# PHENOLIC CONTENT AND *IN VITRO* INHIBITORY EFFECTS ON OXIDATION AND PROTEIN GLYCATION OF SOME THAI MEDICINAL PLANTS

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**ABSTRACT**

Fifteen medicinal plant extracts were investigated for: total phenolic content and free radical scavenging effect by DPPH and ABTS assays; anti-lipid peroxidation activity by TBARS; and for antiglycation activity. The results revealed that the total phenolic content showed good correlation with free radical scavenging by ABTS (r = 0.721) and anti-lipid peroxidation by TBARS (r = -0.659), but showed no correlation with antiglycation. Three extracts from *Tamarindus indica, Camellia sinensis* and *Artocarpus lakoocha* demonstrated a significant antioxidant effect, and also showed a promising antiglycation effect. The $IC_{50}$ (mg/ml) were 0.9-0.16 for the DPPH method; TEAC values (mg Trolox/mg sample) of 1.72–2.83 for the ABTS method; $IC_{50}$ (mg/ml) of 0.64-1.22 for the TBARS method; and $IC_{50}$ ranging from 0.01 to 3.20 for the antiglycation method. These three herbs were found to possess effective antioxidant and antiglycation activities, and could be further developed for use in anti-aging cosmetics.

**Keywords**: Antioxidant, Glycation, Lipid peroxidation, Phenolic content.

## INTRODUCTION

Phenolic compounds are secondary plant metabolites and are involved in a wide range of specialized physiological functions. They are very important for the normal growth, development and defense mechanisms of plants (Maisuthisakul *et al*., 2007). These compounds are capable of inhibiting free radicals, and hence can retard the aging process. Aging causes pathogenesis of the skin (Vioux-Chagnoleau *et al*., 2006). Theories involving free radicals and glycation processes are commonly used to explain the mechanism of skin aging. Aging proceeds by means of highly complicated biochemical processes in which the involvement of reactive oxygen species (ROS) and free radicals have been implicated. The overproduction of ROS and reactive nitrogen species (RNS) is a common underlying mechanism of aging, as they can damage various cellular components, including proteins, lipids and DNA. Ultraviolet (UV) light produces ROS in the skin, which accelerates aging by damaging DNA, proteins, lipids and other cellular constituents (Rabe *et al*., 2006). The Maillard reaction and its end products, advanced glycation end products (AGEs), are rightly considered to be one of the important mechanisms of aging. The glycation reaction involves a series of non-enzymatic reactions between the carbonyl group on reducing sugars and the amino group on proteins, leading to the formation of AGEs, which are involved in the pathogenesis of diabetic and aging-related complications (Rahbar and Figarola, 2002). The outward manifestations of tissue aging, occurring in the elderly, primarily involve the two major structural proteins of the body: collagen and elastin. Therefore, targeting glycation should have a broad and beneficial effect on aging. Aminoguanidine, a small, hydrazine-like compound, has been synthesized and has become one of the most promising pharmacological interventions for glycation inhibition (Dyer *et al*., 1993; Rusak *et al*., 1997). The use of topical medicinal plants is in favor among dermatologists because of their broad biologic activity. They have been reported to have not only antioxidant but also anti-inflammatory as well as antiglycation effects. In this study 15 medicinal plants, which have long been used in Thai traditional medicine to promote good health, were investigated for free radical scavenging, anti-lipid peroxidation, and antiglycation activities; their total phenolic contents were also determined.

## MATERIALS AND METHODS

### *Plant materials*

Fifteen medicinal plants – *Allium ascalonicum* auct., *Allium sativum* Linn., *Angiopteris evecta* Hoffm., *Artocarpus lakoocha* Roxb., *Camellia sinensis* Kuntze, *Chromolaena odorata* (Linn.) King et Robins., *Citrus indica* Tan., *Curcuma comosa* Roxb., *Garcinia atroviridis* Griff., *Garcinia mangostana* Linn., *Jatropha gossypifolia* Linn., *Morus alba* Linn., *Phyllanthus emblica* Linn., *Tamarindus indica* Linn., and *Terminalia bellerica* (Gaertn.) Roxb. – were obtained from Thai-Lanna Herbal Industry Co., Ltd., Chiang Mai, Thailand.

### *Chemicals*

Bovine serum albumin (BSA); 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH); 2,2-azinobis (3 ethylbenzothiazoline-6-sulphonic acid) (ABTS+); deoxy-

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D-ribose; thiobarbituric acid (TBA); butylated hydroxytoluene (BHT); quercetin; kaempferol; Trolox; gallic acid; D-glucose; and sodium azide were obtained from Sigma Chemical, USA. Other chemicals used in the study were 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) (Wako Pure Chemical Industries, Japan); D-fructose (Merck, Germany); Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) (Dow Chemical, USA); soybean phosphatidylcholine (Degussa, Germany); and cholesterol (lanolin, $C_{27}H_{46}O$; Fluka Chemie, Switzerland)

***Preparation of plant extracts***

All plants were dried at 50ºC and then ground into powder. A powder sample of each plant (100 g) was mixed with 95% ethanol (2,000 ml) in the dark at room temperature and shaken during the 4.5 h extraction time to ensure complete extraction. The extracts were filtered through Whatman No.4 paper and then centrifuged (1500x *g* for 15 min). Ethanol was removed from the supernatants on a rotary evaporator at 50 mm Hg, 50$^o$C, to yield thick and viscous residues as ethanolic crude extracts.

***Determination of total phenolic content***

The total phenolic content of each ethanolic extract was determined using Folin-Ciocalteu reagent (Kähkönen *et al*., 1999). Each sample of extract (0.8 to 0.9 g ± 0.01 mg) was diluted with 5 ml methanol; then the sample solution (200 µl) was transferred into a test tube and mixed thoroughly with 1 ml of Folin-Ciocalteu reagent. After mixing for 3 min, 0.8 ml of 7.5% (w/v) sodium carbonate was added. Each sample mixture was agitated with a vortex mixer, allowed to stand for a further 30 min in the dark, and centrifuged at 3300 X *g* for 5 min. The absorbance of each plant extract and a prepared blank were measured at 765 nm using a Genesys 20 spectrophotometer (Thermo Fisher Scientific, USA). Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from the calibration curve: y = 4.3308x + 0.0.0336, R2 = 0.9994 as shown in fig.1. Data are reported as mean ± standard deviation of three replications.

***Evaluation of free radical scavenging activity using DPPH assay***

The experiment was carried out according to the method of Blois (1958) with a slight modification. Briefly, 20 µl of each sample solution dissolved in 95% ethanol was mixed with 180 µl of 167 µM DPPH in methanol. The mixtures were incubated at 37°C for 30 min, and then the absorbance was measured at 540 nm using a DTX 880 multimode detector (Beckman Coulter, USA). A control solution (containing only DPPH) underwent the same process. All measurements were performed in triplicate. The percentage of DPPH radical scavenging activity of each plant extract was determined at five sample concentrations within the range of 10–90% reduction in absorbance, as calculated in Eq. (1):

DPPH radical scavenging activity (%)

$$= [A_o - (A_1 - A_s)]/A_o \times 100 \quad (1)$$

where $A_o$ is the absorbance of the solution, $A_1$ is the absorbance in the presence of the plant extract in DPPH solution, and $A_s$ (which is used for error correction arising from unequal color of the sample solutions) is the absorbance of the sample extract solution without DPPH. The $IC_{50}$ value (mg/ml) of each extract was estimated by sigmoid non-linear regression using SigmaPlot 2000 Demo.

***Evaluation of free radical scavenging activity using ABTS assay***

ABTS radical cat ions ($ABTS^+$) were produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. After the addition of 1.0 ml of diluted $ABTS^+$ solution to 10 ml of antioxidant compounds or Trolox standards (final concentration 0-15 mM) in ethanol, absorbance readings were taken at 30°C exactly 30 min later. Determinations of percentage inhibition by spectrophotometry at 734 nm were carried out in triplicate.

***Lipid peroxidation assay***

This assay was modified from the method of Prabhakar (2007). A solution of soybean phosphatidylcholine (247.36 mg) and cholesterol (30.92 mg) in chloroform (20 ml) was dried under vacuum in a rotary evaporator (<50°C) to yield a thin, homogenous film, which was placed in a desiccator for 24 h. The film was then dispersed in phosphate buffered saline (PBS) solution (pH 7.2, 20 ml) in a water bath (50°C). The mixture was sonicated to obtain a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 60 µl of AAPH to the mixture containing liposome (600 µl). The reaction mixture was incubated at 50°C for 24 h. After incubation, 250 µl of thiobarbituric acid (0.6% w/v), 100 µl of Triton X-100 (3% v/v) and 500 µl of BHT (20% v/v) were added to terminate the reaction. The samples were heated at 90°C for 30 min, and then allowed to cool. The absorbance of the upper organic layer was measured by a multimode detector at 540 nm.

***Protein glycation assay***

Protein glycation was assayed following Kim and Kim (2003) as modified from McPherson. Bovine serum albumin (20 mg/ml) was incubated with D-fructose (235 mM) and D-glucose (235 mM) in potassium phosphate buffer (200 mM; pH 7.4). Ethanol (98%) was used to dissolve the sample extracts. All of the reagents and extracts were filtered through No. 4 filter paper, and each of the mixtures was incubated at 60°C for 6 d. Fluorescence intensity was measured by a multimode detector at an excitation of 370 nm and an emission of 440 nm. Ethanolic solution of aminoguanidine was used as a known inhibitor (Gillery, 2006).

All results are presented as mean ± standard deviation (SD). Data correlations were obtained by Pearson correlation, using the SPSS program version 12.0. A value of $P < 0.01$ was considered statistically significant.

## RESULTS AND DISCUSSION

The results of testing the total phenolic content, free radical scavenging, anti-lipid peroxidation, and antiglycation activities of 15 medicinal plants, as well as standard substances, are presented in table 1.

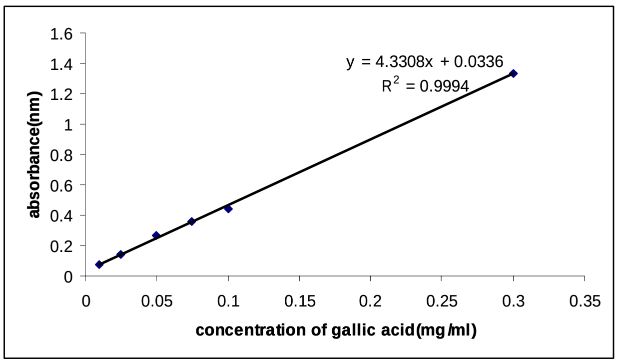


**Fig. 1**: Linear correlation of absorbance (nm) versus total phenolic content.

### *Total phenolic content*

The results revealed that *C. sinensis* contained the highest phenolic content, followed by *A. lakoocha* and *T. indica*. Their GAE values were 0.56±0.02, 0.48±0.01, and 0.44±0.04, respectively. The extract of *G. atroviridis* presented the lowest phenolic content (0.04±0.03); *A. ascalonicum* (0.05±0.01) and *C. indica* (0.07±0.01) also showed low phenolic content, as shown in table 1 and fig. 2. Phenolic content might be considered to correlate with their activities.

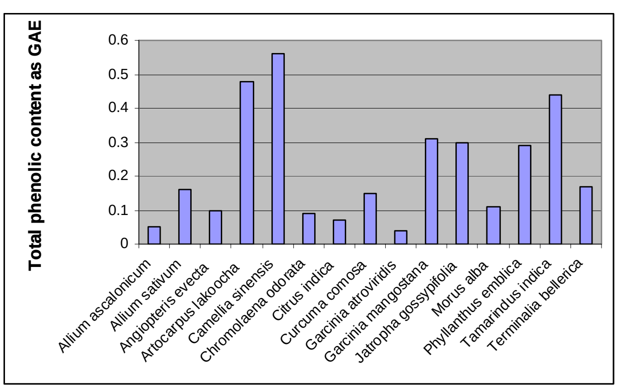


**Fig. 2**: Total phenolic contents of 15 medicinal plant extracts, determined using Folin-Ciocalteu reagent

### *Free radical scavenging activity using DPPH assay*

The results showed that *P. emblica* extract exhibited the highest activity in scavenging DPPH radicals, followed by *T. indica, C. sinensis, T. bellerica* and *A. lakoocha* (fig. 3). These results were comparable to kaempferol; the best standard for inhibiting this reaction was quercetin, followed by Trolox (table 1). No significant correlation was found between phenolic content and DPPH at $P <$ 0.01 (data not shown).

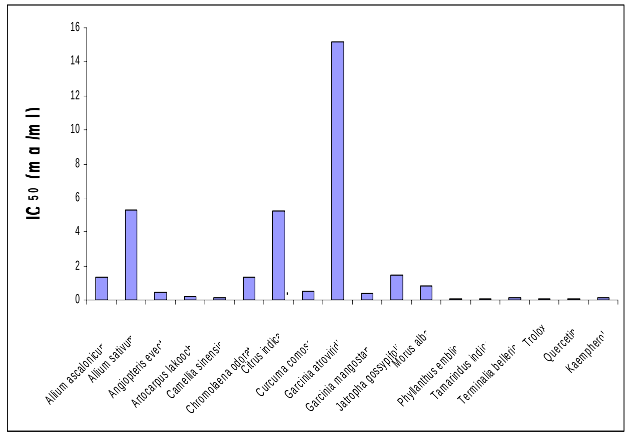


**Fig. 3**: Anti-free radical activity ($IC_{50}$) of ethanol extracts from medicinal plants determined by DPPH assay

### *Free radical scavenging activity using ABTS assay*

Results were expressed as Trolox equivalent antioxidant capacity (TEAC). *P. emblica* demonstrated the highest free radical scavenging activity, followed by *C. sinensis* and *T. indica*, all of which were higher than gallic acid (table 1 and fig. 4). They were also superior to chlorogenic acid and quercetin, whereas the extracts from *A. lakoocha* and *T. bellerica* were comparable to Trolox. Additionally, their total phenolic contents were significantly correlated with antioxidant activity: r = 0.721 ($P < 0.01$), as shown in fig. 5.

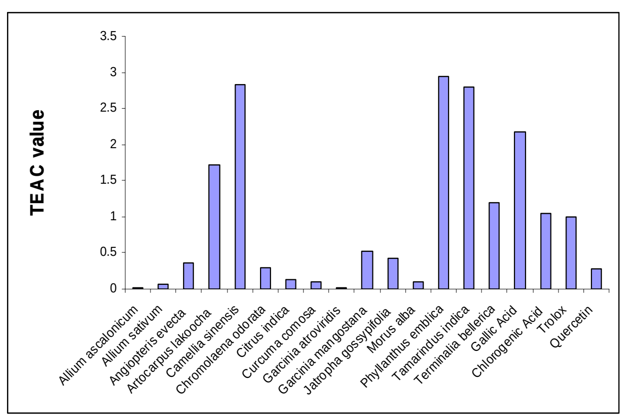


**Fig. 4**: Anti-free radical activity (ABTS) of ethanol extracts from various plant parts of herbs.

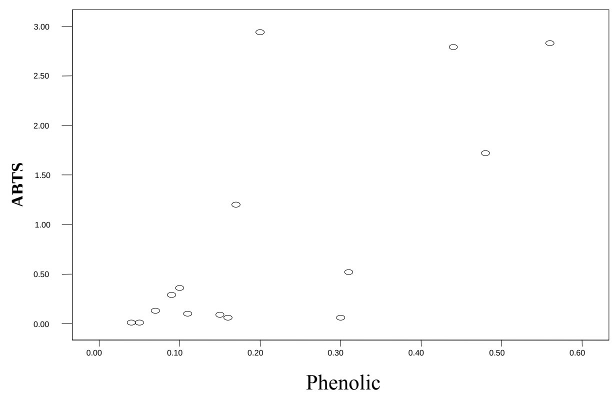


**Fig. 5**: Correlation between total phenolic content and anti-oxidant activity (ABTS) of 15 Medicinal plants.

***Lipid peroxidation assay***

The results were presented as $IC_{50}$ (mg/ml). It was found that *J. gossypifolia, T. indica, C. sinensis, T. bellerica* and *A. lakoocha* possessed high anti-lipid peroxidation, with $IC_{50}$ of 0.42, 0.64, 0.87, 1.11 and 1.22 mg/ml, respectively (table 1, and fig. 6). These results were comparable to quercetin and kaempferol, and better than Trolox. Their total phenolic content (GAE) showed a significant inverse correlation with the $IC_{50}$ value from the TBARs test: r = -0.659 ($P$ < 0.01) (fig. 7). This result indicates that total phenolic content is correlated with anti-lipid peroxidation activity as measured by the TBARS test.

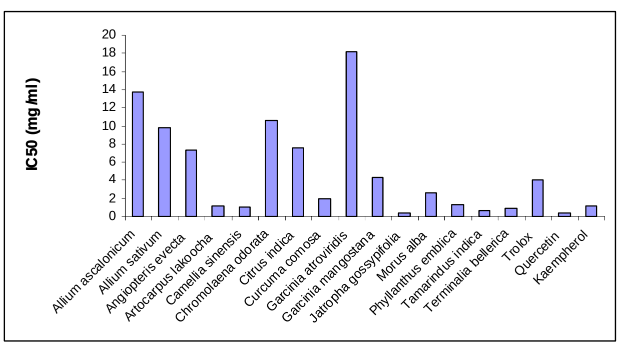


**Fig. 6**: Anti-lipid peroxidation ($IC_{50}$) of ethanol extracts from medicinal plants.

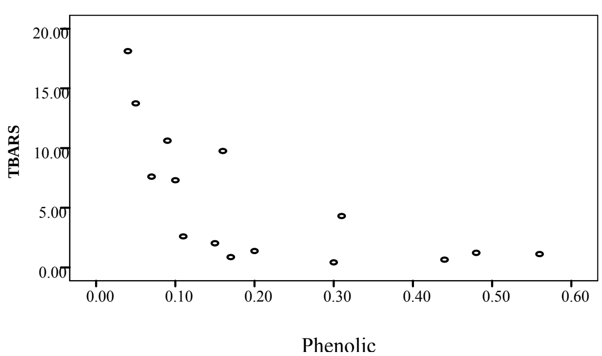


**Fig. 7**: Correlation between phenolic content and $IC_{50}$ of TBARS (mg/ml) in fifteen Thai Medicinal plants.

***Protein glycation assay***

The results revealed that *T. indica* exhibited the highest antiglycation activity, followed by *P. emblica, T. bellerica* and *C. sinensis*, respectively (table 1 and fig. 8). Moreover, all these extracts exhibited $IC_{50}$ values that were lower than aminoguanidine. No statistically significant correlations (at $P$ < 0.01) were found in total phenolic content and glycation tests (data not shown).

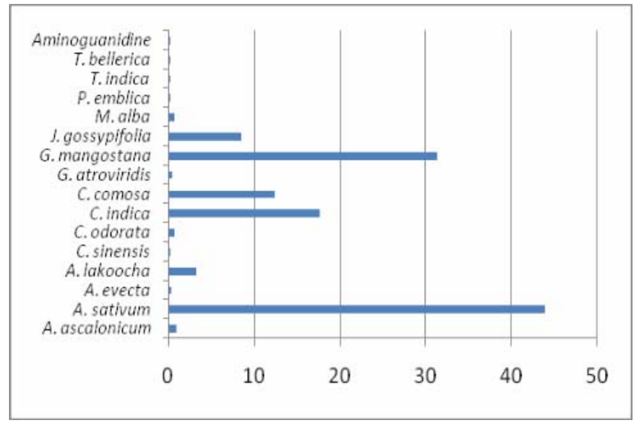


**Fig. 8**: Anti glycation ($IC_{50}$, mg/ml) of ethanol extracts from medicinal plants and standard

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals, or by preventing the decomposition of hydroperoxides into free radicals (Maisuthisakul *et al*., 2007). Results from this study support previous findings that *C. sinensis* contains a high phenolic content and possesses antioxidant activity. The constituents of *C. sinensis* include large amounts of (-)-epigallocatechin, (-)-epicatechin, (+)-gallocatechin, and (+)-catechin and their derivatives, which have been shown to have positive effects on human health (Flutter and Gao, 2004). Our findings showed that the free radical scavenging activity of *C. sinensis* extract in the DPPH assay was comparable to that of Trolox and kaempferol. Its lipid-peroxidation inhibition activity in the TBARS assay was equivalent to kaempferol, and its antiglycation activity was comparable to aminoguanidine.

It has previously been reported that the seed coat extract of *T. indica* contains a polyphenolic flavonoid that displays antioxidant properties. An inhibitory effect of the seed coat extract of *T. indica* on nitric oxide production *in vivo* and *in vitro* has also been reported (Komutarin *et al*., 2004). *A. lakoocha* also had a high phenolic content and exhibited substantial antioxidant activity, comparable to chlorogenic acid (about twice that of Trolox by ABTS assay) and kaempferol (DPPH and TBARS assays). Additionally, the extracts from *G. atroviridis*, *A. ascalonicum*, *A. evecta*, *C. odorata* and *T. bellerica* exhibited promising antiglycation activity, although they

**Table 1**: Total phenolic content and antioxidant, anti-lipid peroxidation, and antiglycation activities of 15 medicinal plants and 6 standard substances.

| Medicinal plants | Part of sample | Total phenolic content Calculated GAE (0.05 mg/ml) based on sample weight (0.1 mg/ml)[a] | DPPH $IC_{50}$ (mg/ml)[a] | ABTS TEAC[a] | TBARS $IC_{50}$ (mg/ml)[a] | Glycation $IC_{50}$ (mg/ml)[a] |
|---|---|---|---|---|---|---|
| *Allium ascalonicum* | Bulb | 0.05±0.01 | 1.33±0.03 | 0.01±0.04 | 13.74±0.01 | 0.90±0.08 |
| *Allium sativum* | Bulb | 0.16±0.03 | 5.30±0.04 | 0.06±0.03 | 9.75±0.04 | 43.92±0.06 |
| *Angiopteris evecta* | Rhizome | 0.10±0.03 | 0.42±0.01 | 0.36±0.04 | 7.30±0.04 | 0.28±0.07 |
| *Artocarpus lakoocha* | Heartwood | 0.48±0.01 | 0.16±0.01 | 1.72±0.04 | 1.22±0.02 | 3.20±0.06 |
| *Camellia sinensis* | Leaf | 0.56±0.02 | 0.10±0.02 | 2.83±0.05 | 1.11±0.01 | 0.04±0.05 |
| *Chromolaena odorata* | Leaf | 0.09±0.02 | 1.33±0.03 | 0.29±0.03 | 10.61±0.03 | 0.60±0.08 |
| *Citrus indica* | Fruit | 0.07±0.01 | 5.20±0.06 | 0.13±0.02 | 7.60±0.02 | 17.64±0.10 |
| *Curcuma comosa* | Rhizome | 0.15±0.02 | 0.50±0.03 | 0.09±0.02 | 2.02±0.03 | 12.30±0.08 |
| *Garcinia atroviridis* | Fruit | 0.04±0.03 | 15.20±0.02 | 0.01±0.03 | 18.12±0.03 | 0.32±0.07 |
| *Garcinia mangostana* | Fruit Peel | 0.31±0.02 | 0.38±0.01 | 0.52±0.05 | 4.30±0.04 | 31.33±0.07 |
| *Jatropha gossypifolia* | Rhizome | 0.30±0.01 | 1.45±0.04 | 0.42±0.03 | 0.42±0.03 | 8.40±0.06 |
| *Morus alba* | Leaf | 0.11±0.03 | 0.85±0.03 | 0.10±0.03 | 2.60±0.02 | 0.62±0.07 |
| *Phyllanthus emblica* | Fruit | 0.29±0.05 | 0.06±0.02 | 2.94±0.03 | 1.37±0.03 | 0.02±0.05 |
| *Tamarindus indica* | Seed Coat | 0.44±0.04 | 0.09±0.02 | 2.79±0.06 | 0.64±0.04 | 0.01±0.06 |
| *Terminalia bellerica* | Fruit | 0.17±0.02 | 0.12±0.06 | 1.20±0.04 | 0.87±0.03 | 0.02±0.06 |
| Gallic acid | - | - | - | 2.18±0.02 | - | - |
| Chlorogenic acid | - | - | - | 1.05±0.03 | - | - |
| Trolox | - | - | 0.09±0.01 | 1.00±0.00 | 4.01±0.02 | - |
| Quercetin | - | - | 0.06±0.02 | 0.28±0.04 | 0.38±0.03 | - |
| Kaempherol | - | - | 0.13±0.02 | - | 1.21±0.02 | - |
| Aminoguanidine | - | - | - | - | - | 0.05±0.02 |

[a]Mean±SD

were determined to have low phenolic content. Their antiglycation effect may be due to other constituents contained in the extracts; this requires further investigation.

All of the results indicate that the extracts from *C. sinensis*, *P. emblica, T. indica*, *A. lakoocha* and *T. bellerica* may prove to be a promising source of anti-aging ingredients for skin-care products.

## CONCLUSION

The results from DPPH, ABTS and TBARS assays of some plant extracts indicated high antioxidative activities, especially the ethanolic extracts of *T. indica, T. bellerica, P. emblica, A. lakoocha* and *C. sinensis*. Total phenolic contents presented as GAE were also the highest among these plant extracts, which confirmed that phenolic compounds play an important role in exhibiting antioxidative activity. In addition to their high antioxidative activities, which were comparable to those of quercetin and kaempferol and greater than that of Trolox, these plant extracts also exhibited protein glycation inhibitory activity comparable to that of aminoguanidine. The results also indicated that extracts from *C. sinensis*, *T. indica* and *A. lakoocha* have the potential for providing anti-aging properties in skin care products. However, this *in vitro* evaluation should be corroborated with an *in vivo* study. Since the active compounds are phenolic with low molecular weight and are lipophilic, they are expected to exhibit good penetration of the stratum corneum barrier of the skin. An *in vivo* activity test could confirm skin penetration. The findings of this study warrant further development of anti-aging cosmetic products based on some of these plant extracts.

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## REFERENCES

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, **181**: 1198-1200.

Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR and Baynes JW (1993). Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest*., **91**: 2463-2469.

Flutter B and Gao B (2004). MHC class I antigen presentation – recently trimmed and well presented. *Cell Mol. Immunol*., **1**(1): 22-30.

Gillery P (2006). Oxidative stress and protein glycation in diabetes mellitus. *Ann. Biol. Clin*. (Paris), **64**(4): 309-314.

Kähkönen MP, Hopia AI, Vuorela HJ, Rauha J-P, Pihlaja K, Kujala TS and Heinonen M (1999). Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.*, **47**(10): 3954-3962.

Kim HY and Kim K (2003). Protein glycation inhibitory and antioxidative activities of some plant extracts *in vitro. J. Agric. Food Chem.*, **51**(6): 1586-1591.

Komutarin T, Azadi S, Butterworth L, Keil D, Chitsomboon B, Suttajit M and Meade BJ (2004). Extract of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages *in vitro* and *in vivo. Food Chem. Toxicol*., **42**(4): 649-658.

Maisuthisakul P, Suttajit M and Pongsawatmanit R (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chem., 100: 1409-1418.

Prabhakar KR, Veerapur VP, Bansal P, Parihar VK, Reddy Kandadi M, Bhagath Kumar P, Priyadarsini KI and Unnikrishnan MK (2007). Antioxidant and radioprotective effect of the active fraction of Pilea microphylla (L.) ethanolic extract. *Chem. Biol. Interact.*, **165**(1): 22-32.

Rabe JH, Mamelak AJ, McElgunn PJ, Morison WL and Sauder DN (2006). Photoaging: mechanisms and repair. *J. Am. Acad. Dermatol*., **55**(1): 1-19.

Rahbar S and Figarola JL (2002). Inhibitors and breakers of advanced glycation endproducts (AGEs): A review. *Curr. Med. Chem. – Immunol., Endocr. Metab. Agents*, **2**: 135-161.

Rusak G, Krajačić M and Pleše N (1997). Inhibition of tomato bushy stunt virus infection using a quercetagetin flavonoid isolated from *Centaurea rupestris* L. *Antiviral Res*., **36**: 125-129.

Vioux-Chagnoleau C, Lejeune F, Sok J, Pierrard C, Marionnet C and Bernerd F (2006). Reconstructed human skin: from photodamage to sunscreen photoprotection and anti-aging molecules. *J. Dermatol. Sci*. (Supplement), **2**(1): S1-S12.