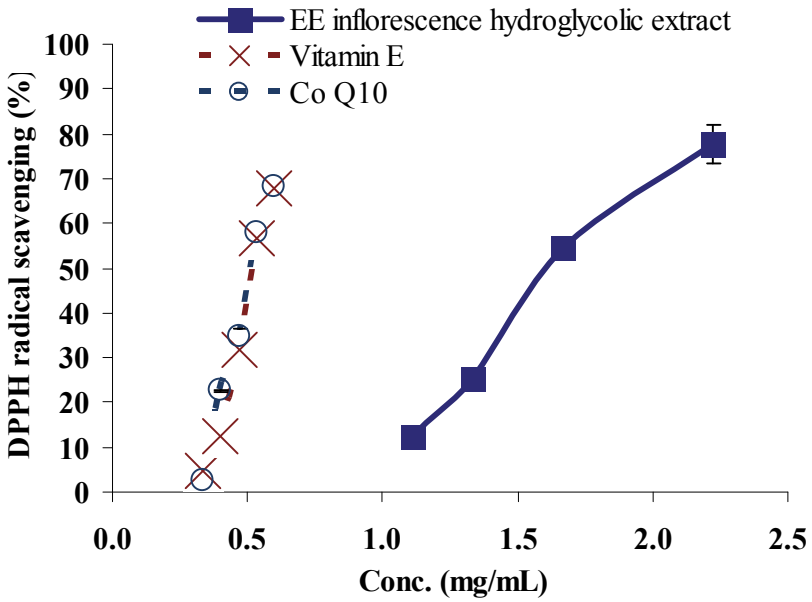


Figure 1 DPPH radical scavenging potential of EE inflorescence HG extract (■), Vitamin E acetate (×) and Co Q10 (○).

Figure 1 indicates that the DPPH radical scavenging activity of the HG extract of EE inflorescence occurred in a concentration-dependent manner. The scaven-ging potential of EE inflorescence extract (SC_{50} = 1.86 ± 0.03 mg/ml) was remarkable when compared with vitamin E acetate (SC_{50} = 0.53 ± 0.01 mg/ml) and CoQ10 (SC_{50} = 0.52 ± 0.01 mg/ml). The EE inflorescence extract also demonstrated a capacity to scavenge ABTS free radical, although its potency was lower than the positive reference, Trolox (Figure 2). The ability of the extract to scavenge ABTS free radical was dependent on concentration. The order of scavenging potential as judged by the half-inhibition concen-tration (SC_{50}) was Trolox (0.32 ± 0.12 mg/mL) > EE inflorescence extract (3.81 ± 0.12 mg/mL). These results therefore indicated that EE inflorescence HG extract has excellent antioxidant activities.

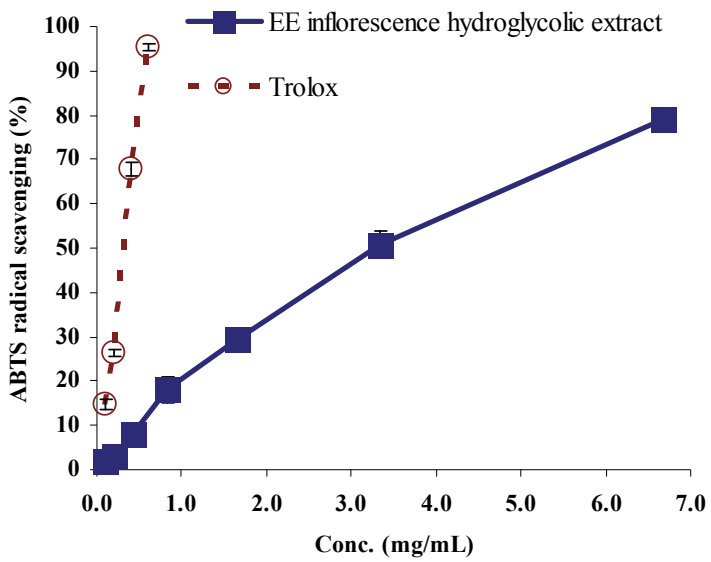


Figure 2 ABTS radical scavenging activity of EE inflorescence HG extract (■) and Trolox (○).

Table The formulas of the lotion base (A) and the lotion containing EE inflorescence HG extract (B)

Ingredients	**A**	**B**
Carbomer	0.3	0.3
Glycerin	3.0	3.0
Cetyl alcohol	2.0	2.0
Glyceryl stearate/PEG-100stearate	0.5	0.5
Polysorbate 60	1.0	1.0
Sorbitan stearate	0.2	0.2
Dimthicone	1.0	1.0
Isopropyl myristate	1.0	1.0
Mineral oil	0.5	0.5
Phenoxyethanol	0.6	0.6
Chlorphenesin	0.2	0.2
EE fluorescence hydroglycolic extract	-	2.0
Sodium hydroxide (fro adjusting pH to 5.5)	qs	qs
Water qs.	100	100

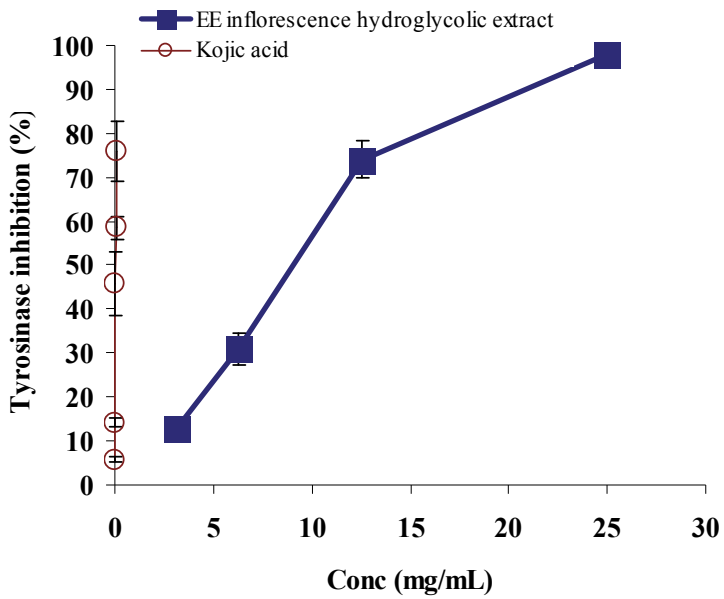


Figure 3 Tyrosinase inhibitory activity of EE inflorescence HG extract (■) and KA (○).

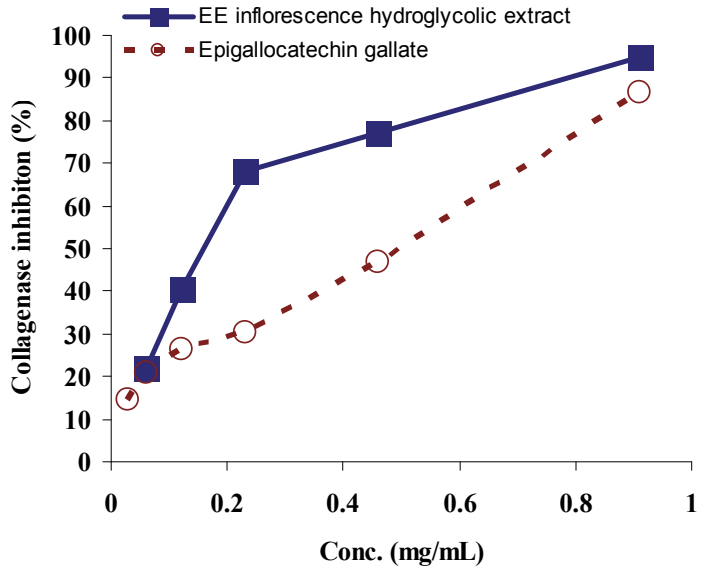


Figure 4 Collagenase inhibitory activity of EE inflorescence HG extract (■) and EGCG (○).

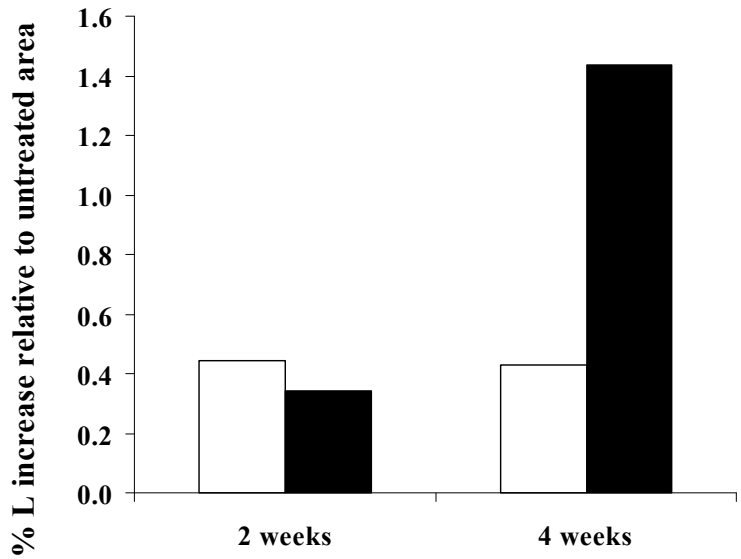


Figure 5 Whitening/lightening potentials of lotion containing EE inflorescence HG extract (dark column) compared to lotion base (clear column) evaluated by observing the increasing of L* after 2- and 4- week of applications on volunteer's forearms.

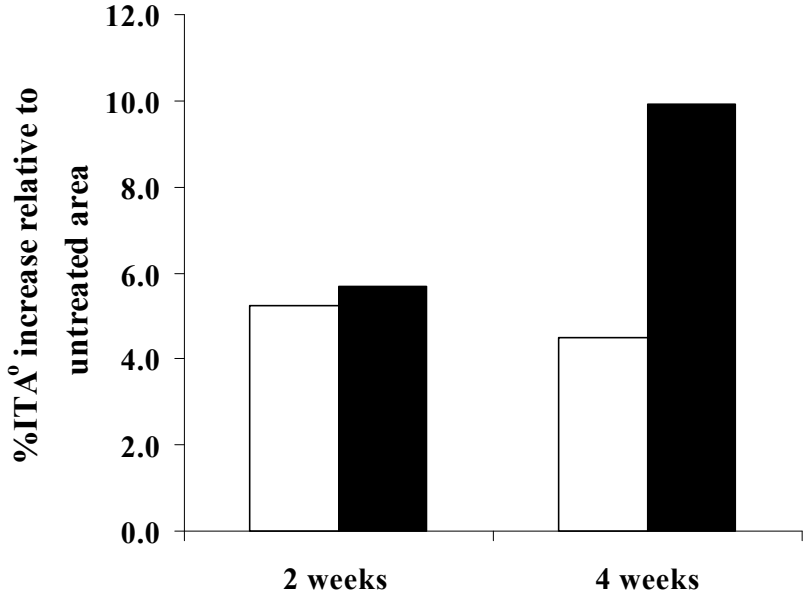
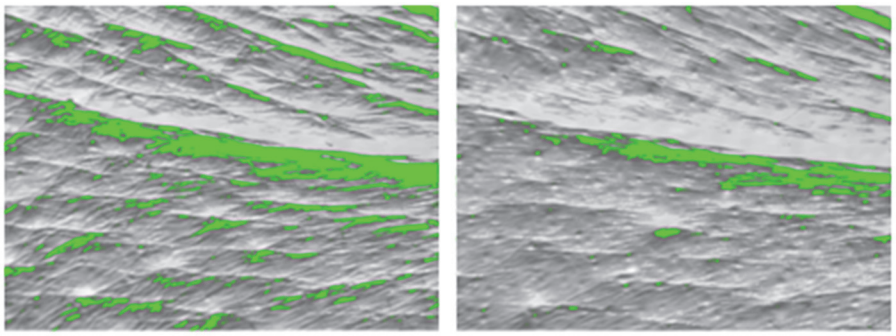


Figure 6 Whitening/lightening potentials of lotion containing EE inflorescence HG extract (dark column) compared to lotion base (clear column) evaluated by observing the increasing of % ITA° after 2- and 4-week of applications on volunteer's forearms.



Before **4 weeks**

Figure 7 Effects of lotion containing EE Inflorescence HG extract on wrinkle reduction. Analysis was performed using skin replicas taken from the eye skin of human volunteer. Analysis of the replica images was performed using Skin-visiometer software.

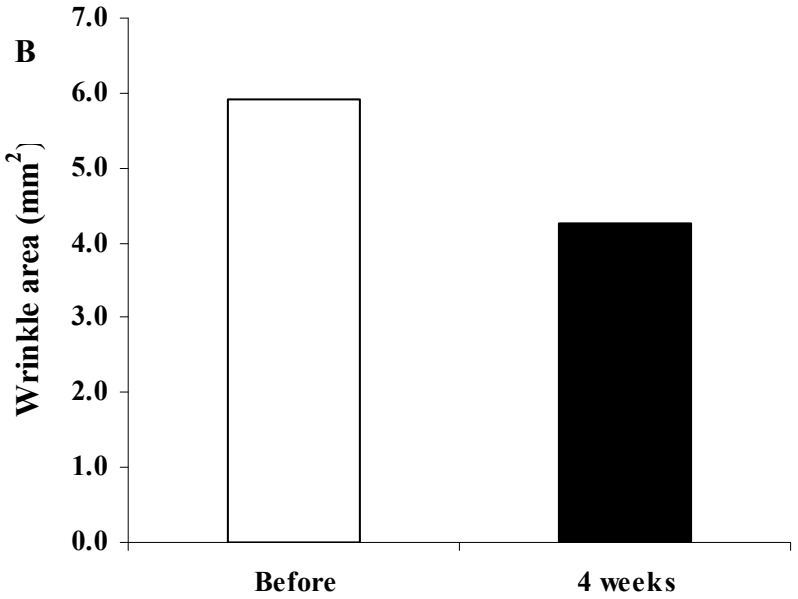


Figure 8 Analysis of the replica images was performed using skin-Visiometer software for the determination of (**A**) wrinkle depth, and (**B**) wrinkle area. Data in the text are reported as percentage reduction of wrinkle depth and wrinkle area after 4 weeks of applications with lotion containing EE inflorescence HG extract.

The HG extract of EE inflorescence was studied for their skin whitening property through tyrosianse inhibition (Figure 3). It can be seen that the extract clearly demonstrated a concentration-dependent inhibitory activity against tyrosinase. Although the anti-tyrosinase potency of EE inflorescence extract (IC_{50} = 10.16 ± 0.73 mg/ml) was lower compared with that of a well-known tyroisnase inhibitor, KA (IC_{50} =0.05

± 0.01 mg/mL), this result indicated a capacity for tyrosinase inhibition of "the natural whitener".

Collagenase is known to be a major enzyme responsible for dehydration and wrinkle formation on the skin surface (Wahab *et al*, 2014). The inhibitory effect of EE inflorescence HG extract on this enzyme is shown in Figure 4. It is clearly shown that the inhibition of collagenase activity was more pronounced in the presence of EE inflorescence extract compared with EGCG. The order of potency as judged from IC_{50} value was EE inflorescence extract (0.22 ± 003 mg/mL) > EGCG (0.46 ± 0.02 mg/mL). This suggests the potent anti-collagenase action of EE inflorescence HG extract which could therefore contribute to anti-wrinkle effect.

***In vivo* study on skin whitening of lotion containing EE inflorescence HG extract on human volunteers:** The skin whitening or lightening effects of EE inflorescence HG extract on volunteer's skin was evaluated by determining the skin color change with Chromameter. It was observed that L* and ITA° values indicated by the areas with the lotion with and without extract compared to the non-applied area after 2-week of applications were in the range of 0.3-0.5 % and 4.0-6.0 %, respectively, which was not statistically significantly different. However, the remarkable increase of L* and ITA° values could be observed on the areas applied with lotion containing EE inflorescence HG extract after 4-week applications as shown in Figures 5 and 6. From these figures, it could be seen that the increase in L* and ITA° values of the skin applied with the lotion containing EE inflorescence extract were significantly different ($p < 0.05$) from the unapplied area. The L* and ITA° values of the skin applied with the aforementioned lotion after 4-week application increased to be 1.44 and 9.92 %, respectively. The results mentioned above implied that the HG extract of EE inflorescence could promote the skin to be whiter/lighter.

***In vivo* study on skin wrinkles reducing capability of lotion containing EE inflorescence HG extract on human volunteers:** The *in vivo* anti-wrinkle effect of lotion containing EE HG extract on skin of human volunteers was evaluated by using skin visiometer. Figure 7 shows thick and deep wrinkles formed along with fine lines of human volunteer skin before test. Interestingly, the same figure reveals that the thickness and depth of wrinkles was alleviated after 4 weeks of treatment with lotion containing EE inflorescence extract.

In order to quantitatively analyze the wrinkle alleviation potential of lotion with the EE inflorescence extract, the depth and area of wrinkles were determined. As shown in Figure 8, the depth and area of wrinkles was significantly decreased around 14.4 and 28 %, respectively after 4 weeks of such lotion application ($p < 0.05$). The obtained results above revealed that the HG extract of EE inflorescence possessed the high potential capacity to reduce skin wrinkles.

Conclusion

This study demonstrated that the HG extract of EE containing phenolic compounds exhibited remarkable *in vitro* antioxidant, anti-tyrosinase and anti-colla-genase activities. The *in vivo* study revealed that the lotion containing this extract produced significant skin whitening and anti-wrinkle effects in volun-teer's skin.

It is suggested that the performance of HG extract of EE can be a good active ingredient in topically used products for promotion of skin whitening and alleviation of skin aging. However, whether this ingredient can be formulated into the internally used products for same benefits are needed further investigation.

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