International Journal of Phytomedicine 6 (2014) 93-102
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Mutagenicity, antimutagenicity and tyrosinase inhibition activity of hydroglycol extracts from *Terminalia chebula* Retzius, *Terminalia bellerica* Roxb and *Rafflesia kerrii* Meijer

Wijittra Nittayajaiprom[1], Padchanee Sangthong[2*], Sirirat Chancharunee[1,2*], Angkana Wipatanawin[1,2], Pimphaka Wanasawas[3] and Malyn Chulasiri[3]

***Corresponding author:**

Padchanee Sangthong
Sirirat Chancharunee

[1]Bio-organic Synthesis and Molecular Biochemistry, Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

[2]Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

[3]Research and Development Division, SJI, Bangkok, Thailand

Abstract

The hydroglycolic extracts from *Terminalia chebula* Retzius, *Terminalia bellerica* Roxb and *Rafflesia kerrii* Meijer were investigated for total phenolic content (TPC), cytotoxicity, mutagenicity, antimutagenicity and antityrosinase for safety assessment as novel botanical-based cosmeceutical ingredients. These plant extracts showed TPC between 4.90 ± 0.02 and 112.40 ± 0.08 mg GAE g^{-1} of extract when using the Folin-Ciocalteu method. The cytotoxicity study revealed that the 50% cytotoxicity dose (CD_{50}) towards normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines was 5.43 ± 0.18 - 39.39 ± 0.14 mg mL^{-1} and 4.35 ± 0.33 - 58.23 ± 0.18 mg mL^{-1}, respectively. In genotoxicity investigation, it was found that all extracts were not mutagenic at the concentrations up to 87.34 mg 0.1 mL^{-1} when tested with *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of metabolic activation (S9 microsomal fraction). The extracts were further tested for antimutagenic activity against 2-aminoanthracene (2-AA) and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) which were used as the tested mutagens. Interestingly, all hydroglycolic extracts exhibited the inhibitory effect on the mutagenicity after being induced by 2AA and AF-2 in *S. typhimurium* strains TA98 and TA100 in the presence and absence of metabolic activation. All plant extracts were further investigated for tyrosinase inhibitory activity. Results showed that all extracts possessed tyrosinase inhibitory activity with 50% inhibitory concentration values (IC_{50}) of 1.27 ± 0.49 - 39.96 ± 0.21 mg mL^{-1}. Overall studies including their antimutagenicity and antityrosinase activities suggest that the hydroglycolic extracts of these three plants may be used as potential candidates for skin-care cosmeceutical ingredients.

Keywords: *Terminalia chebula* Retzius, *Terminalia bellerica* Roxb, *Rafflesia kerrii* Meijer, genotoxicity, tyrosinase inhibition activity.

Introduction

Exposure to UV radiation can induce a number of biological responses, including cell membrane and DNA damage, sunburning, photoaging, melanogenesis and skin cancer. To protect and reduce the harmful effects of UV radiation, synthetic and botanical-based cosmeceutical products are recommended to be used as active photoprotectives. In recent years, naturally occurring compounds derived from plant extracts have gained considerable attention as protective agents due to their low mammalian toxicity [1-3]. In this work, plant extracts of *Terminalia chebula* Retz., *Terminalia bellerica* Roxb and *Rafflesia kerrii* Meijer were investigated for novel properties in cosmeceutical applications.

T. chebula Retz. belongs to the family Combretaceae. It is found in the Southern Asia, India including Thailand. The fruits are used as a traditional medicine [4,5]. *T. chebula* Retz. extract has been reported to have strong antianaphylactic action, as well as antiinflammatory, anticancer, and analgesic properties [5-7]. The *T. chebula* Retz fruits have been found to contain chemically active compounds such as chebulagic acid and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) which are reported to have antiinflammatory and inhibitory effects on elastase and hyaluronidase, respectively [8,9].

T. bellerica Roxb belongs to the same family as *T. chebula* Retz., Combretaceae. Its fruits are also used in traditional medicine and proven to have biological activities including antimicrobial, antidiabetic, antioxidant, anti–HIV, and antiproliferative (against cancer cells) acitivities [10-13]. Chemically, the fruit shows the presence of galic acid, ellagic acid, belleric acid, β-sitosterol, ethyle gallate, and galloyl glucose [13-14].

Rafflesia kerrii Meijer is in the family of *Rafflesiaceae*. It is found in Southeast Asia such as in the rainforests of Southern Thailand, Malaysia, Indonesia and Philippines. In Thai traditional medicine, a decoction of the flower buds is used to help restore the female uterus after giving birth. It is also used to treat fever and

backache. Recently, the structural determination of the five compounds in *R. kerrii* Meijer was reported [15]: Four hydrolyzable tannins i.e., 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucopyranoside, 1,2,6-tri-O-galloyl-*β*-D-glucopyranoside, 1,4,6-tri-*O*-galloyl-*β*-D-glucopyranoside and 1,2,4-tri-*O*-galloyl-*β*-D-glucopyranoside, and one phenylpropanoid glucoside were isolated from 95% aqueous ethanol extract.

In the present study, the *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts were investigated with the expectation for use in cosmeceutical industry. These plants were extracted with hydroglycol which is the colorless and odorless solvent. This solvent has a boiling point higher than 180 C and a freezing point lower than 30 C. Additionally, hydroglycol possesses humectant and antimicrobial properties. Its safety has made it be able to be used in foods, medicines, and cosmetics [16]. The hydroglycolic extracts from fruits of *T. chebula* Retz. and *T. bellerica* Roxb and flowers of *R. kerrii* Meijer were examined for total phenolic content (TPC), cytotoxicity, mutagenicity, antimutagenicity, and antityrosinase activity.

Material and Methods

Chemicals and Reagents

Chemicals and reagents used for total phenolic content were Folin-Ciocalteu reagent, sodium carbonate (Carlo Eebo, Italy) and gallic acid (Prolabro, France). For cytotoxicity assay, RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin, streptomycin (Gibco, England) and *3-(4,5*-dimethylthiazol-*2*-yl)-*2, 5*-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) were used; while Oxoid nutrient broth (Criterion, USA), Bacto agar (Difco, USA), biotin, glucose-6-phosphate, histidine hydrochloride, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), 2-aminoanthracene (2-AA) and 2-(2-furyl)-3-5-nitro-2-furyl) acrylamide (AF-2; Sigma-Aldrich, USA) were used in mutaganicity and antimutagenicity tests. For antityrosinase assay, mushroom tyrosinase, L-3, 4-dihydroxyphenylalanine (L-Dopa; Sigma-Aldrich, USA), ascorbic acid and kojic acid (Sigma-Aldrich, USA) were used.

Plant Extraction

The fruits of *T. chebula* Retz. and *T. bellerica* Roxb and flowers of *R. kerrii* Meijer were obtained as the powder from CR Pure Light, Thailand. Its product specification, material data sheets and the voucher specimens were kept in Research & Development Division, SJI, Thailand. Briefly, these extracts were obtained from the extraction of dried powders by macerating in 70% hydroglycol (the first two former plants), and 50% hydroglycol (the latter plant) for 48 h. The extracted plants were then filtered and all filtrates were kept as if according to the method described previously at 4°C [16].

Total Phenolic Content (TPC) Analysis

TPC of the tested plant extracts were analyzed by the Folin-Ciocalteu method as described previously [17] with slight modification. One hundred microliters of each extract at different concentrations (0.1- 5.0 mg mL^{-1}) was mixed thoroughly with 4 mL of distilled water and 0.5 mL of 2 N Folin-Ciocalteu reagent. The mixtures were pre-incubated at room temperature for 5 min before adding 0.4 mL of 20% sodium carbonate and incubated for 30 min. Absorption was measured at 765 nm using the UV–visible spectrophotometer model Lamda 25. Gallic acid was used as a standard for the calibration curve and TPC was expressed as mg of gallic acid equivalent per mg of the extract. The experiments were done in triplicate.

Cytotoxicity Assay

The cytotoxic effect of the tested plant extracts was studied using the normal mouse fibroblast (L929) and mouse melanoma (B16F10) cell lines by MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [18]. These two cell lines were purchased from the American Type Culture Collection (ATCC), USA. The cell culture facility was kindly provided by Dr. Nisa Chawapun, Department of Radiology, Faculty of Medicine, Thailand. The fibroblast cell lines were separately seeded in 96-well plates at a concentration of 1 10^5 cells mL^{-1} and cultured at 37°C in an atmosphere of 5% CO_2 for 48 h. The RPMI 1640 medium containing the plant extracts at different concentrations (0.008 - 87.340 mg mL^{-1}) was then added in and incubated at 37°C for 48 h. Twenty microliters of MTT solution was finally added and the cells were incubated at the same temperature for another 4 h. Afterwards, the mixture was removed and 100 μL of DMSO was added into each well to solubilise formazan crystal product. The plates were measured using the microplate reader at 550 nm and 620 nm as test and reference wavelengths, respectively. The 50% cytotoxic dose (CD_{50}) or the concentration of the tested plant extracts affected 50% of viable cells was calculated and compared with that of the untreated control. The experiments were done in triplicate.

Mutagenicity Assay

The mutagenicity of the tested plant extracts were performed by Ames test [19]. The tester strains were *Salmonella typhimurium* TA98 for detection of base-pair substitution mutation of *hisD3052* and *S. typhimurium* TA100 for detection of frameshift mutation of *hisG46* [20]. Both strains were kindly provided by Prof. Dr. Usanee Viniketkummuen, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand. The mouse liver microsome S9 (S9 microsomal fraction) for metabolic activation test were prepared from mouse liver which were previously induced by phenobarbital and 5,6-benzoflavone followed the methods as

described previously [21]. The Ames assay was performed using the pre-incubation technique propounded by Mortelmans and Zeiger *et al* [20]. The tested mixture consisted of 50 µL of the plant extracts at different concentrations (0.088 - 87.43 mg mL^{-1}) and 100 µL of the bacterial culture (approximately 1–2 $x10^9$ cells), to which was then added with either 500 µL of the S9 mix (for metabolic activation) or 0.2 M sodium phosphate buffer, pH 7.4 (for non-metabolic activation). The mixture was pre-incubated at 30 C in a shaker water bath for 30 min before added with 2 mL of molten top agar (45ºC) containing 0.5 mM of histidine-biotin, mixed well and poured onto the minimal glucose agar plate. The plates were incubated at 37 C for 48 h. The criteria to interpret the results were regarding to the regulatory guidelines OECD test guideline No. 471(1997) [22]. The numbers of revertant colonies were counted and compared with negative controls (50% hydroglycol, 70% hydroglycol and DMSO), and positive controls (2-AA for metabolic activation and AF-2 for non-metabolic activation). The experiments were performed in triplicate and two plates were used for the calculation of the average of the revertant colonies.

Antimugenicity Assay

The antimutagenicity assay was done using the modified Ames test. The reaction mixture consisted of 50 µL of mutagen (2-AA for metabolic activation and AF-2 for non-metabolic activation), 50 µL of the plant extracts at different concentrations (10 - 80 mg mL^{-1}), 500 µL of S9 mix or 0.2 M sodium phosphate buffer, pH 7.4, and 100 µL of bacterial culture (approximately 1–2 $x10^9$ cells). After pre-incubating for 30 min, the mixture was added with 2 ml of molten top agar (45ºC) containing 0.5 mM of histidine-biotin, mixed well and poured onto the minimal glucose agar plate. The plates were incubated at 37 C for 48 h. The numbers of revertant colonies were counted and compared with negative controls (50% hydroglycol, 70% hydroglycol, and DMSO), and positive controls (2-AA and AF-2). The experiments were performed in duplicate. The numbers of revertants were used to calculate the % inhibition of mutagenesis using the formula 100-[(A-C/B-D) x100], where A is the number of revertant colonies induced by the mutagen plus the extract, B is the number of revertant colonies induced by the mutagen, C is the number of spontaneous revertants induced by the solvent, and D is the number of spontaneous revertants (DMSO).

Antityrosinase Activity Evaluation

The plant extracts were tested for antityrosinase activity by dopachrome method using L-3,4-dihydroxyphenylalanine (L-Dopa) as a substrate regarding to Chan *et al* [17]. The mixture contained the plant extracts at different concentrations (0.1 - 80.0 mg mL^{-1}), 0.1 mM potassium phosphate buffer, pH 6.8, and 0.06 mg mL^{-1} of mushroom tyrosinase solution. The mixture was incubated at 37 C for 5 min and then added with 2.5 mM L-Dopa before incubated at 37 C for 5 min. The tyrosinase activity was measured under a UV–visible spectrophotometer at 475 nm. Ascorbic acid and kojic acid were used as the positive controls. Hydroglycol at 50 and 70% were used as the negative controls. The experiments were done in triplicate. The percentage inhibition of tyrosinase was calculated as the 50% inhibitory concentration (IC_{50}), or the concentration of the tested extracts that could inhibit tyrosinase activity at 50%. The percentage inhibition of tyrosinase was calculated using the following formula: % tyrosinase inhibition = [(A-B)-(C-D)/A-B] x 100, where A is the absorbance of the control (L-Dopa mixed with tyrosinase in buffer), B is the absorbance of the blank (L-Dopa in buffer), C is the absorbance of the reaction mixture, and D is the absorbance of the blank of C (L-Dopa mixed with the test sample without adding tyrosinase in buffer).

Statistical Analysis

The experiment was performed at least in duplicate. The results were expressed as the mean ± standard deviation (S.D.). The statistical analysis was performed by Student *t*-test using the SPSS 11.5 statistical software. The *p* value of <0.05 was considered to be significantly different.

Results & Discussion

Table 1 showed that *R. kerrii* Meijer extract had the highest TPC. The amount was shown to be 112.40 ± 0.08 mg GAE g^{-1} of wet weight, followed by *T. chebula* Retz. and *T. bellerica* Roxb which were revealed to have TPC at 101.18 ± 0.07 and 94.37 ± 0.03 mg GAE g^{-1} of wet weight, respectively. The same Table revealed the cytotoxic effects of *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts on fibroblast cells. The cytotoxic doses expressed as 50% cytotoxicity (CD_{50}) of these extracts were 5.43 ± 0.18 - 39.39 ± 0.21 and 2.00 ± 0.18 - 26.47 ± 0.47mg mL^{-1} for normal mouse fibroblast (L929) and mouse melanoma (B16F10) cell lines, respectively. The extract from *T. bellerrica* Roxb seemed to be most toxic as its CD_{50} towards both cell lines were 5.43 ± 0.18 and 2.00 ± 0.18 mg mL^{-1} or the lowest CD_{50}, respectively, while *R. kerrii* Meijer showed the least toxicity as its CD_{50} towards both cell lines were 39.39 ± 0.21 and 26.47 ± 0.47 mg mL^{-1} or the highest CD_{50}, respectively. The CD_{50} of *T. chebula* Retz. towards L929 and B16F10 cell lines were 11.96 ± 0.80 and 4.35 ± 0.33 mg mL^{-1}, respectively.

In mutagenicity test, all studied plant extracts were not mutagenic towards *S. typhimuri*um strains TA98 and TA100 in either metabolic or non-metabolic activation. Table 2 revealed the extracts from *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer did not show the dose responding manner with the number of *his+* revertant colonies. Besides, they could not induce these revertant colonies to have the number of higher than 2 folds of the revertant colonies from spontaneous mutation shown by negative controls even the tested concentration was up to 87.34 mg 0.1 mL^{-1} in the presence or absence of metabolic activation. The extracts from *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer were investigated for antimutagenicity against 2-AA and AF-2 in *S. typhimurium* strains TA98 and TA100.

Results are illustrated in Table 3 and Figure 1.

Table 1. TPC and cytotoxic effect on mouse fibroblast cell lines of extracts

Plant extracts	TPC[a] (mg GAE g^{-1} of extract)	CD_{50}[b] (mg mL^{-1})	
		L929 cell line	B16F10 cell line
T. chebula Retz.	101.18 ± 0.07	11.96 ± 0.80	4.35 ± 0.33
T. bellerica Roxb	94.37 ± 0.03	5.43 ± 0.18	2.00 ± 0.18
R. kerrii Meijer	112.40 ± 0.08	39.39 ± 0.21	26.47 ± 0.47

Note: [a]Estimated as mean ± standard division of the triplicate experiments. [b]Expressed as the mean ± standard division of triplicate experiments. The data were analyzed by Bio DataFit software v1.02 from Chang Biosciences Inc.

Table 2. Mutagenicity of *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts towards *S. typhimurium* strains TA98 and TA100

Concentrations (mg 0.1 mL^{-1})	No. *His+* revertant colonies/plate[a]			
	TA98		TA100	
	Absence of S9 Mix	Presence of S9 Mix	Absence of S9 Mix	Presence of S9 Mix
DMSO	38.67 ± 4.93*	41.67 ± 4.51*	122.33 ± 8.08*	147.67 ± 10.79*
50% Hydroglycol	28.00 ± 7.94*	43.00 ± 2.00*	122.33 ± 17.62*	146.00 ± 8.72*
70% Hydroglycol	58.00 ± 8.00*	68.33 ± 3.51*	124.00 ± 7.00*	149.67 ± 3.79*
T. chebula Retz.				
0.088	62.00 ± 3.46*	62.33 ± 3.21*	104.33 ± 4.62*	115.00 ± 6.24*
0.873	58.00 ± 5.57*	58.67 ± 1.15*	118.67 ± 6.66*	132.00 ± 7.55*
8.734	50.67 ± 7.57*	56.33 ± 2.89*	109.67 ± 9.61*	131.33 ± 8.96*
87.34	57.33 ± 7.51*	61.00 ± 4.36*	125.00 ± 12.17*	133.33 ± 4.51*
T. bellerica Roxb				
0.088	51.00 ± 7.94*	65.67 ± 4.04*	114.00 ± 3.00*	109.67 ± 2.31*
0.873	50.00 ± 4.00*	58.33 ± 6.66*	113.00 ± 11.00*	113.00 ± 4.00*
8.734	55.33 ± 9.87*	64.00 ± 4.58*	119.33 ± 6.51*	123.00 ± 6.00*
87.34	58.67 ± 9.61*	68.33 ± 3.79*	112.00 ± 6.08*	136.33 ± 7.77*
R. kerrii Meijer				
0.088	36.33 ± 1.15*	42.00 ± 1.00*	120.00 ± 7.81*	136.00 ± 4.00*
0.873	35.33 ± 3.51*	47.00 ± 1.00*	119.33 ± 10.02*	130.67 ± 8.08*
8.734	34.33 ± 1.15*	48.00 ± 4.36*	112.33 ± 4.93*	127.67 ± 7.02*
87.34	43.67 ± 2.89*	55.00 ± 2.65*	115.00 ± 5.29*	133.00 ± 6.08*
AF-2 0.10 µg 0.1 mL^{-1}	360.00 ± 32.74	NA	638.00 ± 51.16	NA
2-AA 1.00 µg 0.1 mL^{-1}	NA	461.00 ± 33.87	NA	1290.33 ± 7.64

Note: [a] Expressed as the mean ± standard division of triplicate experiments. NA: not applicable, *Statistically significant with respect to the positive controls by Student *t*-test ($p<0.05$).

Table 3. Inhibition of mutagenicity by *T. chebula* Retz., *T. bellerica* Roxb, and *R. kerrii* Meijer extracts towards *S. typhimurium* strains TA98 and TA100

Concentrations (mg 0.1 mL^{-1})	No. *His*$^{+}$ revertant colonies/plate[a]							
	TA98				TA100			
	Absence of S9 Mix	% Inhibition of mutagenesis	Presence of S9 Mix	% Inhibition of mutagenesis	Absence of S9 Mix	% Inhibition of mutagenesis	Presence of S9 Mix	% Inhibition of mutagenesis
DMSO	25.50 ± 0.71	-	37.50 ± 2.12	-	84.50 ± 2.12	-	93.00 ± 4.24	-
50% Hydroglycol	31.00 ± 1.41	-	31.50 ± 2.12	-	85.00 ± 7.07	-	82.50 ± 17.68	-
70% Hydroglycol	27.50 ± 4.95	-	28.50 ± 0.71	-	88.50 ± 0.71	-	85.00 ± 14.14	-
T. chebula Retz.								
10.00	331.00 ± 39.60	3.50	470.50 ± 10.61*	16.21	512.00 ± 8.49	5.89	396.50 ± 12.02*	35.10
20.00	313.50 ± 13.44	9.06	287.00 ± 24.04*	51.00	460.00 ± 4.24*	17.44	205.50 ± 28.99*	74.90
40.00	277.00 ± 9.90*	20.67	54.00 ± 11.31*	95.17	454.00 ± 8.49*	18.78	124.50 ± 0.71*	91.77
60.00	270.00 ± 12.73*	22.89	40.00 ± 1.41*	97.82	405.00 ± 14.14*	29.67	118.50 ± 7.78*	93.02
80.00	266.50 ± 0.71*	24.01	30.50 ± 0.71*	99.62	406.00 ± 5.66*	29.44	104.50 ± 4.95*	95.94
T. bellerica Roxb								
10.00	279.50 ± 14.85	19.87	551.50 ± 9.19	0.85	520.00 ± 8.49	4.11	412.50 ± 2.12*	31.77
20.00	273.50 ± 6.36*	21.78	498.50 ± 2.12*	10.90	413.00 ± 16.97*	27.89	343.00 ± 14.14*	46.25
40.00	288.50 ± 7.78*	17.01	122.50 ± 7.78*	82.18	440.50 ± 13.44*	21.78	144.00 ± 5.66*	87.71
60.00	273.50 ± 0.71*	21.78	52.50 ± 0.71*	95.45	420.00 ± 5.66*	26.33	142.50 ± 6.36*	88.02
80.00	224.50 ± 16.26*	37.36	33.50 ± 0.71*	99.05	335.50 ± 20.51*	45.11	103.50 ± 2.12*	96.15
R. kerrii Meijer								
10.00	338.00 ± 4.24	2.38	340.50 ± 14.85*	41.42	513.00 ± 12.73	4.89	448.50 ± 19.09*	23.75
20.00	325.00 ± 2.83*	6.25	109.00 ± 8.49*	85.31	511.00 ± 9.90	5.33	138.00 ± 19.80*	88.44
40.00	307.50 ± 2.12*	12.08	56.00 ± 5.66*	95.36	497.50 ± 14.85	8.33	107.00 ± 8.49*	94.90
60.00	230.50 ± 4.95*	36.57	46.50 ± 0.71*	97.16	476.50 ± 7.78*	13.00	101.00 ± 12.73*	96.15
80.00	222.00 ± 5.66*	39.67	36.50 ± 0.71*	99.05	464.00 ± 11.31*	15.78	91.50 ± 17.68*	98.13
AF-2 (µg 0.1 mL^{-1})								
0.02	NA	-	NA	-	534.50 ± 7.78	-	NA	-
0.10	340.00 ± 12.73	-	NA	-	NA	-	NA	-
2-AA (µg 0.1 mL^{-1})								
0.50	NA	-	NA	-	NA	-	573.00 ± 14.14	-
1.00	NA	-	565.00 ± 8.49	-	NA	-	NA	-

Note: [a] Expressed as the mean ± standard division of triplicate experiments. NA: not applicable, *Statistically significant with respect to the positive controls by Student *t*-test (p<0.05).

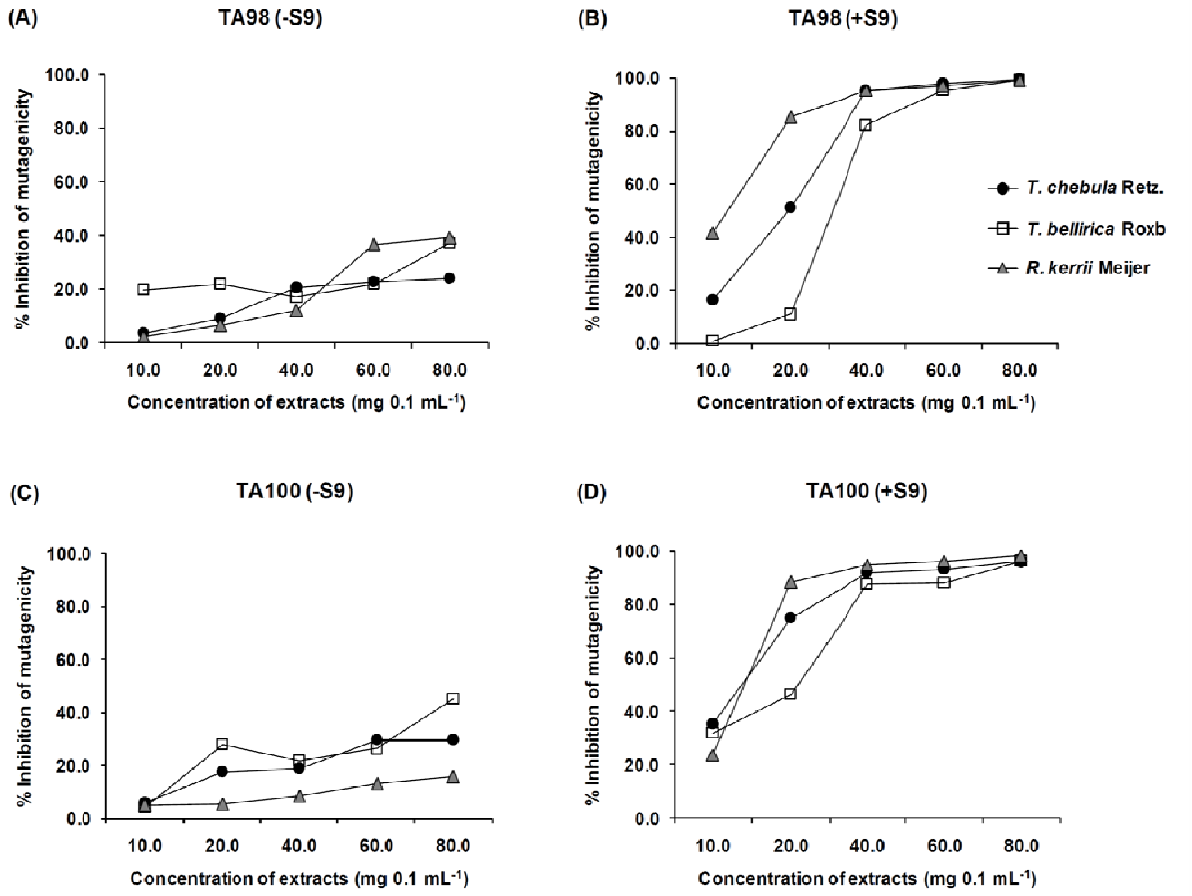

Figure 1: The antimutagenic activities of *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts at different concentrations in *S. typhimurium* treated with AF-2 and 2-AA in the absence and presence of metabolic activation. (A), (B): % inhibition of mutagenesis of extracts in the absence and presence of metabolic activation in *S. typhimurium* strain TA98. (C), (D): % inhibition of mutagenesis of extracts in the absence and presence of metabolic activation in *S. typhimurium* strain TA100.

It could be seen that mutagenesis induced by above mutagens in both strains was reduced by all of these extracts either in the presence or absence of metabolic activation. The % inhibition of metagenesis by the studied extracts increased with the increasing dose regardless in the presence or absence of metabolic activation. In *S. typhimurium* strain TA98 with metabolic activation, the extracts from *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer showed better % inhibition of mutagenesis than non-metabolic activation. The same result was found in strain TA100. In the presence of metabolic activation, the mutagenesis inhibition by these extracts was over 50%, while under non-metabolic activation, the mutagenesis inhibition shown by the extracts from *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer was less than 50%. Comparing between these two strains under the metabolic activation, the antimutagenic potentials of all extracts were observed to be higher in *S. typhimurium* strain TA100 than TA98. When the plant extracts were compared, the extract from *T. bellerica* Roxb seemed to have the highest antimutagenic potential expressed in both strains of *S. typhimurium* induced by AF2 (without metabolic activation). The % inhibition of mutagenesis in strains TA98 and TA100 was 19.87 – 37.36 and 4.11 – 45.11, respectively. While the extract from *R. kerrii* Meijer showed the highest potential induced by 2-AA (with metabolic activation) in both strains. Such mutagenesis inhibition values in strains TA98 and TA100 were 41.42 – 99.05 and 23.75 – 98.13, respectively.

Table 4. Tyrosinase inhibitory activity of plant extracts on mushroom tyrosinase

Plant extracts and tyrosinase inhibitor	% Tyrosinase inhibitory activity [a]	IC_{50} (mg mL^{-1}) [a]
T. chebula Retz.	77.04 ± 3.61	39.98 ± 0.33
T. bellerica Roxb	74.90 ± 3.87	20.05 ± 0.25
R. kerrii Meijer	94.49 ± 0.98	1.92 ± 0.44
Ascorbic acid	98.95 ± 0.90	0.032 ± 0.43
Kojic acid	98.66 ± 0.34	0.019 ± 0.10

Note: [a]Expressed as the mean ± standard division of triplicate experiments. The data of IC_{50} were analyzed using Bio DataFit software v1.02 from Chang Biosciences Inc.

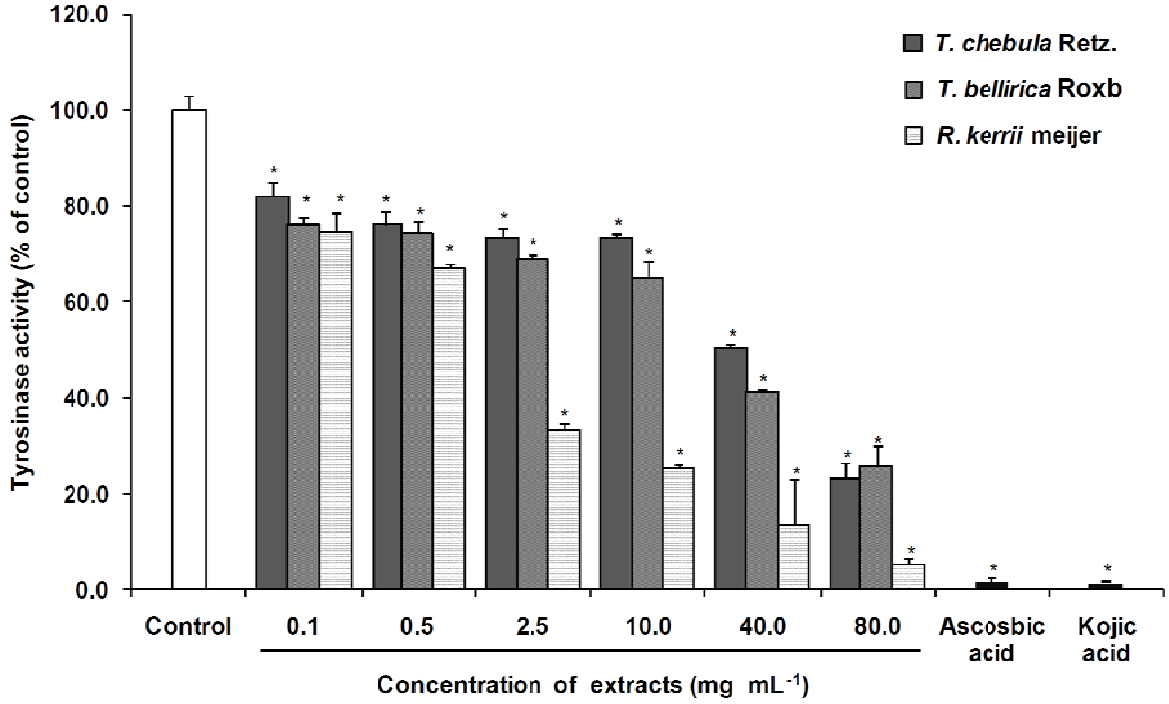

Figure 2: The effect of plant extracts on the mushroom tyrosinase activity. *Statistically significant with respect to the controls by Student *t*-test (p<0.05).

The aforementioned plant extracts were further investigated for antityrosinase activity. All of these plant extracts showed tyrosinase inhibitory activity as demonstrated in Table 4 and Figure 2. The extract from *R. kerrii* Meijer produced the most pronounced activity. Its tyrosinase inhibitory activity was shown to be 94.49 ± 0.98%, followed by the extracts from *T. chebula* Retz. and *T. bellerica* Roxb by which their inhibition activities towards tyrosinase were 77.04 ± 3.61% and 74.90 ± 3.87%, respectively. When the % tyrosinase inhibitory activities of each extract were calculated into IC_{50}, it can be seen from Table 4 that the extracts from *R. kerrii* Meijer had the most appreciable IC_{50} value which was 1.92 ± 0.44 mg mL^{-1}, while the extracts from *T. chebula* Retz. and *T. bellerica* Roxb showed the IC_{50} values of 39.98 ± 0.33 and 20.05 ± 0.25 mg mL^{-1}, respectively.

T. chebula Retz. and *T. bellerica* Roxb have been well-known as the ingredients in triphala of Ayurvedic medicines and a number of Thai traditional medicines. Regarding to indications of use, one benefit is for rejuvenation to make these ingredients be interested for application in pharmaceuticals and cosmetics. The extracts from these two plants that have been widely studied on the biological activities are now popularly used in pharmaceutical and cosmetic industries [23-25]. For *R. kerrii* Meijer, its biological potentials have not been fully investigated especially the activities relating to cosmeceutical use. In the present study, *R. kerrii* Meijer was mainly focused to see its possibility in application as a cosmetic ingredient like that has been successful from the aforementioned plants. The hydroglycolic extract of *R. kerrii* Meijer was carried out on its biological activities by comparing with the hydroglycolic extracts of *T. chebula* Retz. and *T. bellerica* Roxb. All extracts were examined for TPC, cytotoxicity, mutagenicity, antimutagenicity, and antityrosinase. Results from TPC analysis revealed *R. kerrii* Meijer had the highest content in comparison to *T. chebula* Retz. and *T. bellerica* Roxb. Regarding to published reports, *R. kerrii* Meijer is in the presence of hydrolyzable tannins and phenylpropanoid [15], whereas *T. chebula* Retz. and *T. bellerica* Roxb contain phenolic groups of lignans, flavones, flavonls, phenylpropanoid, and tannin [13,14,26,27].

When cytotoxicity was considered, *R. kerrii* Meijer extract showed the lowest cytotoxic effect upon normal mouse fibroblast L929 and melanoma mouse fibroblast B16F10 cell lines. The extract from *T. bellerica* Roxb seemed to have the highest cytotoxicity. There were reports revealed that *T. bellerica* Roxb was toxic on hepatocellular carcinoma and lung cancer cell lines [28] and exhibited antiproliferative effects in several cancer cell lines including breast cancer and prostate cancer cell lines [12]. The extract from *T. chebula* Retz. had less cytotoxic effect than *T. bellerica* Roxb on the above tested cell lines. Some previous findings showed *T. chebula* Retz. was cytotoxic on several malignant cell lines including human breast cancer, mouse breast cancer, human osteosarcoma, human prostate cancer, and human prostate cell lines [7].

In addition to cytotoxicity study, the genotoxicity of the hydroglycolic extracts from *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer was also performed using Ames test to show the mutagenic potentials. All of these three plant extracts were demonstrated to be non-mutagenic in either the presence or absence of metabolic activation towards *S. typhimurium* strains TA98 and TA100. On the contrary and interestingly, all these extracts showed impressive inhibitory effects upon mutagenesis induced by 2-AA and AF-2 which are strong mutagens in either the presence or absence of metabolic activation towards both tested bacterial strains. *R. kerrii* Meijer extract provided the best antimutagnicity upon 2-AA induced mutagenesis as the % inhibition of mutagenesis was highest in comparison to other two studied plant extracts. This plant extract was also able to inhibit the mutagenesis induced by AF2 shown in both TA98 and TA100. From the above results, this study may be implied that the *R. kerrii* Meijer extract possesses the actives that can act on AF-2 which is the direct mutagen but have to be metabolized to provide the metabolites to act on 2-AA which is the indirect mutagen. Since the extract was effective on both *S. typhiumurium* strains TA98 and TA100, it seems that the actives and their metabolites from *R. kerrii* Meijer can support the inhibition of base-pair substitution mutation of *hisD3052* (in TA98) and frameshift mutation of *hisG46* (in TA100) induced by 2-AA and AF2. The extracts from *T. chebula* Retz. and *T. bellerica* Roxb also provided the similar antimutagnic potentials. They showed the better inhibitory effects in 2-AA induced mutagenesis than AF2 and such effects were shown in both TA98 and TA100. Evidences revealed that 2-AA induced mutation by forming adducts on the C8 position guanine in DNA in the presence of microsomal activation, while AF-2 induced mutagenicity by direct action without being activated by hepatic microsomal enzymes to damage DNA [27, 29-31]. There were studies showed hydrolyzable tannin from the fruit of *T. chebula* Retz. could inhibit the mutagenicity of 2-aminofluorene and 4-nitro-*O*-phenylenediamine [27]. The existing hydrolysable tannin compounds in *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer [15,27,32] tends to play an important role in protecting DNA directly by these compounds themselves or indirectly through their metabolites from microsomal enzyme activation or binding with electrophilic metabolites of mutagen.

In cosmetic industry, the products containing herbs or plant originated ingredients are preferred. The expected activities from these natural sources in addition to antiaging are whitening. To fulfill the whitening effect on skin, it is important for the ingredients to have the potentials in inhibiting melanin synthesis or accumulation. Actually, melanin is good for skin in being the major contributor to skin pigmentation in protecting skin from damaging by solar radiations. In mammalian melanin synthesis, tyrosinase, a copper-containing monooxygenase, is the important enzyme involving in the process. Abnormal tyrosinase production elevates the level of melanin accumulation. Therefore, tyrosinase inhibitors as well as depigmenting agents can help reduce such skin hyperpigmentation issue [33-35]. In this study, *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts were investigated for their inhibitory effect on mushroom tyrosinase. Surprisingly, all three extracts had the potentials in inhibiting this enzyme. Again, *R. kerrii* Meijer extract showed the most appreciable activity. Its IC_{50}

was very low (1.27 ± 0.49 mg mL^{-1}), while the IC_{50} of other two plant extracts was much higher (ranged between 20.05 ± 0.05 and 39.96 ± 0.21 mg mL^{-1}). The ability of all these plants in inhibiting melanin synthesis is likely to be due to the phenolic compounds as phenol derivatives containing 2 or 3 hydroxyl groups have been evidenced to have the oxidation/reduction potential to reduce melanin biosynthesis. Besides, the phenolic compounds isolated from galls of *T. chebula* Retz. had the ability to inhibit mushroom tyrosinase and melanin synthesis with the inducing of α-MSH on B16 mouse melanoma cell line [35].

Conclusions

The finding of the present study suggests the possibility of the studied plants especially *R. kerrii* Meijer in addition to *T. chebula* Retz. and *T. bellerica* Roxb in form of hydroglycolic extracts to be applied for use in botanical-based cosmeceutical use regarding to their low cytotoxicity and non-mutagenicity, but potentials in inhibition of mutagenesis and tyrosinase.

Authors' contributions

1) MC, SC, PS, WN, PW and AW have made substantial contributions in conception, design and acquisition of data.
2) SC carried out the investigation of spectroscopy properties of hydroglycolic *T. chebula* Retz., *T. bellerica* Roxb and *R. kerrii* Meijer extracts and revised the manuscript.
2) PS carried out the total phenolic contents (TPC) and antityrosinase activity of all the hydroglycolic plant extracts and drafted the manuscript.
3) MC and PW have been prepared all hydroglycolic plant extracts and revised the manuscript.
4) AW participated in cytotoxicity assay in cell lines and revised the manuscript.

5) WN carried out the mutagenictity and antimutagenicity studies, performed the statistical analysis and drafted the manuscript.
All authors read and approved the final manuscript. All contributors who do not meet the criteria for authorship should be listed in an acknowledgements section.

Acknowledgments

This study was supported by the Thailand Research Fund Master Research Grants (TRF-MAG), Center for Innovation in Chemistry (PERCH-CIC), and S&J International Enterprises Public Company Limited. We would to thank Dr. Nisa Chawapun, Faculty of Medicine, Chiang Mai University, Thailand, for the cell culture facility and Prof. Dr. Usanee Vinitketkumnuen, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, for providing the *S. typhimurium* strains TA98 and TA100.

Conflict of interest

We have no conflict of interest to declare.

References

[1]. Afaq F, Mukhtar H. Photochemoprevention by botanical antioxidants. Skin Pharmacol Appl Skin Physiol. 2002; 15:297-306.

[2]. Svobodova A, Psotova J, Walterova D. Natural phenolics in the prevention of UV-induced skin damage. A review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2003; 147:137-145.

[3]. Matsumura Y, Ananthaswamy HN. Toxic effects of ultraviolet radiation on the skin. Toxicol Appl Pharmacol.2004; 195:298-308.

[4]. Barthakur NN, Arnold NP. Nutritive value of the chebulic myrobalan (*Terminalia chebula* Retz.) and its potential as a food source. Food Chem. 1991;40:213-219.

[5]. Chattopadhyay RR, Bhattacharyya SK. *Terminalia chebula*: An update. Pharmacogn Rev. 2007;1:151-156.

[6]. Shin TY, Jeong HJ, et al. Inhibitory action of water soluble fraction of *Terminalia chebula* on systemic and local anaphylaxis. J Ethnopharmacol. 2001;74:133-140.

[7]. Saleem A, Husheem M, Harkonen P, Pihlaja K. Inhibition of cancer cell growth by crude extract and the phenolics of *Terminalia chebula* Retz. fruit. J Ethnopharmacol 2002;81:327-336.

[8]. Reddy DB, Reddy TC, et al. Chebulagic acid, a COX-LOX dual inhibitor isolated from the fruits of *Terminalia chebula* Retz., induces apoptosis in COLO-205 cell line. J Ethnopharmacol.2009;124:506-512.

[9]. Kim SJ, Sancheti SA, et al. Effect of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose on elastase and hyaluronidase activities and its type II collagen expression. Acta Pol Pharm. 2010;67:145-150.

[10]. Elizabeth KM. Antimicrobial activity of *Terminalia bellerica*. Indian J Clin Biochem.2005;20:150-153.

[11]. Sabu MC, Kuttan R. Antidiabetic and antioxidant activity of *Terminalia belerica* Roxb. Indian J Exp Biol. 2009;47:270-275.

[12]. Kaur S, Michael H, Arora S, Harkonen PL, Kumar S. The *in vitro* cytotoxic and apoptotic activity of Triphala an Indian herbal drug. J Ethnopharmacol. 2005;97:15-20.

[13]. Valsaraj R, Pushpangadan P, et al. New anti-HIV-1, antimalarial, and antifungal

compounds from *Terminalia bellerica*. J Nat Prod. 1997;60:739-742.

[14]. Pfundstein B, El Desouky SK, Hull WE, Haubner R, Erben G, Owen RW. Polyphenolic compounds in the fruits of Egyptian medicinal plants (*Terminalia bellerica, Terminalia chebula* and *Terminalia horrida*): characterization, quantitation and determination of antioxidant capacities. Phytochem. 2009;71:1132-1148.

[15]. Kanchanapoom T, Kamel MS, Picheansoonthon C, Luecha P, Kasai R,Yamasaki K. Hydrolyzable tannins and phenylpropanoid from *Rafflesia kerrii* Meijer (*Rafflesiaceae*). J Nat Med. 2007;61:478-479.

[16]. Chulasiri M, Wanaswas P, et al. Utilizing hydroglycolic extract from myrobalan fruits to counteract reactive oxygen species. Int J Cosmet Sci. 2011;33:371-376.

[17]. Chan EWC, Lim YY, et al. Antioxidant tyrosinase inhibition properties of leaves and rhizomes of ginger species. Food Chem. 2008;109:477-483.

[18]. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63.

[19]. Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res. 1983;113:173-215.

[20]. Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res. 2000;455:29-60.

[21]. Kulwat C, Lertprasertsuke N, Leechanachai P, Kongtawelert P, initketkumnuen U. Antimutagenicity and DT-diaphorase inducing activity of *Gynostemma pentaphyllum* Makino extract. J Med Invest. 2005;52:145-150.

[22]. Organization for Economic Co-operation and Development (OECD). Bacterial reverse mutation test. Test No. 471. In: *OECD* guidelines for testing of chemicals. OECD, Paris (1997).

[23]. Baumann L. Botanical ingredients in cosmeceuticals. J Drugs Dermatol. 2007;6:1084-1088.

[24]. Lee KT, Kim BJ, Kim JH, Heo MY, Kim HP. Biological screening of 100 plant extracts for cosmetic use (I): inhibitory activities of tyrosinase and DOPA auto-oxidation. Int J Cosmet Sci. 1997;19:291-298.

[25]. Kim BJ, Kim JH, Kim HP, Heo MY. Biological screening of 100 plant extracts for cosmetic use (II): anti-oxidative activity and free radical scavenging activity. Int J Cosmet Sci. 1997;19:299-307.

[26]. Burapadaja S., Bunchoo A. Antimicrobial activity of tannins from *Terminalia citrina.* Planta Med. 1995;61:365-366.

[27]. Kaur S, Grover IS, Singh M. Antimutagenicity of hydrolyzable tannins from *Terminalia chebula* in *Salmonella typhimurium*. Mutat Res. 1998;419:169-179.

[28]. Pinmai K, Chunlaratthanabhorn S, Ngamkitidechakul C, Soonthornchareon N, Hahnvajanawong C. Synergistic growth inhibitory effects of *Phyllanthus emblica* and *Terminalia bellerica* extracts with conventional cytotoxic agents: doxorubicin and cisplatin against human hepatocellular carcinoma and lung cancer cells. World J Gastroenterol. 2008;14:1491-1497.

[29]. Hodek P, Trefil P, Stiborova M. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem Biol Interact. 2002;139:1-21.

[30]. Webster RP, Gawde MD, Bhattacharya RK. Protective effect of rutin, a flavonol glycoside, on the carcinogen-induced DNA damage and repair enzymes in rats. Cancer Lett. 1996;109:185-191.

[31]. Carino-Cortes R, Hernandez-Ceruelos A, Torres-Valencia JM, Gonzalez-Avila M, Arriaga-Alba, M. and Madrigal-Bujaidar, E. Antimutagenicity of *Stevia pilosa* and *Stevia eupatoria* evaluated with the Ames test. Toxicol In Vitro. 2007;21:691-697.

[32]. Madani A, Jain SK. Anti-Salmonella activity of *Terminalia belerica*: *in vitro* and *in vivo* studies. Indian J Exp Biol. 2008;46:817-821.

[33]. del Marmol V, Beermann F. Tyrosinase and related proteins in mammalian pigmentation. FEBS Lett. 1996;381:165-168.

[34]. Chang TS. An updated review of tyrosinase inhibitors. Int J Mol Sci. 2009;10:2440-2475.

[35]. Kim M, Park J, Song K, Kim HG, Koh J-S, Boo YC. Screening of plant extracts for human tyrosinase inhibiting effects. Int J Cosmet Sci. 2012;34:202-208..