Extraction, Purification, and Characterisation of the Flavonoids from *Opuntia milpa alta* Skin

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Abstract

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The flavonoids contained in *Opuntia milpa alta* skin were extracted, purified, and identified. Based on single factor experimental results, the extraction parameters were optimised by using orthogonal design and variance analysis. The results show that the maximum yield of the flavonoids (5.55 mg/g dry weight) can be obtained with 80% ethanol (v/v), extraction temperature of 90°C, and the solvent to raw material ratio of 25:1. After purification with AB-8 resin, the main components of the extract were characterised as isorhamnetin 3-*O*-(2, 6-dirhamnosyl)glucoside and isorhamnetin 3-*O*-D-rutinoside by employing liquid chromatography-mass spectrometry and ultraviolet-visible spectrometry.

Keywords: *Opuntia milpa alta* skin; flavonoids; extraction; LC-MS; isorhamnetin 3-*O*-(2, 6-dirhamnosyl)glucoside; isorhamnetin 3-*O*-D-rutinoside

Many studies have shown that natural antioxidants from plant sources can effectively inhibit oxidation of food and reduce the risk of age-dependent diseases (Burda & Oleszek 2001; Zou *et al*. 2004). Flavonoids, abundant in fruits, vegetables, teas, medicinal plants, have attracted the greatest attention and have been studied extensively, because they are a kind of highly effective antioxidants with a lower toxicity than synthetic antioxidants such as BHA and BHT (Pekkarinen *et al*. 1999).

Opuntia milpa alta, a member of the Cactaceae family, is a tropical or subtropical plant originally grown in South America and widely distributed in Mexico, which was brought to China in 1979 as an important nutrient and food source (Habibi *et al*. 2005). Besides many essential nutritional components, *Opuntia milpa alta* contains phenolic compounds, a large group of biologically active non-nutrients. Arcoleo *et al*. (1961) first reported the presence of isorhamnetin in flowers of *O. ficus indica*. Other reports indicated that the plants of the Cactaceae family contain flavonol 3-*O*-glycosides (quercetin, kaempferol, and isorhamnetin), dihydroflavonols, flavonones, and flavanonols (Burret *et al*. 1982; Meyer & McLaughlin 1982; Miller & Bohm 1982). Many kinds of flavonoids have been identified in *Opuntia cactus*, their types and contents varying with the variety, tissue type, and maturation (Wallace 1986). Nearly all reports on flavonoids found in *Opuntia cactus* dealt with the extraction from the floral tissue (Joseph 2004). Although the peel makes up about 40% of the whole cladodes weight and is subsequently the major by-product, it is either wasted or used as forage (Sirohi *et al*. 1997). The detailed data on the extraction of flavonoids from *O. ficus-indica* skin and its components is scarce. One of

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the major needs within the cactus industry is the development of new processed *O. ficus-indica* products to utilise *O. ficus-indica* as well as the skin by-products.

The purpose of this study was to find appropriate conditions for the extraction of flavonoids from *O. ficus-indica* skin and to validate the skin flavonoid components, thus to obtain an informative profile which may serve as a basis for further utilisation of *O. ficus-indica* skin. The results, furthermore, will be important as an indication of the skin flavonoids as a new source of bioactive flavonoids.

MATERIALS AND METHODS

Materials and preparation of samples. *Opuntia milpa alta* was obtained from Shanhai LinHui Biotechnology Ltd. (Shanghai, China). All chemicals and solvents used were of analytical grade.

The *O. milpa alta* cladodes were carefully hand-peeled, and the peels (with a thickness of about 3–4 mm) were cut into small pieces and dried in a ventilated oven at 60°C. After drying, the peel fragments were ground for a few minutes in a domestic coffee grinder and sieved. All the samples tested were placed in a big Soxhlet extractor and refluxed with ether at 60°C for 8 h to remove oil and chlorophyll, and then were air dried for 12 hours. After all ether was evaporated, the peel fragments were ground and separated by sieves of different sizes, the particles obtained between the 20-mesh (0.84 mm) and 28-mesh (0.6 mm) were selected and stored in vacuum packaged in polyethylene pouches at –20°C until the extraction.

Determination of total flavonoids content. A modified method (Chen 1998) was used: 1 ml of a diluted solution containing flavonoids, 0.7 ml of 5% (w/w) $NaNO_2$, and 10 ml of 30% (v/v) ethanol were combined and stirred for 5 min, and then 0.7 ml of 10% $AlCl_3$ (w/w) was added and the mixture was stirred up. Six minutes later, 5 ml of 1 mol/l NaOH was added. Subsequently, the solution was diluted to 25 ml with 30% (v/v) ethanol prior to the measurement. After 10 min standing, the absorbance of the solution was measured at 500 nm with a Unico WFJ2000 spectrophotometer (Unico, Shanghai, China). The contents of flavonoids were expressed in mg rutin per g dry weight basis by comparison with rutin standard curve, and the yield of flavonoids was calculated using the following formula:

$$Y = (6.404A + 0.2806)\ BV/W\ (mg/g)$$

where:
A – absorbance (500 nm)
B – dilution factor
W – dry weight of cactus skin precisely measured (g)
V – volume of the extracting agent (ml)

Experimental design. On the basis of single-factor experiment for the flavonoid production, proper ranges of ethanol concentration, extraction temperature, and solvents to raw material ratio were preliminarily determined. A three level, three variable orthogonal design was used to determine the best combination of the extraction variables for the production of cactus skin flavonoids. The independent factors and the dependent variables used in this design are listed in Table 1.

Table 1. Factors and levels in extraction experiment of flavonoids from *O. ficus-indica* skin

Factor	Level		
	1	2	3
A – ethanol concentration (%)	70	80	90
B – extraction temperature (°C)	70	80	90
C – ratio of solvents to raw material (mg/g)	15	20	25

Purification and identification of the main flavonoids. The crude extracts were filtered, and ethanol was subsequently evaporated. The solution was poured onto a column (400 × 2.5 cm i.d.) packed with pretreated AB-8 resin. After complete absorption of the solution, the column was washed with enough distilled water to remove carbohydrates, and further washed with 65% ethanol to elute flavonoids. The eluate abundant in flavonoids was collected and then concentrated at 40°C with a Laborata 4000 rotary evaporator (Heidolph, Schwabach, Germany) until the formation of sediment. After having been collected and vacuum-dried at 40°C, the sediment thus obtained represented the solid-state product of flavonoids from *O. ficus-indica* skin.

Reversed phase chromatography (Lichrospher C18, 5 μm, 250 mm × 416 mm, Schwabach, Germany) was used to separate the flavonoids and collect the main components according to their retention times (RT). The mobile phase for HPLC

consisted of CH_3COOH (v/v) (A) and 0.5% CH_3CN (v/v) (B) using a linear gradient of 0–80 min, 5% A–70% A. The injection volume was 50 µl. The flow rate was 0.8 ml/minutes. The wavelength for the detection was set at 350 nm. The main flavonoids in the product were identified by ultraviolet-visible spectra and electrospray ionisation mass spectra.

The purified sample (about 10 mg) was dissolved in methanol and diluted to 25 ml for identification. UV analysis was performed on a TU-1800PC spectrophotometer (Purkinje, P. R. China). The mass spectra were obtained with an ZMD 4000 ion-trap LC/MS system (Waters Corp., Milford, USA). The ESI/MS was used to determine the molecular weights of the main flavonoids in the final product by scanning from 100 m/z to 1000 m/z.

RESULTS AND DISCUSSION

The effect of the volume fraction of ethanol on the yield of the flavonoids

Figure 1 shows the extraction yield of flavonoids using various volume fractions of ethanol (50–90%) when other extraction conditions were as follows: temperature 70°C, extraction time 3 h, solvent to raw material ratio 20:1, and the extraction number 2. The extraction yield ranged from 3.3 mg/g to 4.2 mg/g with the increasing concentration. The highest yield of flavonoids was 4.02 mg/g obtained with 80% ethanol. But increasing the concentration from 80% to 90% decreased the yield of flavonoids in which different components polarity reduced yield of flavonoids.

Effect of extraction temperature on the yield of flavonoids

The extraction temperature was respectively set at 60°C, 70°C, 80°C, and 90°C to investigate the influence of the extraction temperature on the yield of flavonoids when other extraction conditions were as follows: volume fraction of ethanol 80%, extraction time 3 h, solvent to raw material ratio 20:1, and the extraction number 2. Figure 2 shows the effect of the extraction time on the yield of flavonoids. The results indicated that the extraction yield tended to increase gradually with the rise of temperature in the range of 50°C~80°C. It seems probable that at the greater speed of the molecules movement at higher temperature flavonoids diffused more quickly from the cells into the extracting agent. However, as flavonoids can be oxidised at temperatures surpassing 80°C, the yield of the extracted flavonoids started to decrease gradually.

Effect of extraction time on the yield of the flavonoids

Here, extraction time was respectively set at 3 h, 4 h, 5 h, 6 h, and 7 h to examine the influence of the extraction time on the yield of flavonoids when

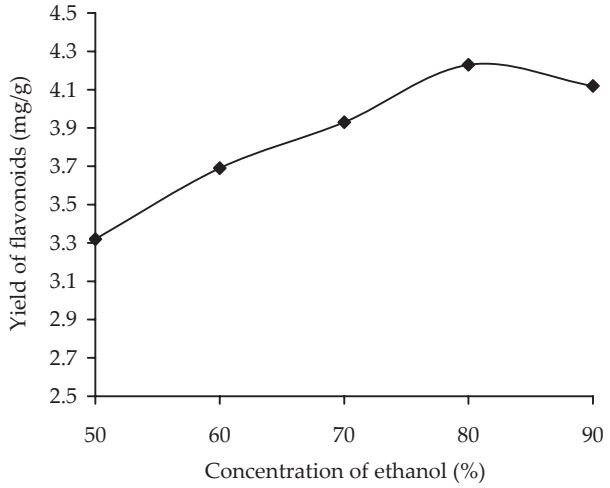

Figure 1. Effect of ethanol solution concentration on the yield of flavonoids

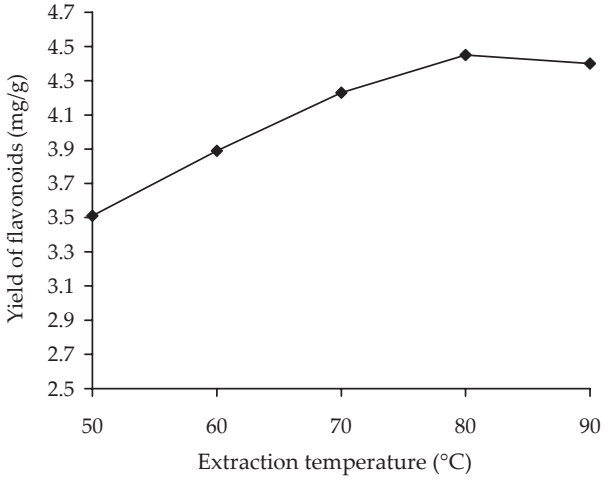

Figure 2. Effect of extraction temperature on the yield of flavonoids

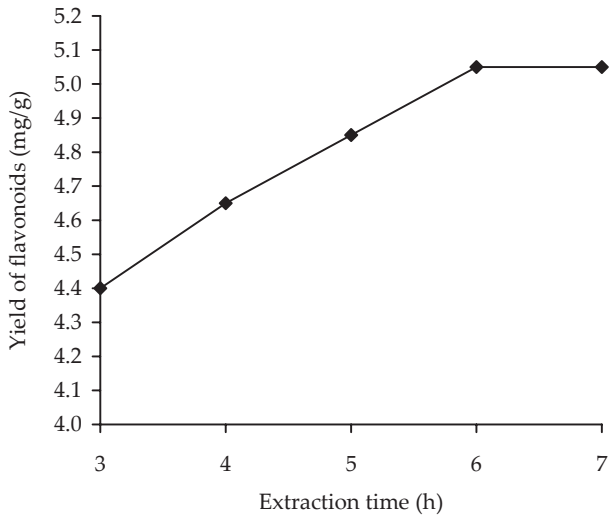

Figure 3. Effect of extraction time on the yield of flavonoids

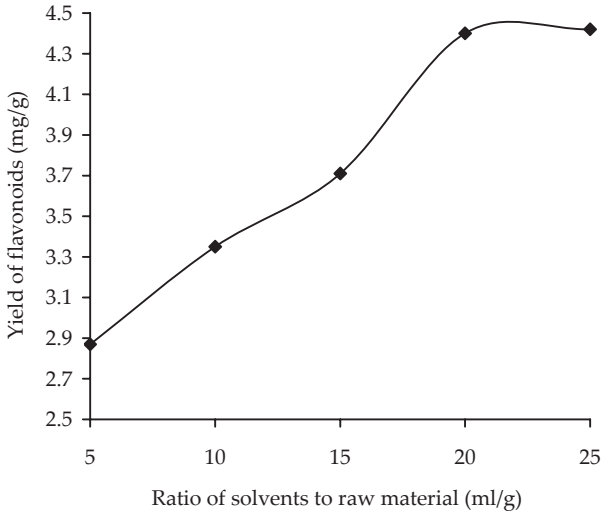

Figure 4. Effect of solvents to raw material ratio on the yield of flavonoids

other extraction conditions were as follows: volume fraction of ethanol 80%, extraction temperature 80°C, solvent to raw material ratio 20:1, and the extraction number 2. Figure 3 shows the effect of the extraction time on the yield of flavonoids. The results indicated that the yield of flavonoids increased slightly when the extraction time was between 3 h and 6 hours. The yield was close to the peak value (5.1 mg/g) at the extraction time of 6 hours. Beyond this point, the yield of flavonoids remaind stable with increasing the extraction time. Therefore, the optimum extraction time appeared to be 6 hours.

Effect of solvents to raw material ratio on the yield of flavonoids

In this part, water to raw material ratio was set at 5, 10, 15, 20, and 25 in order to investigate the influence of water to raw material ratio on the yield of flavonoids when other reaction conditions were the same as those described in section "Effect of extraction time on the yield of the flavonoids" except the extraction time of 3 h (Figure 4). We could observe that, as the ratio of water to raw material was in the range of 5–25, the extraction yield rapidly increased with the increasing ratio; however, as the ratio continued to increase, the yield increased only slowly. Consequently, we concluded that a high extraction yield could be obtained with the solvent to raw material ratio in the range of 20–25.

Orthogonal design results and variance analysis

On the basis of the effects of single factor as described in the above chapters, using the relationships in Table 1 when the extraction duration was set to 6 h, the actual levels of the variables in each of the experiments in the orthogonal design were calculated and the experimental results obtained are given in Table 2. The results of the range analysis show the degree of affecting the flavonoids yield to be in the order C > B> A. The examination of the data given in Table 2 revealed that the $A_2B_3C_3$ procedure parameter may be the best under these experimental condition. The optimum extract procedure was validated, and the yield of flavonoids reached 5.55 mg/g by repeated $A_2B_3C_3$ procedure. This yield of flavonoids was higher than the flavonoids content (4.5 mg/g dry weight, 46 mg/100 g fresh weight) in the skin (Xue *et al*. 1999). Previous reports showed the total phenolic content in Mexican cactus pads accounting for 8–9 mg/100 g fresh weight (Stintzing & Carle 2005), and flavonoids content in succulent pulp accounting for 1 mg/100 g fresh weight (Xue *et al*. 1999). Flavonoids content of the skin was close to those in white grape and blackberry (Marinova *et al*. 2005). More in-depth studies should be envisaged to make full use of cactus skin.

The single-factor analysis of variance of orthogonal experiment results are shown in Table 3 as

Table 2. Results of orthogonal experiments

Item	A	B	C	Yield of flavonoids (mg/g)
1	1	1	1	3.15
2	1	2	2	4.95
3	1	3	3	5.25
4	2	1	2	5.05
5	2	2	3	5.52
6	2	3	1	3.95
7	3	1	3	5.15
8	3	2	1	3.75
9	3	3	2	5.47
k1	4.45	4.45	3.62	
k2	4.84	4.74	5.16	
k3	4.79	4.89	5.31	
R	0.39	0.45	1.69	

made by SPSS software. The order of the effects of the individual factors on flavonoids extraction was C > B> A. The ratio of solvents to raw material had the greatest effect on the extraction procedure and it was found to be significantly different at 1% level. Equivalent effects were observed with the volume fraction of ethanol and temperature change, even though they were not proved to be of any significant difference at 5% level, yet showed a significant difference at 10% level. The optimum extraction conditions obtained from the statistical analysis were $A_2B_3C_3$. This means that 80% ethanol concentration, 90°C, and the solvent to raw material ratio of 25 present the optimum conditions for flavonoids recovery.

Identification of the main flavonoids

Reversed phase chromatography with gradient elution has been widely used for the separation and characterisation of flavonoids (Ryan *et al.* 1997). Correlative methods identifying flavonoids from *O. ficus-indica* have been introduced (Jeong *et al.* 1999). Based on the experimental conditions described above, flavonoids extracted from

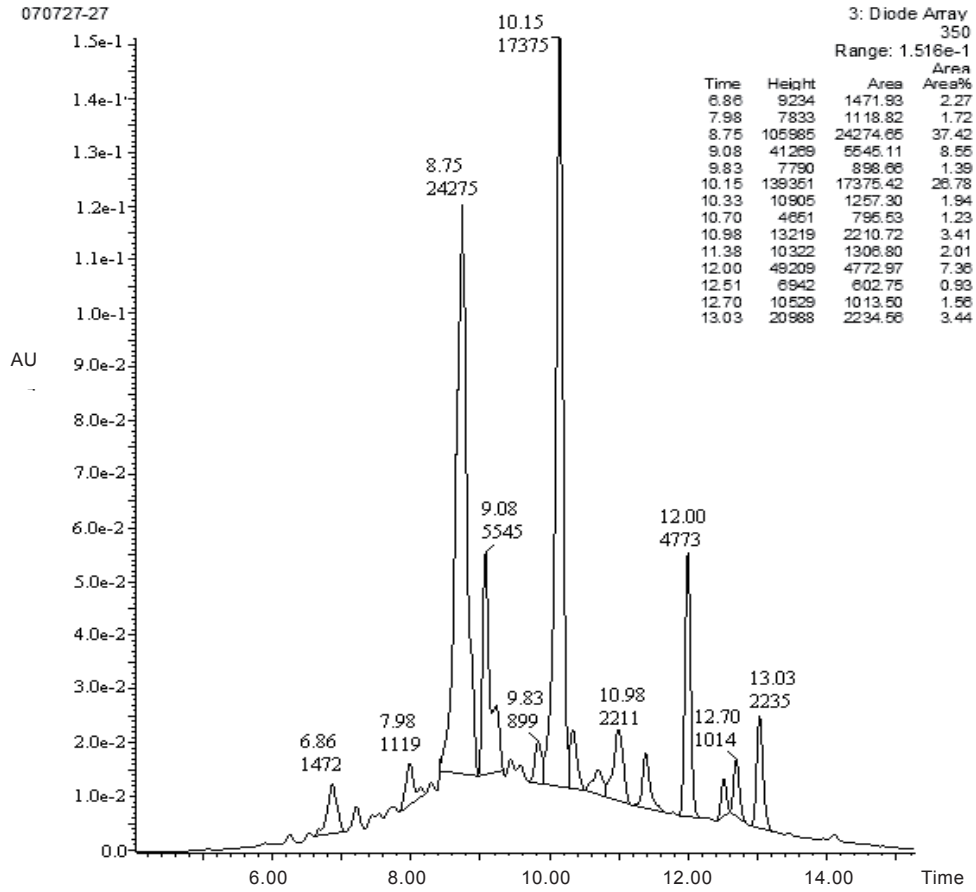

Figure 5. HPLC spectra of flavonoids extracted from *O. ficus-indica* skin

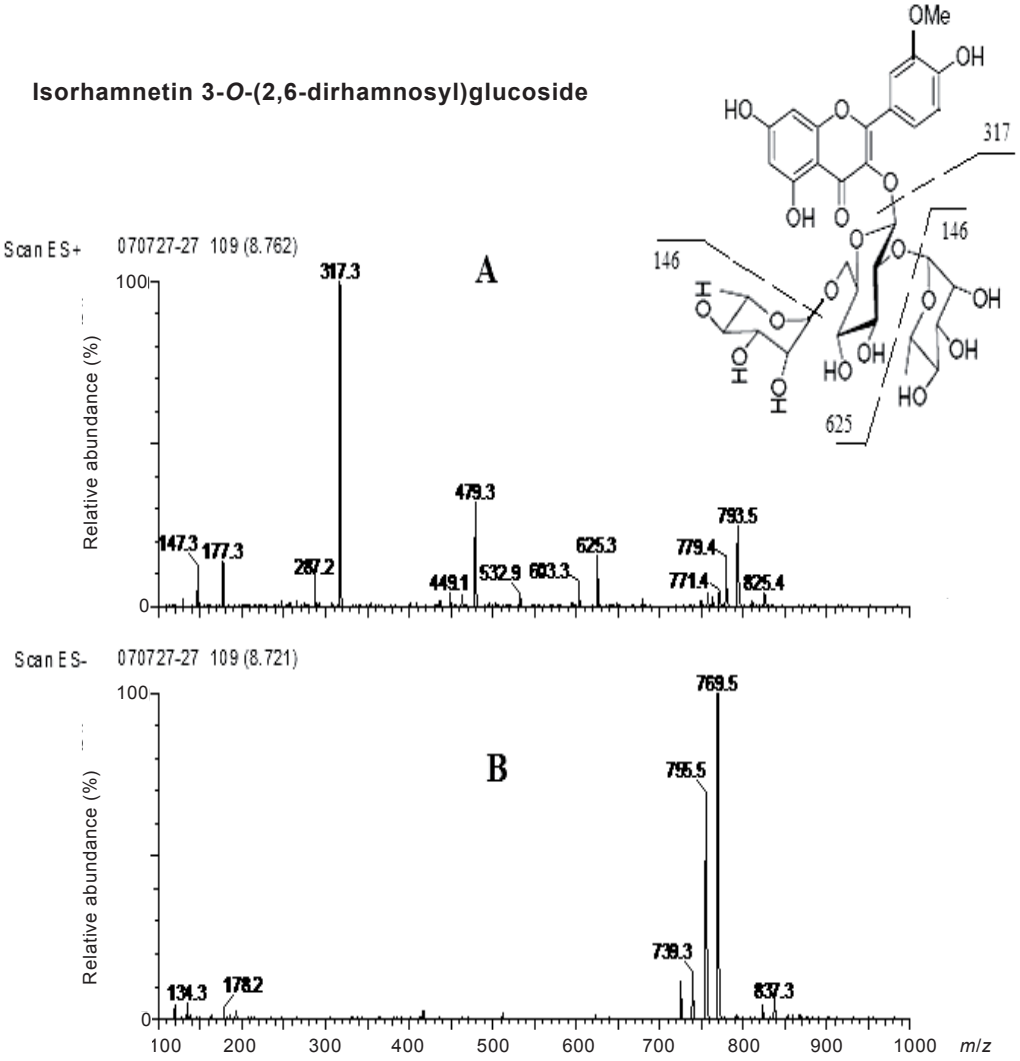

Figure 6. Mass spectrum of compound I

O. ficus-indica skin were separated at 14th min, compound I (peak RT 8.75) and compound II (peak RT 10.15) peak areas occupying 37.42% and 26.78%, respectively (Figure 5).

ESI-MS is a new development of mass spectrometry, which can accurately measure the molecular weights of both small molecules and large biological molecules. In order to determine precisely the molecular weights of the main flavonoids in the final product, two modes of ESI-MS, [M-H]-and $[M+Na]^+$, were executed.

Figure 6 shows ESI-MS ($[M+Na]^+$) and ESI-MS ($[M-H]^-$) of compound I. Combined with Figure 6B, the molecular weight of the compound I was 770.3 from Figure 6A. The fragment ion at *m*/*z* 625.3 $[M-146+H]^+$ was speculated from the fragment ion at *m*/*z* 770.3 on the loss a rhamnosyl group, and the fragment ion at *m*/*z* 479.3$[M-146-146+H]^+$with the loss of two rhamnosyl group. The significant peak at *m*/*z* 317.3 $[M-146-146-162+H]^+$ was supposed as after the loss of two rhamosyl groups and a glucosyl group. These ESI-MS data matched with isorhamnetin3-*O*-(2, 6-dirhamnosyl)glucoside (Sakushima *et al*. 1980; Mauri & Pietta 2000; Ding *et al*. 2009).

The UV spectra of compound I obtained in this study were as follows: UV, λ_{max} (nm) (MeOH) 254.0, 352.6; (MeONa) 271.0, 324, 400; ($AlCl_3$) 277sh, 365,404; ($AlCl_3$/HCl) 278sh, 306sh, 361, 403; (NaAc) 274, 324, 407; (NaAc/H_3BO_3) 256, 271, 360. The UV spectrum in methanol and its changes after the addition of the customary shift reagents suggested that compound I is a flavonol glycoside with free hydroxyl groups at positions C-5, C-7, and C-4', while with shift reagents ($AlCl_3$, $AlCl_3$/HCl) it exhibited band I absorption suggesting the

Table 3. Variance analysis of the orthogonal design

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Significance
A	0.27	2	0.135	13.5	*
B	0.30	2	0.15	15	*
C	5.25	2	2.625	262.5	***
Error	0.02	2	0.01		
Total sum of square	1.70	8		$F_{0.01}$ 2.2 = 99 $F_{0.05}$ 2.2 = 19 $F_{0.1}$ 2.2 = 9	

***significant at 0.001 level; *significant at 0.1 level

presence of a free 4'-hydroxyl group and absence of ortho-dihydroxyl group (Ducrey *et al.* 1995; Markham 1982). Thus, glycosylation sites were suggested to be at C-3. Based on the above evidence, compound I was structurally speculated as isorhamnetin3-*O*-(2, 6-dirhamnosyl)glucoside.

The compound II ESI-MS data (Figure 7) matched with isorhamnetin 3-*O*-D-rutinoside (Chaurasia & Wichtl 1987). Its UV spectra as follows: (MeOH) – 254, 266 sh, 305 sh, 356; ($AlCl_3$) – 268, 279 sh, 300 sh, 369 sh, 402; ($AlCl_3$/HCl) – 267, 275 sh, 300 sh, 359 sh, 399; (NaOMe) – 271, 328,

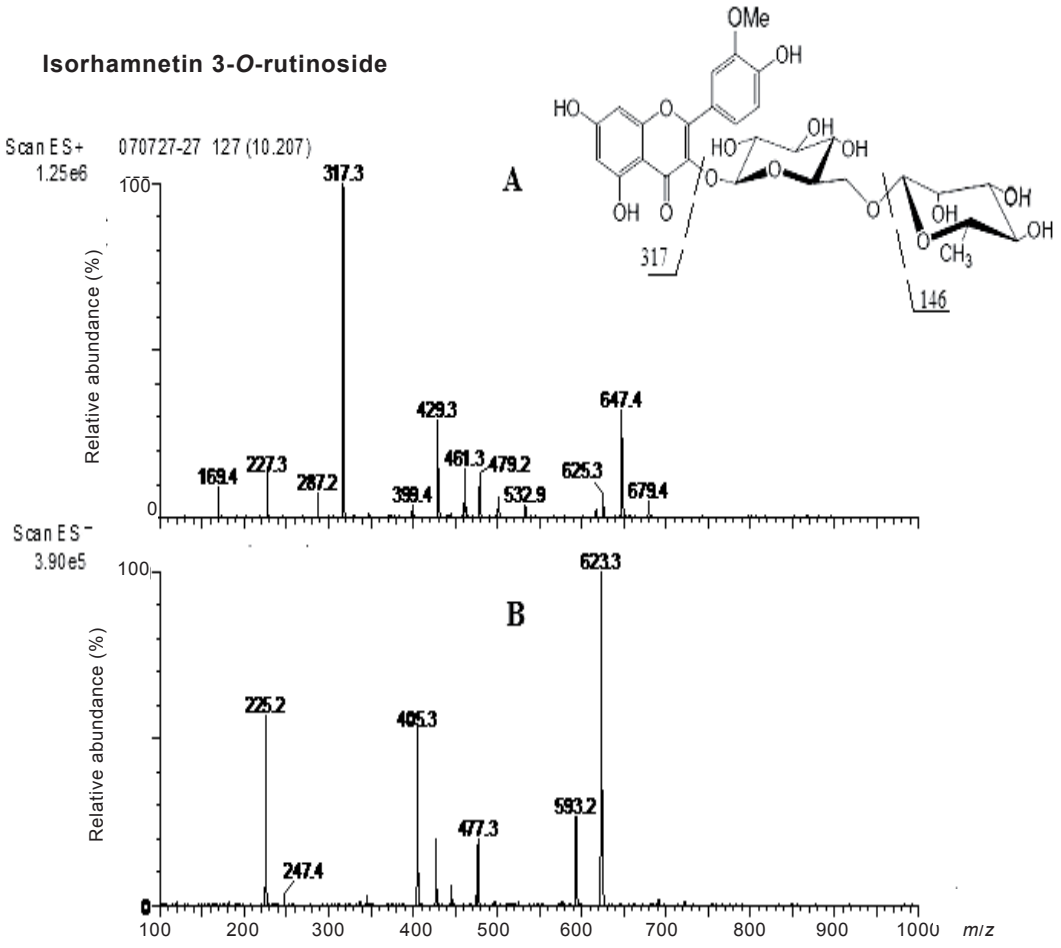

Figure 7. Mass spectrum of compound II

413; (NaOAc): 272, 320, 396; (NaOAc/H_3BO_3) – 254, 267sh, 304 sh, 360 nm.

On comparison with flavonoids UV spectra reported (Babaei 2008), compound II was similarly identified as isorhamnetin3-*O*-D-rutinoside.

Isorhamnetin 3-*O*-D-rutinoside possesses a strong anti-tumour promoting activity (Hideyuki *et al*. 1999) and antimicrobial activity (Agnese *et al*. 2001). Based on its high content of flavonoids and its bioactivity, cactus skin should be further exploited due to its medicinal value and others.

CONCLUSION

The present study indicates that flavonoids in *O. ficus-indica* skin can be produced by employing the extraction with ethanol solutions. As obtained by orthogonal design and variance analysis, the optimal conditions for the production of flavonoids contained in *O. ficus-indica* skin include the following parameters: the volume fraction of ethanol 80%, extraction temperature 90°C, solvents to raw material ratio 25:1. Under these conditions, the experimental yield was 5.55 mg/g. The results of LC-MS and flavone diagnostic reagent indicated that the main components of the extracted flavonoids were isorhamnetin 3-*O*-(2, 6-dirhamnosyl)glucoside and isorhamnetin 3-*O*-D-rutinoside.

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