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ARTICLE

Detection of Puerarin from *Pueraria Mirifica* Tuber and Its Formulated Cosmetic Products

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ABSTRACT

Pueraria mirifica (PM) has traditionally been used to relieve postmenopausal symptoms. Recently, its extract has been developed into various cosmetic products to promote skin rejuvenation and youthfulness. This study investigated the phytochemicals of PM tuber and compared between the tuber flesh and its outer peel. Puerarin which is one of the major isoflavones and being considered as the marker compound was used to determine the presence of PM extract in local cosmetic products. Puerarin could be ionized by a mass spectrometer at both negative and positive modes. The peak ionized at the negative mode showed to have a narrower peak width (0.2 min) and higher signal-to-noise ratio (30) for pueararin (1 mg/L). The results also found PM extract contained many C- and O-glycosylated isoflavones, especially from its peel extract. This explains the peel extract showed to have four times higher antiradical activity than those of flesh extract. Puerarin from the cosmetic products was recovered via successive methanolic sonication and followed by liquid-liquid extraction using ethyl acetate. Puerarin was successfully partitioned from the highly complex chemical mixture of cosmetic products with the recovery ranged from 89.1 % to 115 %. Hence, isoflavones was found to be higher at the outer peels than its tuber flesh. A simple and reliable method has been developed to analyse the presence of PM extract in cosmetic products based on the detection of puerarin after successive extraction via methanolic sonication and ethyl acetate partition.

1. Introduction

Pueraria mirifica (PM) or *P. candollei* var. *mirifica* is a plant can be found in northern Thailand and belongs to the Leguminosae family. The tubers of PM have been consumed by native Thai for generations to relieve postmenopausal symptoms including skin wrinkle, hair and memory losses [1,2]. Many scientific studies have proven the pharmacological activities such as estrogenic [3,4],

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antioxidant [5], antioesteroporosis [6,7], anticancer [8], antimutagenic [9], neuroprotective [10], antidiabetic [11] of PM extracts. Few clinical studies proved that the administration of PM would promote the reduction of low-density lipoprotein [12-14].

Researchers revealed that the predominant phytochemicals in PM are isoflavones or commonly known as phytoestrogens. This is because isoflavones have structural similarities to estrogens with the hydroxyl groups at the C7 and C4 positions as estradiol molecule which could bind to estrogen receptors. Puerarin (8-C-glucoside of daidzein) accounts for about half of the total isoflavone content together with other small amount of isoflavones such as genistin (7-O-glucoside of genistein) and daidzin (7-O-glucoside of daidzein). Puerarin is a marker compound of the *Pueraria* genus [15]. It is a heat sensitive compound and temperature may change the composition of isoflavone conjugates [16]. Possibly, isoflavones may be altered after an extended period of storage even at room temperature [17].

The administration of PM extract at 20-100 mg/day for 6 months, or 100-200 mg/day for 12 months was found to relieve menopause symptoms without significant changes on hepatic, hematologic, and renal functions [1]. A recommended safe dose of PM supplement was 1-2 mg/kg body weight/day or about 50-100 mg/day [1]. However, the long-term effect of administration has not yet been clarified in detail. PM has yet to obtain regulatory approval as a dietary ingredient in Australia, Singapore, United States and European countries due to the lack of safety assessment.

In the present study, the phytochemicals of PM tuber were analyzed and compared between its flesh and peel extracts. Maceration was performed to extract phytochemicals from the PM tuber to avoid thermal degradation of isoflavones. A successive extraction by methanol and ethyl acetate was introduced to recover puerarin from the highly complex mixture of cosmetic products. The presence of puerarin was confirmed using liquid chromatography tandem mass spectrometry.

2. Methodology

2.1 Chemical and Products

The tuber of PM was purchased from the province of Phatthalung, Thailand. The standard chemical, puerarin (≥ 98 %) was purchased from Sigma-Aldrich (St. Louis, USA). Three cosmetic creams containing *P. mirifica* extract were purchased from different regions of local markets in Malaysia. Product A has low viscosity and yellowish cream, whereas Product B and C were white and smooth cream.

2.2 Maceration of *P. mirifica*

The tuber of *P. mirifica* was cleaned and peeled before sliced into thin and small pieces. Both flesh slices and peel were put on trays for oven drying at 65°C for 3 days until completely dryness. The dried samples were ground into powder. Ten grams of samples was macerated in 200 mL methanol (95%) in two different flasks for 5 days. The flasks were swirled on a daily basis and loosened the caps for gas release. The supernatant was decanted, filtered and evaporated to dryness using a rotary evaporator (Heidolph, Laborota 4003, Germany). The dried extracts were kept in a refrigerator for subsequent antiradical and LC-MS/MS analyses (Figure 1).

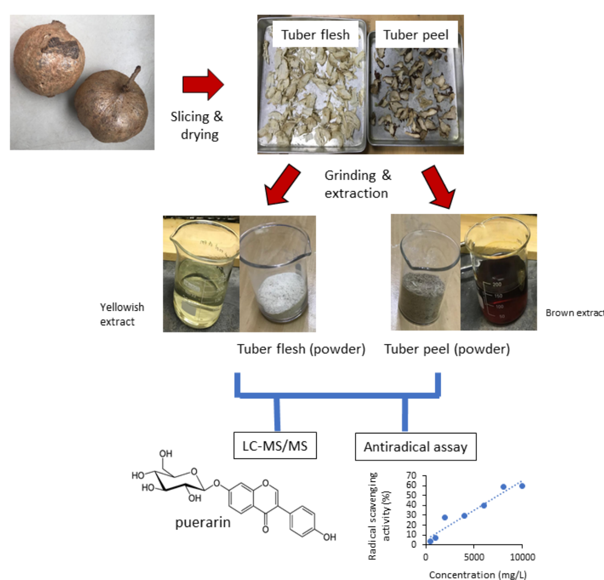

Figure 1. Flow diagram of sample preparation, extraction and analyses for *P. mirifica* tuber

2.3 Puerarin Extraction from Commercial Products

One gram of product creams was weighted and mixed with 5 mL methanol in individual tubes (10 mL). The tubes were vortexed for 1 min and sonicated for 10 min using an ultrasonic bath (Daihan Scientific, South Korea, 50 kHz, 400W) at 30 °C. The mixture was then centrifuged at 6000 rpm for 60 min. The supernatant was decanted and filtered into ceramic evaporating dishes for drying in an oven at 65 °C.

The dried extracts were re-constituted in 5% methanol (20 mL) and poured into a separating funnel (100 mL). Ethyl acetate (30 mL) was added into the funnel and the mixture was vigorously shaked for 5 min. Generated gas during extraction was released from the funnel from time to time. The mixture was left to separate into 2 significant

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layers. Ethyl acetate which was located at the top layer was withdrawn. Another fresh ethyl acetate (20 mL) was added into the remaining aqueous solution for extraction again. The process was repeated for thrice and the collected ethyl acetate was combined for drying. The dried product extract was kept in a fridge at 4 °C for subsequent analysis.

2.4 Determination of Puerarin Recovery

The performance of puerarin extraction from commercial products was determined by spiking a known concentration of standard puerarin into products. One gram of product creams was taken and put into individual tubes. A 1 mL of puerarin (10 mg/L) was spiked into the individual tubes. The tubes were then topped up with methanol to 5 mL. The tubes were then well mixed for 1 min, sonicated for 10 min and centrifuged to harvest the supernatant as explained in the procedures of puerarin extraction from commercial products. The harvested supernatant was oven dried. The experiments without the procedure of puerarin spiking were performed as control (unspiked). The dried weights of product extracts were recorded for unspiked and spiked experiments.

Subsequently, the dried product extracts were re-constituted in 5% methanol (20 mL) and subjected to liquid-liquid chromatography using ethyl acetate as solvent for puerarin recovery. The combined ethyl acetate (60 mL) was dried and then re-constituted in methanol for LC-MS/MS analysis. The recovery of puerarin was determined using Equation (1).

$$\text{Recovery (\%)} = \frac{([P]_1 - [P]_0)}{[P_{spiked}]} \; x\, 100 \qquad (1)$$

$[P]_0$: Puerarin concentration in unspiked product extract (mg/L)

$[P]_1$: Puerarin concentration in spiked product extract (mg/L)

$[P_{spiked}]$: Spiked concentration (2 mg/L)

2.5 LC-MS/MS for Puerarin

A hyphenated system consisted of ultra-high performance liquid chromatography (Waters Acquity UPLC; Milford, MA) and a triple quadrupole and linear-ion trap mass spectrometer (AB SCIEX 4000 QTRAP; Foster City, CA) was used to determine the concentration of puerarin. The strong wash and weak wash solution of the system were 10 and 50% of acetonitrile, respectively. The gradient of mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile) was: 0-5 min, 10% B; 5-15 min, 10-80% B; 15-20 min, 80% B; 20-25 min, 80-10% B; 25-30 min, 10% B for final washing. The separation was carried out in a C18 reversed phase Acquity column (4.6 x 100 mm, 1.7 μm). The flow rate was 0.25 mL/min and the injection volume was 5 μL.

The presence of puerarin was detected using the scan mode of enhanced product ion. The fragmentation pattern was matched to the product ions generated by standard puerarin. Untargeted mass screening was performed by the scan mode of enhanced mass spectra (EMS) linked to information dependent acquisition (IDA) with two parallel modes of enhanced product ion (EPI) at different collision energies ranged from 10 to 40 V. The capillary of ion source was maintained at 400 °C. The voltage was set at 5.0 kV and 4.5 kV for positive and negative ion modes, respectively. Nitrogen was used as ion source gas for nebulization, 40 psi; for drying solvent, 40 psi; curtain gas, 10 psi; collision gas, high; declustering potential, 40 V, and collision exit energy, 10 V. The scan rate was 1000 amu/s. A serial dilution of puerarin standard solution ranged from 0.5 to 10 ppm was prepared for the construction of calibration curve. Sample was filtered with nylon membrane filter (0.22 μm) before injection.

2.6 Antiradical Assay

The radical scavenging activity of samples was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. A 3.5 mL methanolic DPPH (0.1 mM) was added into 0.5 mL sample with different concentrations. The solution was incubated for 30 min and the absorbance of the solution was measured using a UV-Vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) at 517 nm. The DPPH reagent added with methanol only was used as blank. The inhibition of samples was calculated from Equation (2). The inhibition at 50% (EC50) explains the effective concentration of samples to inhibit 50% free radicals generated from the assay.

$$\text{Inhibition (\%)} = \frac{A_b - A_s}{A_b} \times 100 \qquad (2)$$

Where A_b = absorbance of blank,

A_s = absorbance of sample or standard

3. Results and Discussion

3.1. *Pueraria Mirifica* Tuber Extract and Its Antioxidant Capacity

Maceration was conducted for the flesh and peel of PM tuber. The results found to produce yellowish and brown extracts from the flesh and peel samples, respectively. Higher yield of extract (9.4 %) was obtained from the peel samples which also exhibited higher radical scavenging activity (Table 1). As a comparison, the EC50 of peel ex-

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tract was about four times higher than the flesh extract. This was because the higher amount of flesh extract was required to achieve EC50 (7.4303 mg/mL) than that of the peel extract (1.8636 mg/mL). In another word, the antioxidant capacity was about 57.7 % per milligram of peel extract as observed from the concentration dependent inhibitory curve. This value was about four times higher than that of flesh extract (15.6 % per milligram of flesh extract). The result was in good agreement with the findings of Zhang et al. [18] who reported higher free radical scavenging activity exhibited by the outer bark of *Pueraria lobata* roots. The total inhibition of DPPH radicals can be seen at 8 mg/mL of peel extract, whereas only 60% of inhibition was exhibited by flesh extract at the similar concentration (Figure 2). The antioxidant capacity of PM tuber was found to be lower than that of *Pueraria thunbergiana* Benth (16.4 µg/mL) as reported by Son et al. [19]. The researchers reported that higher antioxidant capacity could be due to the high total phenolic and isoflavone content. Many bioactivities reported for the genus *Pueraria* were attributed to isoflavone glycosides, mainly puerarin [15]. Table 1 shows the peel extract possessed higher concentration of puerarin (4.6553 mg/g extract) which was about four times higher than the flesh extract (1.0731 mg/g extract).

Table 1. Yield and antioxidant capacity of Pueraria mirifica tuber flesh and peel extracts

Sample	Extract colour	Extraction yield (%)	Puerarin (mg/g extract)	IC50 (mg/mL)
Tuber flesh	Yellowish	7.1 ± 0.9	1.073080863	7.4303
Tuber peel	brown	9.4 ± 1.8	4.655276066	1.8636

3.2 LC-MS/MS Detection of *Pueraria Mirifica* Tuber Extract

In the subsequent experiments, both flesh and peel extracts were subjected to LC-MS/MS analysis. The fragment ions of puerarin in both negative and positive ion modes are presented in Figure 3. The characteristic ions of m/z 297 and m/z 267 as reported by Li et al. [20] were also detected in this study. The peak width of puerarin was found to be narrower at the negative ion mode (0.2 min) than that of positive ion mode (0.5 min) at 0.5 % peak height. A narrow peak width is always better for peak resolution, even though MS detector does not have difficulty in compound identification with overlaped peaks. Furthermore, the signal to noise ratio of puerarin was higher at the negative ion mode (30) than that of positive ion mode (10.7). Both calibration curves showed to have a good linearity in the range of 0.5 to 10 mg/L with the coefficient correlation of 0.9980 and 0.9563 at the negative and positive ion modes, respectively.

The untargeted mass screening also found to detect many flavonoids, especially isoflavones from PM tuber (Table 2). The detected compounds have mostly higher content in peel extract. In line with the findings of Cheng et al. [21], the outer bark of PM tuber also contained higher isoflavonoids. This also explains peel extract exhibited higher antioxidant capacity due to the presence of isoflavones.

3.3 Recovery of Puerarin from Cosmetic Products

Since the wide application of PM in cosmetic formulation, three commercial cosmetic creams with PM extract as one of the major ingredients was purchased from the local markets of Malaysia. They were extracted by methanol and then back extraction by ethyl acetate using the technique of liquid-liquid extraction. The yield of methanolic

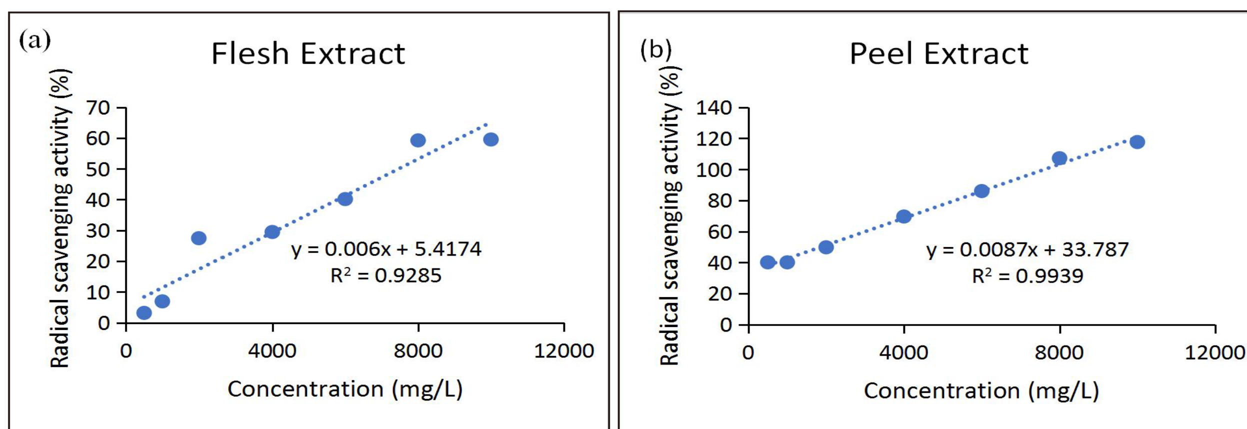

Figure 2. The inhibitory curves of (a) flesh and (b) peel extracts in a concentratuion dependent manner

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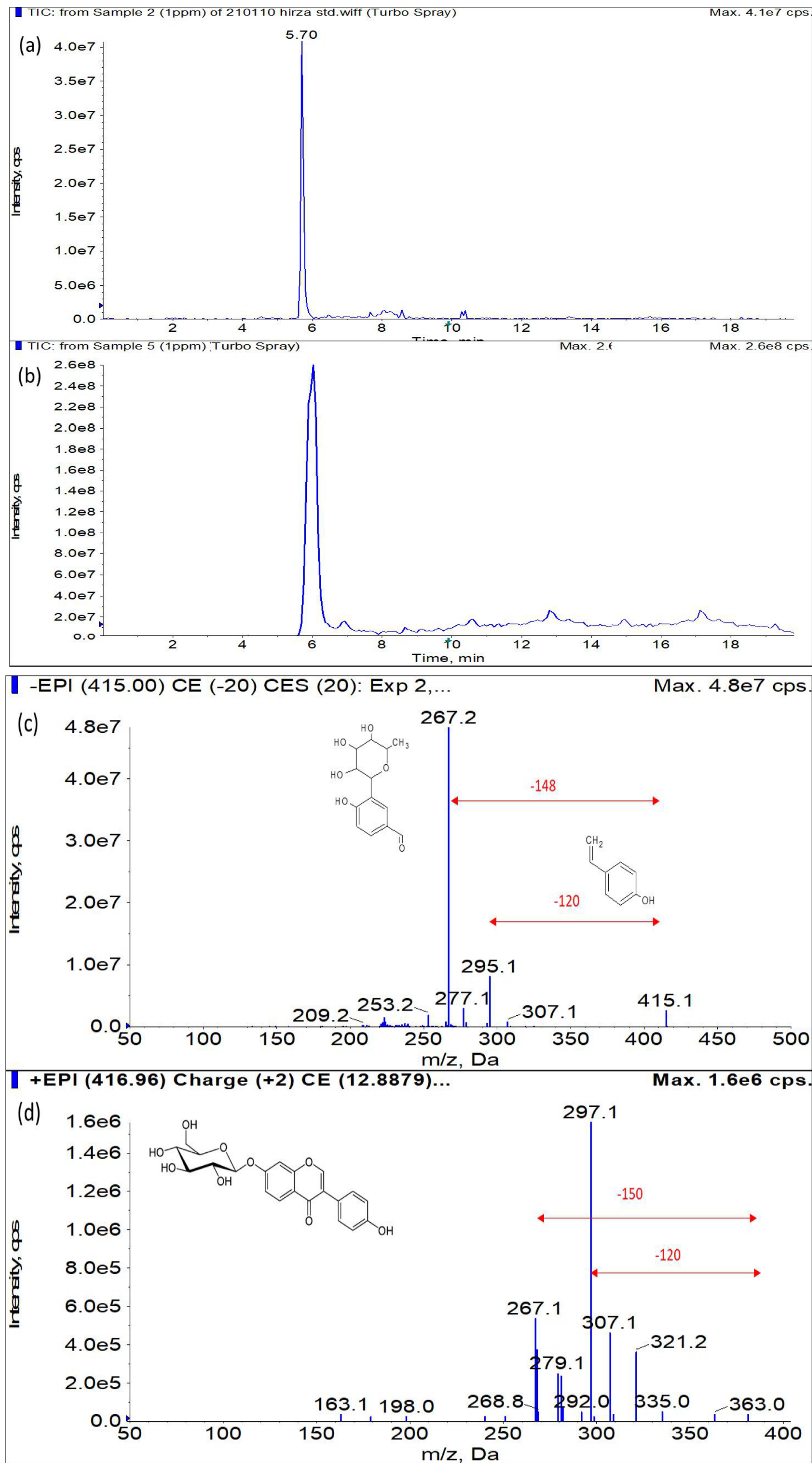

Figure 3. Total ion chromatograms (a and b) and mass spectra of puerarin (c and d) detected at the negative (c) and positive (d) ion modes

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Table 2. Compounds detected at negative and positive modes in the flesh and peel extracts of *Pueraria mirifica* tuber

Rt (min)	Negative ion (m/z)	Fragment ions	Compound	Flesh	Peel	Reference
2.33	133	133/115	malic acid	1.58E+09	1.19E+09	[22]
7.50	253	223/207/195/180/155/135/133	diadzein	1.23E+09	1.76E+09	[23]
8.64	269	269/224/180/159	apigenin	6.19E+08	9.18E+08	[24]
14.30	271	271/253/225	naringenin	3.54E+08	1.06E+08	[25]
11.71	339	339/324/281/253/241/183	glyceollidin	0	1.16E+09	[26]
4.26	415	415/307/297/267bp/253/209	puerarin	1.36E+09	1.48E+09	Standard
3.89	431	431/311/283/255/227/211	vitexin (apigenin-8-C-glucoside)	0	1.03E+09	[24]
4.67	445	445/325/282/254/225	glycitin	0	8.29E+08	[26]
4.99	563	563/323/311/283/239	apigenin-6,8-C-pentoside-hexoside	8.75E+07	1.42E+09	[27]
3.37	577	577/457/294/266/222	puerarin glycoside	0	5.60E+08	[28]
5.28	611	611/564/269bp	apigenin diglucoside	1.02E+09	1.42E+09	[29]
Rt (min)	**Positive ion (m/z)**		**Compound**	**Flesh**	**Peel**	**Reference**
7.26	255	255/199/227/137	daidzein	7.83E+08	1.42E+09	[28]
5.12	255	255/237(-18)/227(-28)/199(-28-28)/137	dihydroxyflavone	2.39E+08	7.98E+08	[29]
8.60	271	271/197/153	apigenin	2.59E+08	0.00E+00	[29]
7.50	285	285/270/229/213/139	calycosin	0	5.49E+08	[29]
8.96	301	301/286/283/240/187/123	pratensein	0	5.45E+08	[29]
6.40	315	315/297/287/237/209/137	dihydroxy-dimethoxyisoflavone	0	3.07E+08	[29]
4.26	417	417/307/297/267bp/163	puerarin (daidzin 8-C-glucoside)	1.25E+09	2.60E+09	Standard
3.67	433	433/397/367/313/283/267/255/165	3′-hydroxy-puerarin	0	1.45E+09	[30]
5.15	433	433/397/367/313/283/267/255/165	3′-hydroxy-puerarin (isomer)	7.66E+08	1.64E+09	[30]
4.52	447	447/351/327/297/269/255/165/	apigenin C-glucuronide	0	1.64E+09	[30]
4.00	549	549/417/307/297/267	puerarin pentoside	4.25E+08	2.76E+09	[28]
3.32	579	579/417/307/297/267bp/239	puerarin glycoside	1.66E+08	1.23E+09	[28]
1.96	579	579/417/307/297/267bp/239	puerarin glucoside isomer	0	7.41E+08	[28]
4.10	579	579/447/429/393/381/327/297/269	swertisin pentoside	0	8.76E+08	[31]

Table 3. Puerarin content in cosmetic products and their radical scavenging activity

Product	Yield of methanolic extract (%)	Yield of ethyl acetate extract (%)	Puerarin (μg/mL)	DPPH (%)	Puerarin recovery from spiked experiments (%)
A	2.8 ± 0.7	20.6 ± 7.7	0.3095 ± 0.0254	32.1 ± 2.4	115.8
B	3.8 ± 0.6	32.4 ± 9.8	0.4353 ± 0.0341	3.1 ± 0.01	96.0
C	5.6 ± 0.6	38.1 ± 8.2	0.5255 ± 0.0309	4.4 ± 0.4	89.1

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and ethyl acetate extract, as well as the recovered puerarin concentration are listed in Table 3. Lipophilic puerarin could be partitioned and recovered from the complex aquoues mixture into the organic phase of ethyl acetate [32]. It was found that the concentration of puerarin was ranged between 0.3095 to 0.5255 μg/mL (Table 3). The measured radical scavenging activity was varied from 3.1 % to 32.1 % independent on the detected concentration of puerarin in the products. This was because the cosmetic products contained a mixture of plant extracts which might be extracted out together with puerarin, and thus contributing to the synergistic antioxidant capacity.

A spiked experiment was also conducted to reveal the performance of puerarin extraction from commercial cosmetic products. Puerarin could be recovered from the highly complex product mixture effectively (Table 3). A good recovery ranged from 89.1 % to 115.8%, was obtained for puerarin. The results revealed that the recovery of puerarin was strongly affected by the texture of sample matrices. Products with thick or liquidified texture should be well homogenized prior to analyses.

4. Conclusions

The concentration of puerarin was found to be about 4 times higher in the peel extract of PM tuber than its flesh extract. The higher concentration puerarin in the peel extract was also showed to have higher radical scavenging activity. The detection which was performed by a triple quadrupole mass analyser exhibited better identification power at the negative ion mode. Successive extraction by methanolic sonication and ethyl acetate partition was found to be effective in recovering puerarin from the highly complex cosmetic products.

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