Appraisal of biological activities and identification of phenolic compound of African marigold (*Tagetes erecta*) flower extract

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Abstract: The flowers of African marigold (*Tagetes erecta* L), a medicinal plant widely cultivated in Thailand, were subjected to evaluation for total phenolics, DPPH scavenging and thiobarbituric acid-reactive substance (TBARs) assays as well as tyrosinase inhibitory activity. In preliminary studies, the ethyl acetate (EA) extract obtained by continuous extraction showed the highest activities with highest phenolic content among all extracts. Bioassay-guided fractionation of EA extract led to isolation of a flavonoid identified as quercetagetin. Interestingly, it was found that quercetagetin exhibited potent DPPH scavenging activity with IC_{50} of 3.70 µg/ml which is about 2-3 times higher activity than standard quercetin (IC_{50} 5.07 µg/ml) and trolox (IC_{50} 9.93 µg/ml). Moreover, it exhibited tyrosinase inhibitory activity on L-tyrosine (IC_{50} 89.31 µg/ml), higher than α- and β–arbutins (IC_{50} 157.77 and 222.35 µg/ml) and slightly higher (IC_{50} 128.41 µg/ml) than ellagic acid (IC_{50} 151.1 µg/ml) when using L-DOPA as substrate. Testing with skin fibroblasts, all the extracts and quercetagetin demonstrated no toxic effect. These finding strongly indicate that African marigold flower is a promising source of natural antioxidative and tyrosinase inhibitory substances with safe to skin.

Keywords: *Tagetes erecta*, quercetagetin, antioxidant activity, tyrosinase inhibitory activity.

INTRODUCTION

For decades, many Thai herbs and flowers have been investigated for active phytochemicals by many researchers to evaluate the biological activities such as antimicrobial, anti-inflammatory, antioxidant and used as health promoting products (Tereschuk *et al*., 1997; Abad *et al*., 1999; Lorenzo *et al*., 2002). For cosmetic applications, they also investigated their antioxidant, tyrosinase inhibitory and anti-inflammatory activities involving the prevention of skin aging or skin whitening. These activities have been attributed to various phenolic compounds. *Tagetes* is a genus of annual and perennial mostly herbaceous plant in the sunflower (Compositae or Asteraceae) family and is native to North and South America, but nowadays some species have become naturalized around the world (Soule, 1996). *Tagetes erecta* L. (TE). The African marigold, an ornamental plant is native to Mexico and America, has widely been cultivated throughout Thailand for medicinal, ceremonial and decorative purposes. In Thailand, this plant is commonly called "Dow Ruang", literally translated as "star glittering". Numerous traditional uses of various parts of the plant was reported for example, infusion of leaves has been served for anti-inflammatory, antioxidant, fungicidal, nematocidal and insecticidal activities (Parejo *et al*., 2005; Li *et al*., 2011). There are few studies that mentioned about the antioxidant activity of other marigolds: I. Parejo *et al* (2003) found *T. maxima* Kuntze exhibited strong radical scavenging and antioxidant activities. Previous phytochemical studies of TE, generally focused on the essential oils from flowers and on the carotenoids or monoterpene contents, but they are few reports on the phenolic composition of this plant (Guinot *et al*., 2008). This report focuses on the extraction and isolation of marigold flower extracts including identification of a major flavonoid compound identified as quercetagetin (1) together with their biological activities and safety for cosmetic application.

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MATERIALS AND METHODS

Plant material

The African marigold (*T. erecta*) flowers were cultivated and collected from Chiang Mai Province, the northern part of Thailand during January-March and was authenticated by Associated Professor Omboon Luanratana, the expert researcher at Mahidol University, Thailand. The voucher specimen (No.0212090-0212091) has been deposited at the Herbarium Section, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand.

Chemicals and reagents

Standard quercetagetin was purchased from Extrasynthese, France. 2,2-Diphenyl-1-picryl hydrazyl (DPPH), 2-thiobarbituricacid 98%) TBA), *t*-octylphenoxypolyethoxyethanol)Triton X-100), 2,6-di-*tert*-butyl-4-methylphenol (BHT), Trolox, 2-thiobabituric acid 98% (TBA) quercetin, ascorbic acid, Folin-Ciocalteu reagent and gallic acid were purchased from Sigma Chemical Co., (USA). Cholesterol from lanolin ($C_{27}H_{46}O$) was purchased from Fluka Chemie GmbH., Japan. Phosphatidylcholine Epikuron 200 was purchased

from Degussa, Germany, while 2, 2' azobis 2-amidinopropane (dihydrochloride)AAPH) was purchased from Wako Pure Chemical Industries, Japan). Mushroom tyrosinase (product number T3824) and l-tyrosine were purchased from Sigma-Aldrich. L-dopa was purchased from Isotec. l-(+)-Ascorbic acid, acrylamide, Tris (hydroxymethyl)-methlamine, calcium chloride ($CaCl_2$), sodium azide (NaN_3) and sulphorodamine B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and Coomassie® Brilliant Blue G-250 from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from GIBCO (Grand Island, NY, USA).

Extraction, isolation and identification

Dried and powdered marigold (*T. erecta*) flowers (250 g) were separately extracted by Soxhlet apparatus with *n*-hexane (H), ethyl acetate (EA) and ethanol (Et), respectively. All extracts were subsequently filtered and evaporated *in vacuo*. The obtained residues were named as H (yield: 11.23 g=4.49%) EA (yield: 25.65 g=10.26%) and Et (yield: 59.58 g=23.83%) extracts. The most active extract, EA, was subjected for further isolation by vacuum column chromatography (VCC) using three solvents (hexane, ethyl acetate and methanol) to obtained 14 fractions. All the marigold extracts and fractions from EA were investigated for their total phenolic contents, antioxidant and tyrosinase inhibitory activities.

The isolation and purification of active substance from EA extract are as follow: Briefly, 20 g of EA extract was chromatographed on silica gel column and eluted with gradient profile of hexane:EtOAc:MeOH to yield 14 fractions (named as VCC1 to VCC14). Fraction VCC9 (2.55g) was then subjected to a sephadex LH-20 column (2x60 cm, isopropyl alcohol (IPA)) to afford 13 fractions (named as VCC9-1 to VCC9-13). Fraction VCC9-13 (1.28g) was purified by sephadex LH-20 (2x50 cm, acetone) column chromatography to obtain a pale yellow flavonoid 0.96g (0.325% of dried flowers).

The identification of the pale yellow flavonoid was performed using a Bruker Avance 400 FT-NMR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra of the compound in acetone-d_6 were recorded. The melting point of that pure flavonoid and of standard quercetagetin were measured by SMP 3 melting point apparatus (Stuart Scientific). The HPLC analysis of marigold extracts (EA, fraction VCC9) were carried out using a Shimadzu liquid chromatography system consisting of a binary pump (LC-20AD), an autosampler (SIL-20AC) with a 20 µl as injection volume, a column compartment (Inertsil ODS-3, 4.6 × 250 mm, i.d., 5 µm) and a diode-array detector (SPD-M20A). The mobile phase consisted of Acetonitrile [A] (HPLC grade) and 0.01 % trifluoro acetic acid in DI water [B] with a gradient elution from 20-100 % [A]. The flow rate was 1 ml/min. The UV spectra were recorded in the range of 210-400 nm.

Determination of total phenolic content

The marigold extracts were determined for total phenolics by Folin-Ciocalteu assay (Kitzberger *et al*., 2007 and Sendra *et al*., 2009) using a calibration curve with gallic acid. Each filtrated of sample was transferred into an Eppendorf tube that contained distilled water and mixed with the reagent for 3 min then Na_2CO_3 7.5% w/v was added. Vortex mixer was used to agitate the mixtures and incubated for a further 30 min in the dark, then measured the absorbance at 765 nm using spectrophotometer (Shimadzu UV-Vis 2450, Japan). All determinations were done in three replicates. The concentration of total phenolic compounds in all extracts was expressed as gallic acid equivalent (GAE) in milligram per gram dry sample.

Determination of antioxidant activities

Determination of antioxidant activity with 2,2–diphenyl-1-picryl hydrazyl (DPPH) radical scavenging method

The experimental procedure was adapted from Brem *et al*. (2004). Different concentrations of samples dissolved in EtOH were tested with freshly prepared 167µM DPPH$^{\bullet}$ in 180 µL EtOH. Trolox and quercetin served as reference antioxidants. The results were determined after 30 min of reaction time in order to analyze antiradical activities in triplicate and the results are shown as mean ± SD. The disappearance of the free radical DPPH$^{\bullet}$ was measured spectrophotometrically at 520 nm with a microplate reader. The percentage inhibition was calculated by the equation: % Inhibition = $(A_c\text{-}A_s/A_c) \times 100$ where A_c is the absorbance of the control and A_s is the absorbance of test compound. IC_{50} value was then calculated from the graph plotted between inhibition percentages and extract concentration.

Determination of inhibitory effect on lipid peroxidation with thiobarbituric acid reaction species (TBARS) assay

Liposome suspension, consisting of cholesterol (0.25 g), phosphatidylcholine (0.03 g) and 20 ml of 0.2 M potassium phosphate buffer (pH 7.2), was prepared in a sonicator. The extract in ethanol was mixed with a mixture of the sonicated solution (600 µg/ml), and AAPH (0.07M, 60 µl) then incubated for at 50°C. After incubation, the solution (80 µl) was mixed with 0.2% BHT (24 µl), 3% Triton-X (100 µl), 20% acetic acid (500 µl) and 0.6% TBA (250 µl), heated for 30 min and cooled to room temperature. The absorbance of the mixture was measured spectrophotometrically at 540 nm. The percentage inhibition was calculated same as in DPPH assay. IC_{50} values were calculated from the graph plotted between inhibition percentages and extract concentration as: Trolox and quercetin served as reference anti-oxidants.

Mushroom tyrosinase inhibitory assay

The method followed from Pomerantz (1963) with minor modification. The assay was conducted in a 96-well microtitre plate. Each well plate contains 100 µl of sample (EA extract, fraction VCC9, and quercetagetin) 40 µl of 2.5 mM L-dopa or 2.5 mM L-tyrosine solution. After 5 min of incubation at 37°C, the well plate was added 60 µl of mushroom tyrosinase enzyme (50 units/ml in phosphate buffer pH 6.5). Later, the amount of dopachrome produced in the mixture, which was determined by comparison with the blank (solution without enzyme) at 450 nm with DTX 880 multimode detector after incubation at 37°C for 15 min. Ellagic acid, α-arbutin and β-arbutin were used as reference tyrosinase inhibitors. The percentage inhibition of tyrosinase activity was calculated as follows: % inhibition = $[(A_a-A_b)/ A_a] \times 100$. Where A_a=absorbance at 450 nm without test sample and A_b=absorbance at 450 nm with test sample. Sample concentration providing 50% inhibition was calculated as IC_{50} value, from the graph of tyrosinase inhibition percentage against log of sample concentration.

Cytotoxicity test on fibroblasts from human skin

The skin fibroblasts at the 7th passage were cultured in DMEM medium supplemented with FBS 10% v/v, penicillin and streptomycin (100 Unit/ml and 100 mg/ml, respectively) at 5% CO_2, 37°C. Cytotoxicity of quercetagetin, the semi-purified fraction (Fraction VCC9), EA extract and positive control (Ascorbic acid at 0.0001-1 mg/ml) were determined by SRB assay according to the method of Papazisis *et al*. (1997). In 96-well plates, cells were plated at 1.0×10^4 cells/well and left for overnight. Then the fibroblasts were exposed to the samples at 0.0001-1 mg/ml for 24 h. After that the adherent cells were fixed, washed, dyed with SRB and dye was solubilized. Finally the absorbance was measured at 540 nm by a well reader. The assays were done in three independent separate experiments. The percentages of cell viability were calculated as Cell viability (%) = $(A_s\backslash-A_b/A_c-A_b) \times 100$, where A_c was the absorbance of the control, A_s was the absorbance of the sample and A_b. was the absorbance of the medium.

RESULTS

Three crude extracts as hexane (HE), EtOAc (EA) and ethanol (Et) could be extracted from *T. erecta* flowers. Fractions VCC 1 to VCC 14 were obtained from the isolation of EA and then VCC9 was subjected to further isolate until a flavonoid, quercetagetin was purified. The structure elucidation of quercetagetin was completed by NMR spectroscopic technique (table 1) and by comparison spectral data with those of standard quercetagetin.

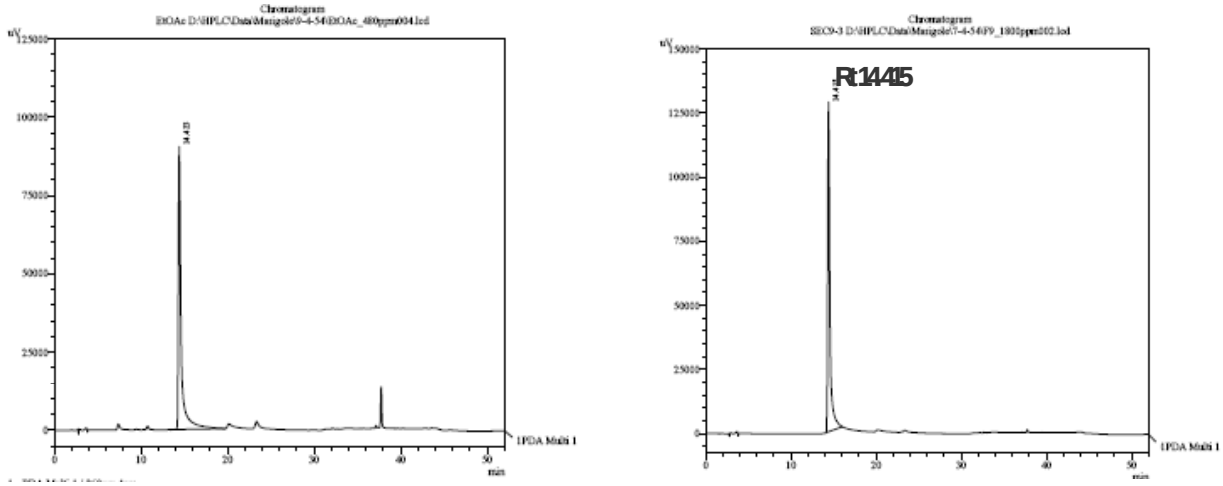
The EA extract, fractions VCC 8 and VCC 9 which revealed the highest antioxidant activity also showed the highest total phenolic contents among all extracts and fractions. Quercetagetin exhibited potent DPPH scavenging activity with IC_{50} of 3.70 µg/ml (Table 2) and also exhibited tyrosinase inhibitory activity on L-tyrosine with IC_{50} 89.31 µg/ml and on L-DOPA with IC_{50} 128.41 µg/ml (table 3).

Cytotoxicity test showed that all the extracts and quercetagetin demonstrated no toxic effect on skin fibroblasts (table 4).

DISCUSSIONS

HPLC analysis and identification of phenolic compound

Fractions VCC 8 and VCC 9 exhibited high antioxidant capacity. Fraction VCC 9 also showed good inhibitory effect on tyrosinase enzyme which is comparable to β-arbutin. Besides, it presented the highest percentage yield



(A) (B)

Fig. 1: HPLC chromatograms of EA extract (A) and VCC9 (B).

among all active fractions, therefore it was chosen for further isolation and identification. The EA extract and fraction VCC 9 were evaluated by HPLC using a photodiode array as detector and quercetagetin as reference standard. The retention time of quercetagetin reference was 14.53 min. The HPLC chromatogram of EA extract showed 27.03% purity of quercetagetin while fraction VCC 9 showed high purity of quercetagetin with 84.11% (fig. 1).

Quercetagetin (1) which is also known as 6-hydroxyquercetin has been commonly found as glycoside form in many plants of Compositae family (D'Agostino *et al*., 1997; Vilegas *et al*., 1999; Parejo *et al*., 2005). The ^{1}H- and ^{13}C-NMR spectra (Table 1) were similar to those of standard quercetagetin.

Quercetagetin (3,3',4',5,6,7-hexahydroxyflavone) (1). Pale yellow powder; melting point could not be detected (Both purified compound and standard quercetagetin were decomposed when heating). UV λ_{max} (MeOH) nm: 207, 260, 359. The chemical structure of quercetagetin isolated from *T. erecta* flower was presented in fig. 2.

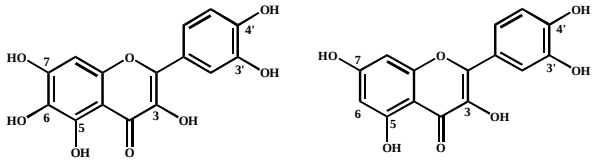


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Fig. 2: Chemical structures of quercetagetin (**1**) isolated from *T. erecta* flower and of quercetin (**2**).

Table 1: ^{1}H- and ^{13}C-NMR data of Quercetagetin (400 MHz) in Acetone-d_6

Carbon	δ_H (ppm), multiplicity (*J*/Hz)	δ_C (ppm)
1	-	-
2	-	147.3
3	-	135.5
4	-	175.7
5	-	146.1
6	-	128.3
7	-	153.2
8	6.63, *s*	94.3
9	-	144.9
10	-	103.4
1'	-	123.1
2'	7.82, *d* (2.0)	115.3
3'	-	145.6
4'	-	149.8
5'	6.99, *d* (9.0)	116.0
6'	7.70, *dd* (9.0; 2.0)	120.5

From the results, most phenolic compounds contained in the EA extract (318.05 mg gallic acid/g extract) compared with HE and Et. While fractions VCC 8 and VCC 9 also expressed the highest total phenolic contents (518.50 and 461.7 mg gallic acid/g extract, respectively) compared with those fractions. In fact, there are many antioxidative experiments which proven that the phenolic compounds were the best free radical scavenger and inhibitor of lipid peroxidation (Atmani *et al*., 2009). From our results (table 2), the EA extract, fractions VCC 8 and VCC 9 which revealed the highest antioxidant activity also showed the highest total phenolic contents among all extracts and fractions. These results strongly indicate that phenolic compounds are major contributors to their antioxidant activity. Therefore, the EA extract and its semi-pure fractions obtained from TE were found to be promising antioxidants for cosmeceutical application as anti-aging. From Table 2, fraction VCC 8 presented a slightly higher phenolic contents than VCC 9, but gave about 2 times less percentage yield and slightly lower antioxidant capacity. Thus, the fraction VCC 9 was then subjected for further investigation. Finally, a pale yellow flavonoid identified as quercetagetin was successfully obtained from fractions VCC 9-13. This compound also exhibited potent DPPH scavenging activity with IC_{50} of 3.70 µg/ml which is about 2-3 times higher activity than that of standard quercetin (5.07 µg/ml) and trolox (9.93 µg/ml). This result is attributed by the high reactivities of hydroxyl substituents in the structures (Heim *et al*., 2002). For the inhibition on lipid peroxidation determined by TBARs assay, the liposome was prepared to represent as cell membrane for the lipid peroxidation reaction. We found that quercetagetin demonstrated IC_{50} of 25.38 µg/ml, about 2 times less activity than that of quercetin (12.94 µg/ml). This may not associated with general rule of antioxidant capacity of flavonols, in which the amount and the -OH position in their structures are the most important for their *in vitro* antioxidant properties. In our study, quercetin which is more lipophilic than quercetagetin, could be better incorporated into liposome and the inhibition of lipid peroxidation was by chain termination and scavenging peroxyl radicals as previously explained (Kulkani *et al*., 2004). Together with the discrepancy in structure-activity relationship caused by diversity and multiple mechanisms of flavonoid actions, and the numerous methods and measurements of oxidative process (Heim *et al*., 2002) are therefore reasonable to explain this result.

Tyrosinase inhibitory activity

Tyrosinase enzyme plays an important role in converting tyrosine to L-dopa (with hydroxylation), L-dopa to dopaquinone (with oxidation) and then to dopachrome, before the polymerizations into melanins, the skin pigment. Compounds with tyrosinase inhibitory activity are widely known as skin whitening agent. The results from the mushroom tyrosinase inhibitory activity test of the marigold extracts (Table 3), were found that the EA extract shows low activity whereas its fractions, especially fractions VCC 7, VCC 8 and VCC 9 expressed the highest activity with IC_{50} ranged from 164.49 to 291.83 µg/ml which were comparable to both α-arbutin

Table 2: Total phenolic contents and antioxidant activities of African marigold extracts and fractions from EA extract

Samples	Total phenolic (mg gallic acid/g extract)	DPPH (IC_{50} µg/ml)	TBARs (IC_{50} µg/ml)
Extract			
Hexane extract, H	10.97±0.24	353.18±1.59	LA
Ethyl acetate extract, EA	318.05±3.18	15.71±0.24	91.48±0.56
Ethanol extract, Et	17.78±1.22	71.09±0.62	263.80±1.98
Fractions of EA (% yield in term of EA)			
VCC1 (0.54)	LA	LA	LA
VCC 2 (0.27)	11.66±0.43	LA	LA
VCC 3 (1.16)	15.61±0.54	LA	LA
VCC 4 (0.57)	12.12±0.33	LA	LA
VCC 5 (0.53)	2.70±0.26	LA	LA
VCC 6 (1.16)	79.70±1.18	LA	LA
VCC 7 (1.12)	260.90±3.32	19.98±1.13	80.28±0.85
VCC 8 (5.10)	518.50±6.45	7.80±0.49	42.73±0.44
VCC 9 (12.77)	461.70±5.12	7.64±0.10	38.95±0.28
VCC 10 (9.52)	307.50±3.28	12.48±2.91	69.18±0.21
VCC 11 (3.05)	277.10±2.59	15.50±4.14	75.17±0.13
VCC 12 (4.42)	313.53±3.43	57.24±0.68	234.78±1.21
VCC 13 (29.16)	120.90±1.36	82.32±0.69	408.68±2.96
VCC 14 (24.80)	49.05±0.63	174.91±25.58	988.02±13.75
Quercetagetin (1)	ND	3.70±0.21	25.38±0.05
Quercetin	ND	5.07±0.18	12.94 ±0.38
Trolox	ND	9.93±0.29	ND

LA – Low activity, ND- Not determined

and β-arbutin (157.77 and 222.35 µg/ml). Interestingly, they could inhibit tyrosinase enzyme in the step of converting L-dopa to dopachrome while α-arbutin and β-arbutin could not. For a pure quercetagetin, it exhibited about 2 times higher activity than α-arbutin and 3 times than β –arbutin for the step of converting L-tyrosine to L-dopa and then dopachrome. In addition, for the step of converting L-dopa to dopachrome, fraction VCC 8 and fraction VCC 9 exhibited IC_{50} ranged from 140.36 to 193.17 µg/ml which were comparable to ellagic acid (IC_{50}=151.1 µg/ml). Moreover, the pure quercetagetin, also showed higher activity than ellagic acid. These implied that antioxidative compounds may promote the tyrosinase inhibitory effect in the step of converting L-dopa to dopachrome due to their antioxidative synergistic. Therefore, the fractions from the EA extract especially fractions VCC 7, VCC 8 and VCC 9 that consisting high amount of total phenolic compounds (table 2) as well as quercetagetin, obtained from marigold were found to be promising inhibitor to tyrosinase enzyme for cosmeceutical application as skin whitening.

Cytotoxicity on normal human skin fibroblasts

The results on viability test on human skin fibroblasts of marigold extracts revealed that all of the extracts and quercetagetin gave cell viability between 85 and 121%, slightly decreasing in % cell viability of quercetagetin and ascorbic acid have been observed at high concentration (1 mg/ml), but not significant. Ascorbic acid which is more powerful and well known antioxidant was always used as a positive control of many antioxidative tests as well as cytotoxicity for skin fibroblasts (Manosroi *et al*., 2011). This indicated that not only the EA extract, but also its semi-purified fraction and pure compound (quercetagetin) show no cytotoxicity effect (table 4). This finding implied that marigold extracts and the consisting flavonoids were safe for cosmetic or topical application.

Table 3: Mushroom tyrosinase inhibitory activity of African marigold extracts and fractions from EA, expressed as IC_{50} (µg/ml)

Samples	IC_{50} (µg/ml) (mean± SD)	
	L-tyrosine as substrate	L-dopa as substrate
α -arbutin	157.77±2.8	NA
β-arbutin	222.35±11.63	NA
EA extract	509.43±57.28	440.59±21.47
VCC7	164.49±3.88	352.22±32.4
VCC 8	186.39±6.95	140.36±7.99
VCC 9	291.83±21.76	193.17±27.35
VCC 10	460.93±19.12	488.85±23.7
VCC 11	390.76±14.19	470.47±29.79
Quercetagetin (1)	89.31±2.06	128.41±3.72
Ellagic acid	ND	151.1±2.23

ND: Not detectable. NA: No activity

CONCLUSION

In this study, quercetagetin, as a major flavonoid

Table 4: Viability of normal human skin fibroblasts tested with EA extract, fraction VCC9 and the pure compound, quercetagetin from African marigold compared with ascorbic acid

Samples	Final concentration (mg/ml)				
	0.0001	0.001	0.01	0.1	1
Normal human skin fibroblasts					
EA extract	113.31±10.68	113.26±24.24	102.24±16.59	114.16±22.81	115.91±25.76
VCC9	113.42±13.16	108.25±29.50	98.68±18.54	121.82±18.74	109.89±31.56
Quercetagetin (1)	102.06±32.01	105.28±16.72	93.56±21.77	87.83±19.11	85.22±21.16
Ascorbic acid	123.86±18.23	93.15±5.82	101.75±19.91	82.35±21.69	76.72±28.06

compound was successively isolated from African marigold flower that possesses potent antioxidant activity compared with quercetin (a typical flavonoid found in mostly plants) and trolox (vitamin E analog) and also exhibited strong tyrosinase inhibitory activity compared to arbutins, well known skin whitening agents. Besides, the EA extract and its fractions which consisting high amount of phenolic contents also revealed high potential in both activities. This study is the first report on antioxidant and tyrosinase inhibitory activities and identification of phenolic compound found in *T. erecta.* Therefore, these finding strongly indicate that marigold flower cultivated in northern Thailand, is a promising source of natural antioxidative and tyrosinase inhibitory substances with no cytotoxic effect on human skin fibroblast and should be further investigated for the development into cosmeceutical products as anti-aging.

ACKNOWLEDGEMENT

The authors are grateful to National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency, Thailand for financial support.

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