Asian Journal of Chemistry; Vol. 27, No. 3 (2015), 957-960

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.17771

Antioxidant Activity and Melanogenesis Inhibitory Effect of Acerola Fruit (*Malpighia glabra L.*) Aqueous Extract and Its Safe Use in Cosmetics

LING WANG[1], FUHENG LI[1,*], CONGFEN HE[2], YINMAO DONG[2] and QI WANG[2]

[1]School of Life Science, Northeast Agricultural University, Harbin, Heilongjiang Province, P.R. China
[2]School of Science, Beijing Technology and Business University, Beijing, P.R. China

*Corresponding author: Tel: +86 451 5519 0002; E-mail: lifuheng1963@126.com

Received: 5 April 2014; *Accepted*: 12 June 2014; *Published online*: 19 January 2015; AJC-16704

Acerola (*Malpighia glabra* L.) fruit is well known as an excellent dietary source of vitamin C and many other phytochemicals. We investigated the effects of aqueous extract of acerola fruit on melanogenesis in B16 mouse melanoma cells and antioxidant activity. Aqueous extract of acerola fruit significantly reduced the cellular melanin level and inhibited tyrosinase activity in a concentration-dependent manner. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging tests indicated that aqueous extract of acerola fruit has potent antioxidative activity. These results suggested that aqueous extract of acerola fruit might be effective in skin-whitening and that its inhibitory effect on melanogenesis is due to a decrease in intracellular tyrosinase as determined by levels of gene expression (mRNA) and enzyme activity. The hemolysis rate after exposure to aqueous extract of acerola fruit was less than 10 %, below the 20 % regulatory limit for the red blood cell test. No allergic reactions were observed in the human skin patch tests. Hence, aqueous extract of acerola fruit could become an effective component in antiaging foods and whitening cosmetics.

Keywords: *Malpighia glabra* L., Antioxidant activity, Tyrosinase, B16 mouse melanoma cell, Skin-whitening agent.

INTRODUCTION

In humans, melanin determines the color of the skin and hair and its major role is protection against UV radiation. However, an excessive accumulation of melanin causes hyperpigmentation such as melasma, post-inflammatory melanoderma and solar lentigo[1,2]. Melanin is synthesized in mammals in the melanosomes of melanocytes. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2)[3]. Tyrosinase is the rate-limiting enzyme for melanogenesis and catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine and the oxidation of 3,4-dihydroxyphenylalanine to dopaquinone, these being the first stages in melanogenesis[4]. Most melanin synthesis inhibitors, such as arbutin, ellagic acid and kojic acid, inhibit melanogenesis by inhibiting tyrosinase activity[5-7]. However, many of the currently used skin-whitening agents exhibit toxicity toward melanocytes and produce adverse side effects. Thus, there is a need for the development of natural depigmentation agents derived from plants.

Acerola (*Malpighia glabra* L.), commonly known as the Barbados cherry or West Indian cherry, is cultivated in South and Central America, as well as in some southern regions of North America[8]. The main appealing feature of acerola is the high vitamin C content, which can vary[9] from 1247.10 to 1845.79 mg/100 g. In addition, acerola fruit is rich in other nutrients such as carotenes, thiamin, riboflavin, niacin, proteins and mineral salts, mainly iron, calcium and phosphorus[10]. The high ascorbic acid content and the presence of anthocyanins highlight the importance of acerola, mainly because of the functional ability that these compounds exhibit to capture free radicals in the human body. A food can be considered functional if it has been demonstrated to beneficially affect one or more target functions in the body, producing adequate nutritional effects in a way that is both relevant to well-being and health and also to reduce the risk of a disease[11].

Previous studies on acerola fruit were mainly focused on its chemical composition and antioxidant capacity[12-14]. Acerola is widely used in the food, health care and beverage industries. However, its use in beauty products has not been well studied and research is needed to address the antioxidant activity and melanogenesis inhibitory effect of acerola fruit extract in cosmetics. Therefore, the aims of this study were to investigate the potential inhibitory effect of aqueous extract of acerola fruit on melanogenesis, to identify the mechanism behind melanin synthesis inhibition and to examine the safety of aqueous extract of acerola fruit for use in cosmetics.

EXPERIMENTAL

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), mushroom tyrosinase, L-tyrosine, L-DOPA and arbutin were purchased from Sigma Chemicals (MO, USA). Dulbecco's modified eagle medium (DMEM), pancreatin, penicillin and streptomycin were purchased from invitrogen (USA). Fetal bovine serum (FBS)was purchased from Hangzhou Sijiqing Biologics, TritonX-100 was purchased from Beijing Dingguo Biotechnology, the Quant One Step RT-PCR Kit was purchased from Beijing Tiangen Biotech, absolute ethyl alcohol and NaOH were purchased from Beijing Chemical Reagent Company and Patch was purchased from Beijing Baiyi Yida Science and Technology Development.

Cell culture: The B16 mouse melanoma cell line was obtained from the Molecular and Cell Biology Laboratory, School of Life Science, Tsinghua University and cultured in a DMEM medium supplemented with 10 % fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin in a 5 % CO_2 atmosphere at 37 °C. The culture medium was changed every two days.

Preparation of acerola fruit extract: Unripe acerola fruits (*Malpighia glabra* L.) were purchased from Guangxi Jinhongguo Agriculture Comprehensive Development. The seeds were removed and pure water was added to bring the ratio of solid to liquid to 1:2 (m/v). The fruit/water mixture was ground in a homogenizer at full speed. The aqueous extraction was performed at 60 °C for 1 h. Aqueous extract of acerola fruit was obtained by centrifugation at 5,000 × g for 10 min to remove the debris, followed by filtration, concentration and freeze drying.

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity: 1,1-Diphenyl-2-picrylhydrazyl radical is a stable organic free radical which can be used to evaluate the antioxidant capacity of samples by spectrophotometry[15]. The antioxidant activity was measured by the degree of scavenging of the DPPH radical at 517 nm. All tests were performed in triplicate. The antioxidant activity of aqueous extract of acerola fruit (0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL) was measured with vitamin C as the positive control at the same concentrations.

Tyrosinase inhibition assay: Tyrosinase is the rate-limiting enzyme in the synthesis of melanin. Inhibition of tyrosinase produces a whitening effect. The rate of tyrosinase inhibition in samples was measured at 475 nm using a UV spectrophotometer[15]. The whitening activity of aqueous extract of acerola fruit (0.1, 0.2, 0.4 and 0.8 mg/mL) was measured with arbutin as a positive control at the same concentrations.

Measurement of melanin content: Melanin content was determined using a modification of the method reported by Hosoi *et al.*[16]. B16 cells were cultured at 1×10^5 cells/well in 24-well plates. After 12 h of incubation, the cells were treated for 24 h with aqueous extract of acerola fruit (0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL, respectively). The control was treated without aqueous extract of acerola fruit. The cells were harvested by trypsinization. After being washed twice with PBS, the samples were air-dried and then dissolved in 1 N NaOH (200 mL) containing 10 % DMSO. Samples were heated at 80 °C for 1 h and then cooled to room temperature prior to analysis. The melanin content was determined by measuring the absorbance at 405 nm[17].

Cellular tyrosinase activity assay: Tyrosinase activity was analyzed by spectrophtometry following the oxidation of 3,4-dihydroxyphenylalanine to DAPA-chrome[18]. B16 cells were cultured at 1×10^5 cells/well in 24-well plates. After 24 h of incubation, the cells were treated with concentrations of aqueous extract of acerola fruit (0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL) for 48 h. Cells treated without aqueous extract of acerola fruit served as the control. The cells were washed with PBS twice and lysed with 100 mM sodium phosphate buffer (pH 6.8) containing 1 % Triton X-100 and 0.1 mM PMSF. The lysate was centrifuged at 13,000 × g for 20 min at 4 °C. After protein quantification and adjustment of the protein concentration with lysis buffer, 100 μL of each lysate was aliquoted into a 96-well plate and 100 μL of 5 mM L-DOPA was then added to each well. Following incubation at 37 °C for 2 h, the absorbance was measured at 475 nm.

Reverse transcription-polymerase chain reaction (RT-PCR): B16 cells were incubated with aqueous extract of acerola fruit (0.1, 0.2 and 0.8 mg/mL) and vitamin C (0.1 and 0.2 mg/mL) for 72 h. Total RNA was then prepared and reverse-transcribed by using random primers and the Quant One Step RT-PCR Kit. The following oligonucleotide primers were used for gene amplification in the PCR: for tyrosinase, 5'-TTC AAAGGGGTGGATGACCG-3' (forward) and 5'-GAC ACATAGTAATGCATCCG-3' (reverse); for β-Actin, 5'-TCA GAAGGACTCCTATGTGG-3' (forward) and 5'-TCT CTTTGATGTCACGCACC-3' (reverse).

Red blood cell test: Potential irritation by aqueous extract of acerola fruit was assayed using the red blood cell test at a final concentration of 20 mg/mL according to a previously published method[19-21]. The test employed 0.4 % SDS as the positive control and PBS as the negative control. The hemolysis ratio was calculated by the following equation: $H(\%) = 100\ \% \times (OD_{530\ nm\ (sample)} - OD_{530\ nm\ (negative\ control)}) / (OD_{530\ nm\ (positive\ control)} - OD_{530\ nm\ (negative\ control)})$. The color interference of the test sample in the red blood cell test can be eliminated by subtracting the background value of the test sample. The sample is normally considered to be non-irritating if the rate is below 20 %.

Human skin patch test: The skin toxicity of aqueous extract of acerola fruit at 5, 10 and 20 mg/mL was determined with the human skin patch test according to the method of Zheng *et al.*[22]. Forty volunteers (20 females and 20 males, 18 to 35 years of age) were chosen; the patches were removed after 24 h and skin responses were evaluated within 0.5 h of patch removal. Five grades were assigned to evaluate the results according to the procedure set out in the Hygienic Standard for Cosmetics[23]. Grade 0: negative effect; Grade 1: suspicious effect, slight erythema; Grade 2: slight positive effect, erythema; Grade 3: positive effect, herpes; Grade 4: serious positive, confluent herpes.

Statistical analysis: The analyses of the data were done using the SPSS Statistics v19.0 statistical package (IBM Corporation, NY, USA). The experimental data were subjected to χ^2 tests; $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Assessment of antioxidant activity: The antioxidative capacity of aqueous extract of acerola fruit increased in a dose-dependent manner (Fig. 1). At a concentration of 0.04 mg/mL,

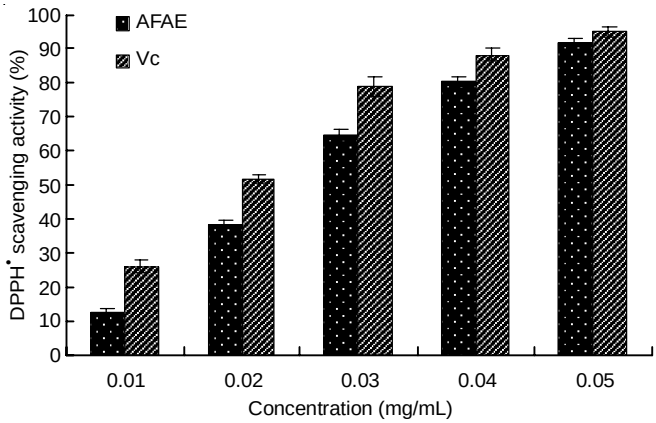

Fig. 1. Antioxidant activity of aqueous extract of acerola fruit (n = 3). Vc = Vitamin C

DPPH radical-scavenging rates for aqueous extract of acerola fruit and vitamin C were 80.5 and 88 %, respectively, with little difference. At a concentration of 0.05 mg/mL, the DPPH radical-scavenging rates of the two samples were almost the same, indicating that aqueous extract of acerola fruit has excellent DPPH-scavenging activity. Chemical analyses showed that the polysaccharide, vitamin C and niacinamide contents in aqueous extract of acerola fruit were 453, 203 and 9.36 mg/g, respectively. Aqueous extract of acerola fruit has a high vitamin C content and vitamin C is a well known antioxidant agent[24]. The synergy of vitamin C with other active ingredients present in aqueous extract of acerola fruit, such as niacinamide and carotenes, *etc*., its excellent antioxidant effect.

Effect of aqueous extract of acerola fruit on tyrosinase activity: Tyrosinase is a rate-limiting enzyme in melanin synthesis. Many melanin synthesis inhibitors reduce melanogenesis by inhibiting tyrosinase activity directly. We examined the direct effect of aqueous extract of acerola fruit on tyrosinase activity by using purified mushroom tyrosinase. The decrease in tyrosinase activity showed dose-dependence after addition of aqueous extract of acerola fruit or arbutin compared with the control (Fig. 2). Aqueous extract of acerola fruit was much more effective in inhibiting the activity of mushroom tyrosinase than was arbutin. This is probably due to the fact that aqueous extract of acerola fruit contains high levels of vitamin C, carotenes, niacinamide and other active ingredients. The effects of vitamin C are greater than those of multivitamins on mushroom tyrosinase inhibition and antioxidation[25]. At the same time, other active ingredients, such as carotenes, can also inhibit tyrosinase activity. Thus, aqueous extract of acerola fruit possesses strong tyrosinase inhibition capacity and can be used as a whitening ingredient in cosmetics.

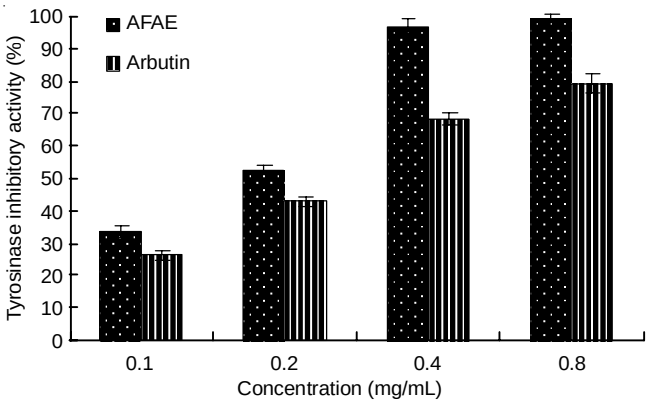

Fig. 2. Inhibition of tyrosinase by aqueous extract of acerola fruit (n = 3)

Effect of aqueous extract of acerola fruit on melanin production: In this study, B16 cells were used as the cell model in which to analyze the inhibitory effect of aqueous extract of acerola fruit on melanogenesis. To investigate the effects on melanin production, the melanin content of B16 melanoma cells treated with aqueous extract of acerola fruit was quantified. Aqueous extract of acerola fruit treatment reduced the melanin content of the B16 cells in a dose-dependent manner at concentrations that did not influence cell viability (Fig. 3).

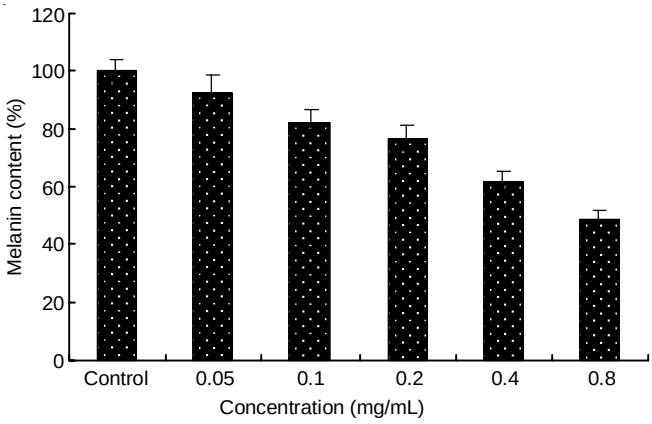

Fig. 3. Effects of aqueous extract of acerola fruit on melanin production (n = 3)

Inhibitory effect of aqueous extract of acerola fruit on B16 cell tyrosinase activity: To examine a possible mechanism behind the anti-melanogenesis effect of aqueous extract of acerola fruit, we measured cellular tyrosinase activity. Cells were treated with various concentrations of aqueous extract of acerola fruit for 48 h and lysates of the treated cells were used as the enzyme source. Tyrosinase activity in the cultured B16 cells was suppressed in a concentration-dependent manner by aqueous extract of acerola fruit treatment (Fig. 4). These results indicated that aqueous extract of acerola fruit is an effective inhibitor of melanogenesis through the direct inhibition of tyrosinase activity, making it a good candidate for a skin-whitening material.

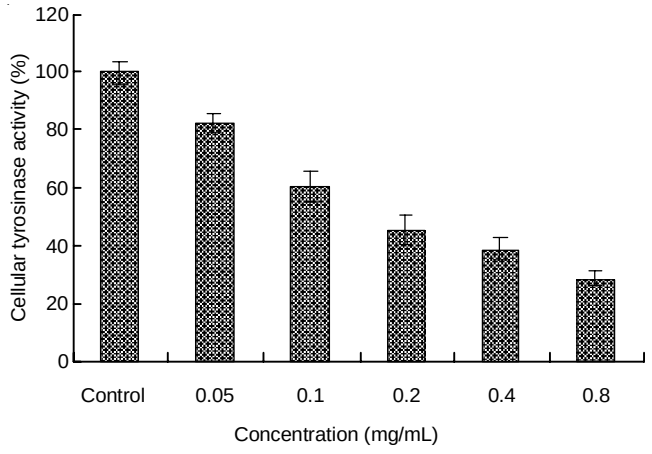

Fig. 4. Effect of aqueous extract of acerola fruit on cellular tyrosinase activity (n = 3)

Effect of aqueous extract of acerola fruit on tyrosinase mRNA levels in B16 cells: The level of tyrosinase mRNA in the B16 cells was determined by RT-PCR. Tyrosinase mRNA levels decreased significantly in a concentration-dependent manner (Fig. 5). In contrast to vitamin C, tyrosinase mRNA expression in the cultured B16 cells was suppressed by aqueous extract of acerola fruit in a concentration-dependent manner. These results indicated that the aqueous extract of acerola fruit inhibited melanogenesis at the transcriptional level and other active ingredients also played a role. Therefore, we suggest that the aqueous extract of acerola fruit inhibited melanogenesis at the transcription level also. Future studies need to be conducted to describe the mechanism of transcriptional repression.

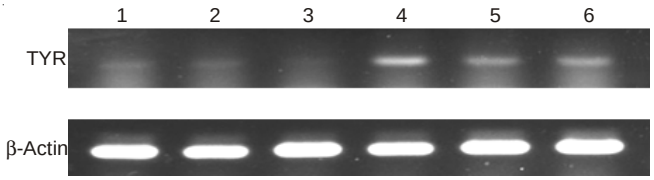

Fig. 5. Effect of aqueous extract of acerola fruit treatment on tyrosinase mRNA level in B16 cells; (1) aqueous extract of acerola fruit (0.1 mg/mL); (2) aqueous extract of acerola fruit (0.2 mg/mL); (3) aqueous extract of acerola fruit (0.8 mg/mL); (4) blank control; (5) Vc (0.1 mg/mL); (6) Vc (0.2 mg/mL). Vc = Vitamin C

Red blood cell tests: Based on current regulations, a sample is deemed to be non-irritating if its red blood cell test results are < 20 %. Our red blood cell test results are shown in Fig. 6, where the rates of hemolysis due to aqueous extract of acerola fruit were all much less than 10 %.

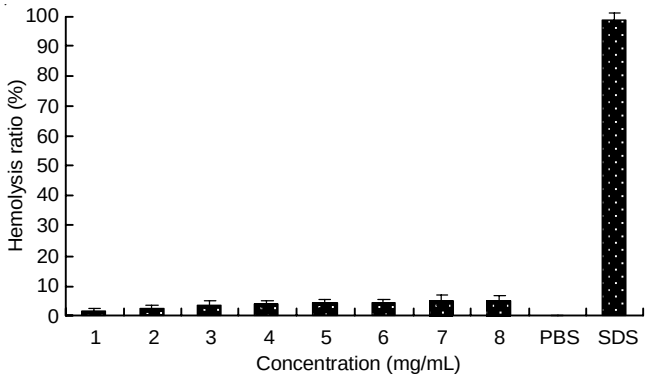

Fig. 6. Red blood cell test results for hemolytic activity of aqueous extract of acerola fruit (n = 3)

Human skin patch tests: The results of the human skin patch test showed that 40 people who took part in the study all had a Grade 0 response; no allergic reactions were observed in the test, demostrating that aqueous extract of acerola fruit did not cause any adverse reactions in human skin. Acerola is the green (unripe) fruit that has a higher potential to be beneficial to DNA, protecting it against oxidative stress[26]. In conclusion, previous studies and the safety evaluation results presented here indicate that aqueous extract of acerola fruit is safe to use in antiaging and whitening products intended for application on human skin.

Conclusion

In this study, we suggest that aqueous extract of acerola fruit has good antioxidant and tyrosinase-inhibiting properties and also a potent melanogenesis inhibitory effect when assayed in B16 mouse melanoma cells. The effect on melanogenesis appears to be due to a decrease in intracellular tyrosinase at the mRNA level. Safety tests show that aqueous extract of acerola fruit is safe and non-irritating to human skin and it thus has excellent potential as an additive for antiaging and whitening agents in cosmetics and personal care products.

ACKNOWLEDGEMENTS

This research was supported by Beijing Key Laboratory of Plant Resources Research and Development.

REFERENCES

1. K. Urabe, J. Nakayama and Y. Hori, in eds.: J.J. Nordlund, R.E. Boissy, V.J. Hearing, R.A. King, W.S. Oetting and J.P. Ortonne, Mixed Epidermal and Dermal Hypermelanoses. The Pigmentary System: Physiology and Pathophysiology, Oxford University Press, New York, pp. 909-913 (1998).
2. M.K. Cullen, Genetic Epidermal Syndromes: Disorders Characterized by Lentigines, In: The Pigmentary System: Physiology and Pathophysiology, Oxford University Press, New York, pp. 760-766 (1998).
3. V.J. Hearing, *J. Invest. Derm. Symp.*, **4**, 24 (1999).
4. V.J. Hearing and M. Jimenez, *Pigment Cell Res.*, **2**, 75 (1989).
5. K. Maeda and M. Fukuda, *J. Pharmacol. Exp. Ther.*, **276**, 765 (1996).
6. H. Shimogaki, Y. Tanaka, H. Tamai and M. Masuda, *Int. J. Cosmet. Sci.*, **22**, 291 (2000).
7. J. Cabanes, S. Chazarra and F. Garcia-Carmona, *J. Pharm. Pharmacol.*, **46**, 982 (1994).
8. P.D. Johnson, in eds.: A.P. Simopoulos and C. Gopalan, Acerola (*Malpighia glabra* L., *M. punicifolia* L., *M. emarginata* D.C.): Agriculture, Production and Nutrition, In: Plants in Human Health and Nutrition Policy, World Rev. Nutr. Diet. Basel, Karger, vol. 91, pp. 67-75 (2003).
9. V.L.A.G. Lima, E.A. Melo, M.I.S. Maciel, F.G. Prazeres, R.S. Musser and D.E.S. Lima, *Food Chem.*, **90**, 565 (2005).
10. S.A. de Assis, D.C. Lima and O.M.M. de Faria Oliveira, *Food Chem.*, **74**, 133 (2001).
11. M. Roberfroid, *Dig. Liver Dis.*, **34**, 105 (2002).
12. A.L. Vendramini and L.C. Trugo, *Food Chem.*, **71**, 195 (2000).
13. T. Mezadri, D. Villaño, M.S. Fernández-Pachón, M.C. García-Parrilla and A.M. Troncoso, *J. Food Compos. Anal.*, **21**, 282 (2008).
14. A.M. Righetto, F.M. Netto and F. Carraro, *Food Sci. Technol. Int.*, **11**, 315 (2005).
15. J. Zhao, J.X. Lai, C.F. He, H. Zhao, Y.D. Yang and Y.M. Dong, *China Surf. Deterg. Cosmet.*, **39**, 402 (2009).
16. J. Hosoi, E. Abe, T. Suda and T. Kuroki, *Cancer Res.*, **45**, 1474 (1985).
17. L.C. Wu, L.H. Chang, S.H. Chen, N.C. Fan and J.A.A. Ho, *LWT- Food Sci. Technol.*, **42**, 1513 (2009).
18. Z.W. Qiao, Y. Koizumi, M.X. Zhang, M. Natsui, M.J. Flores, L. Gao, K. Yusa, S. Koyota and T. Sugiyama, *Biosci. Biotechnol. Biochem.*, **76**, 1877 (2012).
19. J.Y. Xue, X.F. Yang, Y. Yang, M. Zhao, J.M. Huang, X.K. Xiong and X.H. Tan, *China Occup. Med.*, **37**, 469 (2010).
20. A. Lagarto, R. Vega, Y. Vega, I. Guerra and R. Gonzalez, *Toxicol. In Vitro*, **20**, 529 (2006).
21. W.G. Zhang, X.F. Yang, Y. Yang, X. Li, X.K. Xiong, X.P. Xie and X. Tan, *Chin. J. Prev. Med*, **6**, 544 (2010).
22. Y.X. Zheng, G.L. Xing, J. Wang and S.Y. Wang, *Glob. Seabuckthorn Res. Dev.*, **6**, 16 (2008).
23. Ministry of Health of the People's Republic of China Hygienic Standard for Cosmetics, Zhijian Publishing House, Beijing, China, pp. 149, 219 (2002).
24. S.I.R. Franke, D. Pra, J. Silva, B. Erdtmann and J.A.P. Henriques, *Mutat. Res.*, **583**, 75 (2005).
25. Y.K. Choi, Y.K. Rho, K.H. Yoo, Y.Y. Lim, K. Li, B.J. Kim, S.J. Seo, M.N. Kim, C.K. Hong and D.S. Kim, *Int. J. Dermatol.*, **49**, 218 (2010).
26. R. Silva Nunes, V.F.S. Kahl, M. Silva Sarmento, M.F. Richter, L.V. Costa-Lotufo, F.A.R. Rodrigues, J.A. Abin-Carriquiry, M.M. Martinez, S. Ferronatto, A. Barros Falcão Ferraz and J. Silva, *Plant Foods Hum. Nutr.*, **66**, 129 (2011).