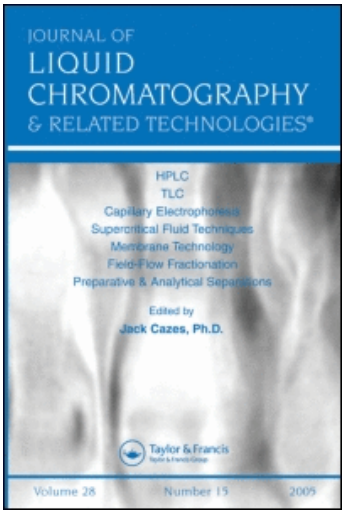

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Identification of Chemical Constituents in the Root of *Isatis Indigotica* Fort. by LC/DAD/ESI/MS/MS

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Abstract: The root of *Isatis indigotica* Fort. (ROCIIT) is a commonly used traditional Chinese medicine for anti inflammatory remedies. A method has been developed for rapid analysis of the constituents of ROCIIT by HPLC/DAD/ESI/MS/MS. Mass spectra were scanned in both positive and negative ion modes with an electrospray ionization (ESI) source. Twelve compounds in ROCIIT, arginine, cytidine, tyrosine, uridine, phenylalanine, guanosine, goitrin, adenosine, isaindigodione, salicylic acid, indigoticalignanoside A, and hydroxyindirubin, were identified. The root of *Baphicacanthus cusia (Nees)* Brem. (ROBCB), a substitute of ROCIIT, was also analyzed. Considerable differences between ROCIIT and ROBCB were revealed.

Keywords: *Isatis indigotica* Fort, LC/MS, Identification, Goitrin

INTRODUCTION

ROCIIT is a famous traditional Chinese medicine for antipyretic and detoxifying purposes. Studies have shown that ROCIIT possessed diverse and significant pharmacological effects such as antibacterial, antivirus,[1,2] antiendotoxin,[3,4] enhancing immunity,[5,6] anticancer,[7,8] and anticoagulant.[9] Some bacteria such as typhoid bacillus, bacillus subtilis, bacillus coli, etc, can be restrained by the aqueous extract of ROCIIT. It was reported that salicylic acid, benzoic acid, and 4 (3H) –quinazolinone, etc, in ROCIIT had antiendotoxin effects in vitro.[10] The antivirus effect of ROCIIT is now recognized widely by the Chinese people.

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It has been revealed that ROCIIT contains a variety of chemical ingredients, including indole compounds,[11–15] quinazolinones,[13,14] sinigrin, amino acids,[16] organic acids,[17] sitosterols,[18] 2-quinolinone derivatives,[19] and compounds of sulfide.

LC/MS is a powerful technique for the identification of known compounds in crude plant extracts. In the present study, cytidine, uridine, guanosine, adenosine, and goitrin extracted from ROCIIT were identified by liquid chromatography tandem electrospray ionization mass spectrometry (LC/DAD/ESI/MS/MS), by comparing their HPLC retention times and UV spectra with corresponding authentic standards. Arginine, tyrosine, phenylalanine, isaindigodione, salicylic acid, indigoticalignanoside A, and hydroxyindirubin were identified by their MS and MS/MS data. The chemical structures of these compounds are shown in Figure 1. Until now, there has no report on the analysis of ROCIIT by the LC/MS method. The rapid identification will be very important and helpful for guiding and supporting the pharmacological investigation.

Moreover, ROBCB was assayed because it is often used as a substitute of ROCIIT in some areas of south China. Considerable differences between ROCIIT and ROBCB were revealed in the present study.

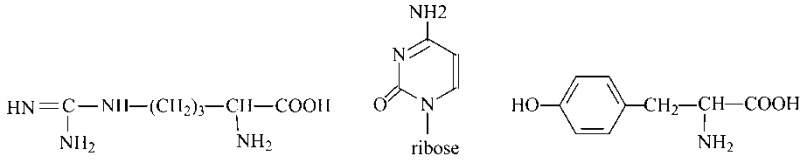
EXPERIMENTAL

Materials and Reagents

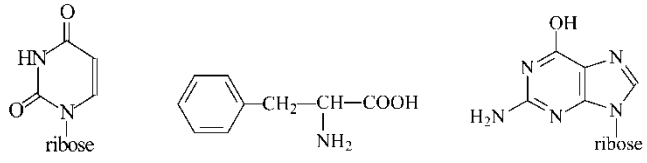
ROCIIT was collected from Anhui province, P. R. China, and ROBCB was collected in Guangdong province, P. R. China. Cytidine, uridine, guanosine, adenosine, and ten amine acids were purchased from the National Institute for the Control of Pharmaceutical and Biological Products P. R. China (Beijing, P. R. China). Goitrin was obtained by HPLC preparation in our laboratory and identified by EI/MS, ^{1}H-NMR and ^{13}C-NMR by comparison with literatures. Water was doubly distilled in house. Methanol (HPLC grade), was purchased from Dikma (ON, USA). Ethanol and formic acid were all of analytical grade (Shenyang, P. R. China).

HPLC/MS System and Conditions

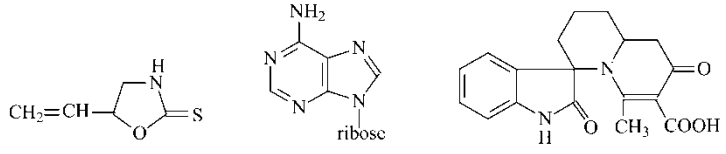
An Agilent 1100 series HPLC system (Waldbronn, Germany) consisted of a pump with a vacuum degasser, a thermostatic column compartment, a diode array detector (DAD), an autosampler, and an Agilent ChemiStation. The separation was performed on a Zarbox Eclipse XDB-C_{18} column (150 $\times$ 4.6 mm, 5 μm, Agilent, USA) with a C_{18} guard column (10 $\times$ 4.6 mm, 5 μm, Tianmei, P. R. China). The mobile phase consisted of solvent A (methanol) and B (0.1% formic acid water) with the gradient elution of A, 3%–10% at 0–10 min and 10% – 80%, at 10 – 30 min. The flow rate was 1.0 mL $\cdot$ min^{-1}. The column



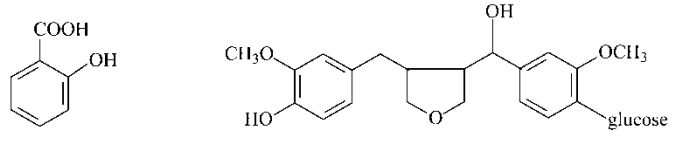
arginine cytidine tyrosine



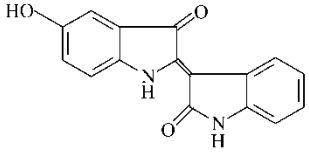
uridine phenylalanine guanosine



goitrin adenosine isaindigodione



salicylic acid indigoticalignanoside



hydroxyindirubin

Figure 1. Structures of constituents in ROCIIT.

oven temperature was 30°C. Samples were detected at 245 nm with an injection volume of 5 μL.

The mass spectra analysis was performed on an Agilent 1100 series SL ion trap mass spectrometer (Agilent, Germany) with an ESI source in both positive and negative ion detection mode. The nebulizer nitrogen gas pressure was set at 0.24 MPa and the drying nitrogen gas rate was set at 10 $L \cdot min^{-1}$. Drying gas temperature was 325°C and the scan range was 60 – 800 m/z.

Sample Solutions Preparation

Dry ROCIIT and ROBCB were crushed to powder before use. The powder (2.0 g) was soaked with water for 12 h, and was decocted 2 h for extraction. The extract was evaporated under reduced pressure to 10 mL. Then, 95% ethanol was added to the concentrated extract and the concentration of ethanol was made to 65%. The solution was stored at 4°C for 24 h and

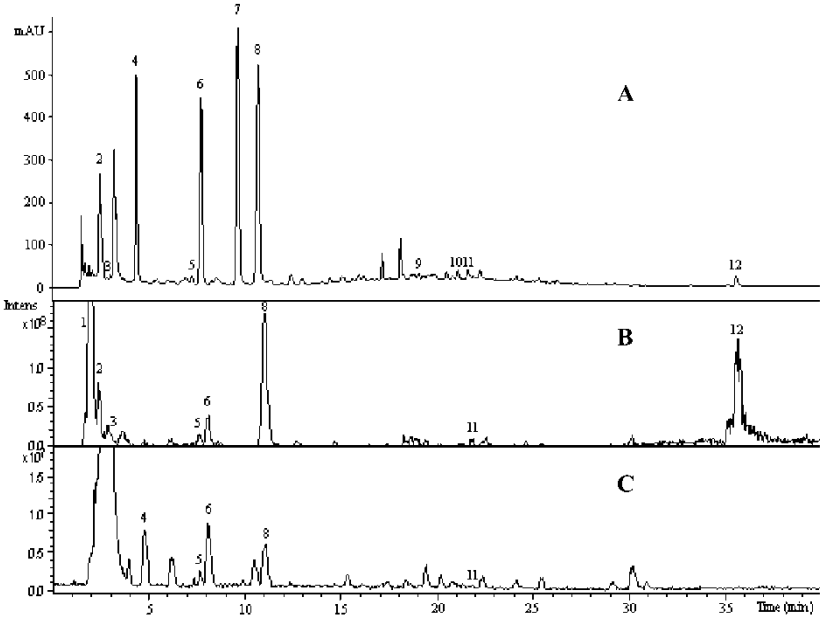


Figure 2. LC/MS chromatograms of the water extraction of ROCIIT. (A) UV chromatogram; (B) positive TIC; (C) negative TIC; 1, arginine; 2, cytidine; 3, tyrosine; 4, uridine; 5, phenylalanine; 6, guanosine; 7, goitrin; 8, adenosine; 9, salicylic acid; 10, isaindigodione; 11, indigoticalignanoside; 12, hydroxyindirubin. Chromatographic conditions: Zabox Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 μm) with a guard column C_{18} (10 × 4.6 mm, 5 μm). Methanol - 0.1% formic acid water was mobile phase, gradient elution. The flow rate was 1.0 $mL \cdot min^{-1}$ at 30°C and detection wavelength was 254 nm, with injection volume of 5 μL.

filtered. The filtrate was evaporated to about 8 mL under reduced pressure, then diluted to 10 mL with water, and filtered through a 0.45 μm membrane filter before injection. Both ROCIIT and ROBCB were prepared in triplicate.

RESULTS AND DISCUSSION

Total Ion Chromatograms and Extracted Ion Chromatograms of ROCIIT

Constituents of the sample were first separated by HPLC, and then were analyzed by ESI/MS/MS. The UV chromatogram, positive and negative total ion chromatograms (TIC) of ROCIIT were shown in Figure 2. Extracted ion chromatograms (EIC) of the constituents of ROCIIT were shown in Figure 3,

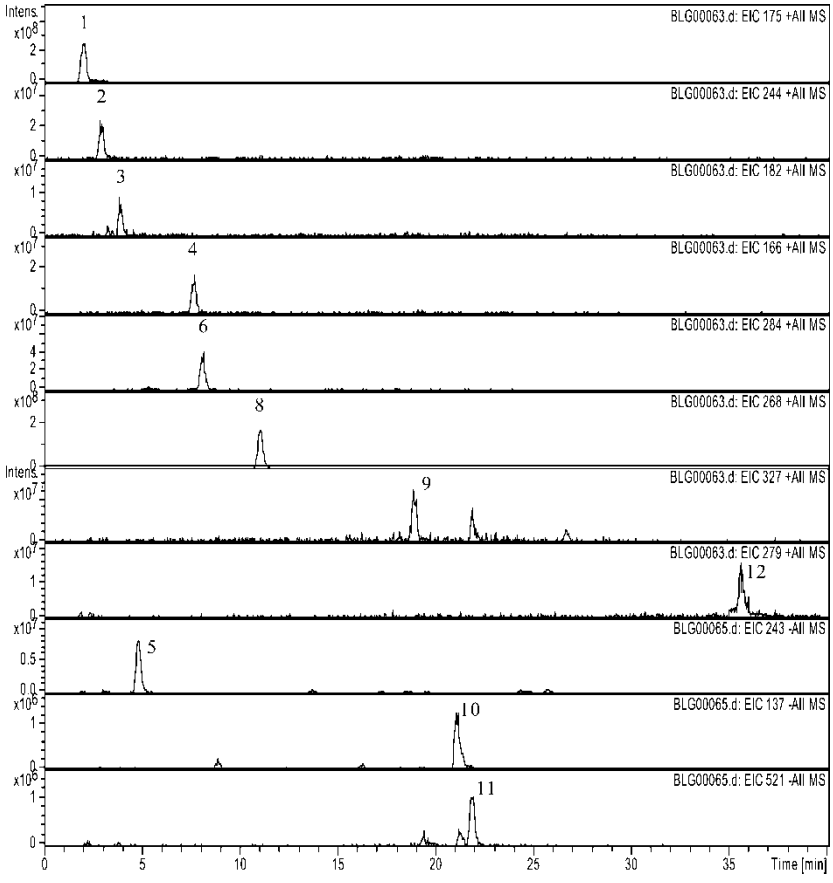
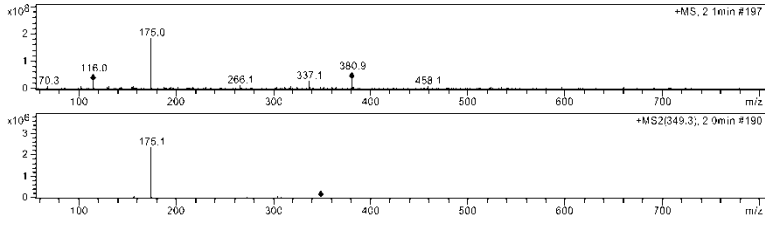
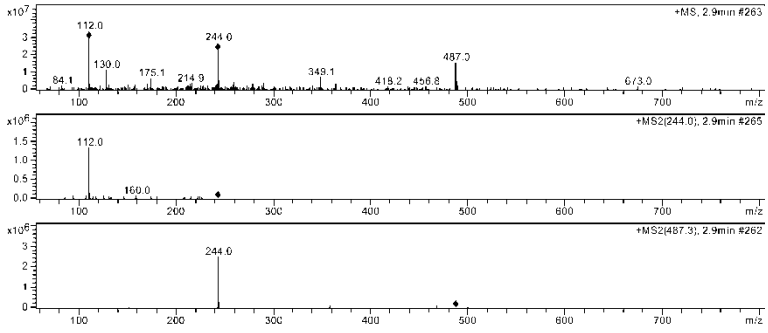


Figure 3. EICs of constituents in ROCIIT. Number 1-12 represented the same constituents shown in Figure 2, except goitrin.

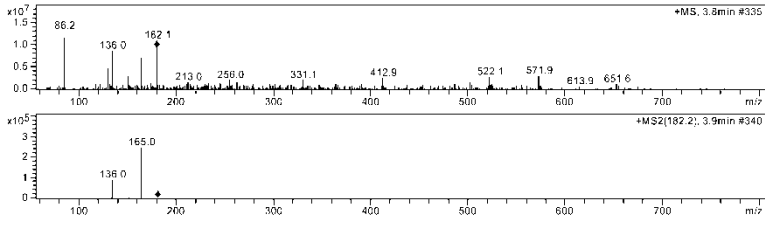
except goitrin. In the positive ESI mode, a protonated molecule of $[M+H]^+$ was mainly formed, and a deprotonated molecule of $[M-H]^-$ in the negative ESI mode. The mass spectra of the constituents were shown in Figure 4. The attributes of MS and MS/MS spectra of the constituents were listed in Table 1.



arginine MS and MS/MS



cytidine MS and MS/MS



tyrosine MS and MS/MS

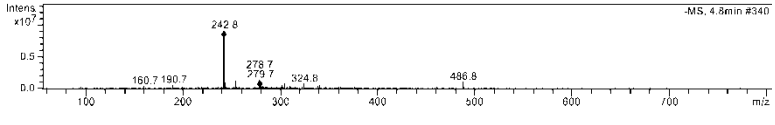
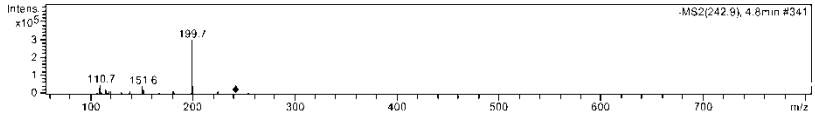
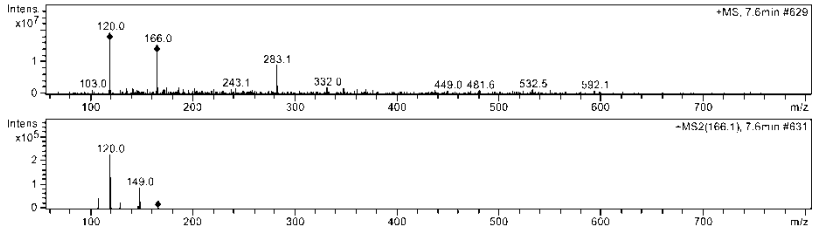


Figure 4. MS and MS/MS spectra of constituents in ROCIIT.

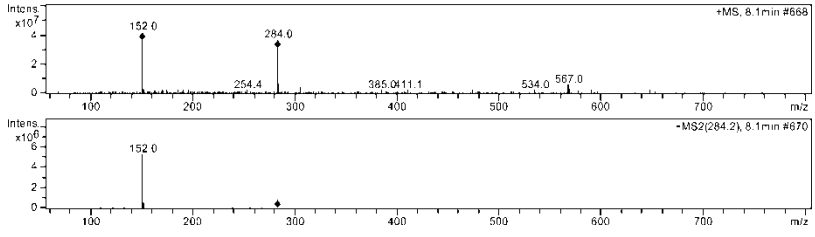
(*Continued*)



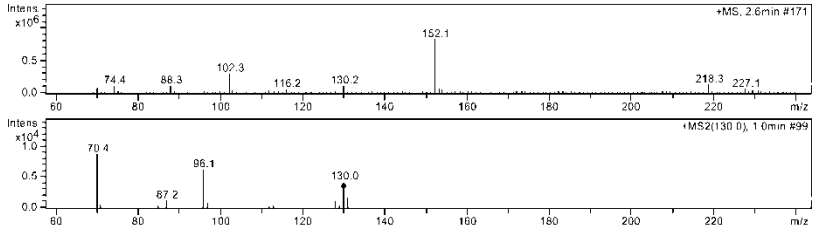
uridine MS and MS/MS



phenylalanine MS and MS/MS



guanosine MS and MS/MS



goitrin MS and MS/MS

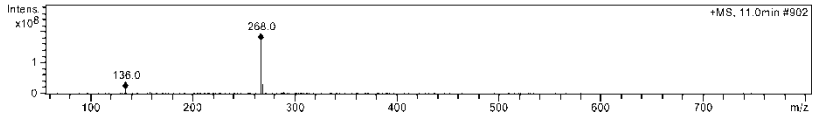
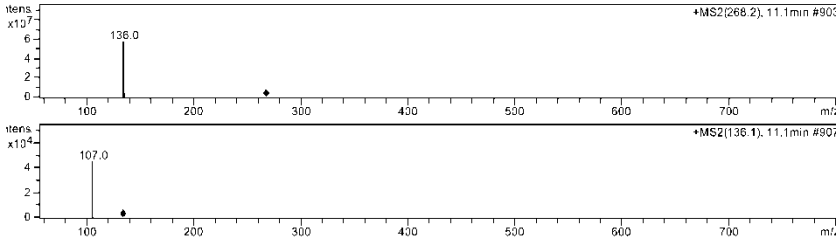
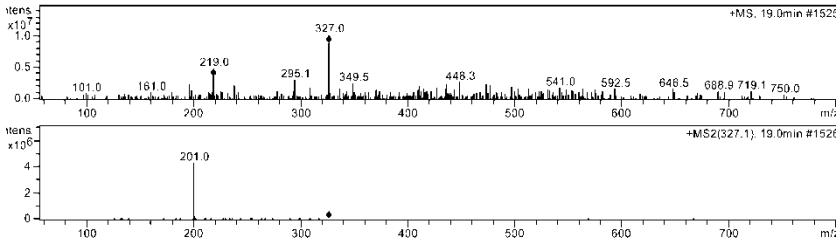


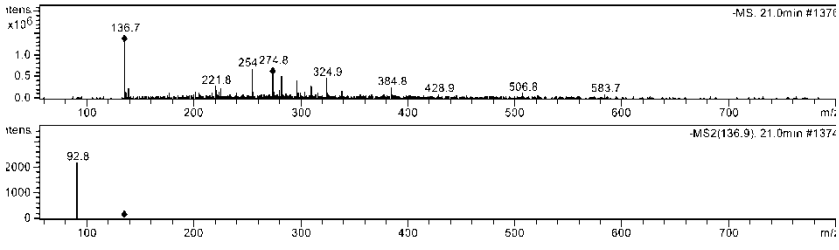
Figure 4. Continued.



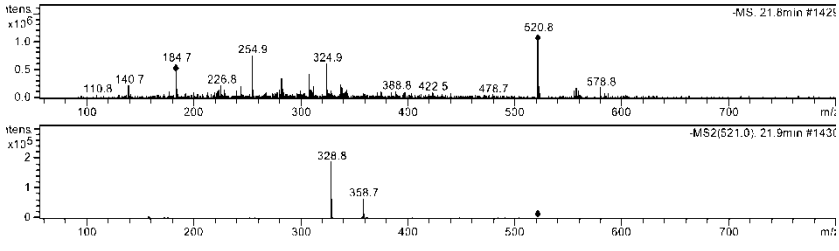
adenosine MS and MS/MS



isaindigodione MS and MS/MS

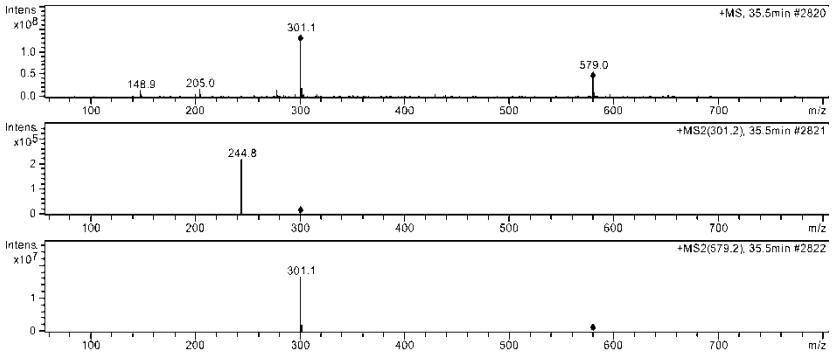


salicylic acid MS and MS/MS



indigoticalignanoside MS and MS/MS

Figure 4. Continued.



hydroxyindirubin MS and MS/MS

Figure 4. Continued.

Ten amino acids were analyzed by ESI/MS/MS with direct infusion to conclude MS and MS/MS regulations, and the result was listed in Table 2. According to the regulation, compounds 1, 3, and 5 were concluded to be arginine, tyrosine, and phenylalanine, respectively. This agreed with our research that ROCIIT was abundant in arginine.

It has also been found that goitrin had good UV absorption but weak response in ESI/MS, and the mass spectra were obtained by direct infusion to MS with goitrin solution. Compound 10 was identified to be hydroxyindirubin and there were 4, 5, 6, and 7-O-substitutes, so 4 hydroxyindirubins may exist. Two peaks were detected at about 35 min in the UV chromatogram, so it was presumed that there were at least 2 hydroxyindirubins in ROCIIT.

Comparison of ROCIIT and ROBCB by UV Chromatograms and TICs

ROBCB was analyzed by the same method, and the typical UV chromatogram and TICs were shown in Figure 5. Considerable differences were shown between the UV chromatograms of ROCIIT and ROBCB. More polar constituents in ROCIIT were detected than those in ROBCB. The differences were also revealed in TICs. There were higher and more peak responses before 16 min in the TICs of ROCIIT than those of ROBCB. The 12 compounds mentioned above were not detected in ROBCB, except for adenosine and hydroxyindirubin in EIC.

Optimization of Extraction Conditions

Samples (2.0 g) were extracted with water, 25%, 50%, 75%, and 95% ethanol, respectively. The samples extracted with water followed the aforementioned

Table 1. Attributes of MS and MS/MS spectra of ROCIIT in both positive and negative ion modes

Peak No.	Compoud	MW	MS (m/z)	Attribution	MS^2 (m/z)	Attribution
1	Arginine	174	175	$[M + H]^+$		
			349	$[2M + H]^+$		
2	Cytidine	243	244	$[M + H]^+$	112	$[M + H\text{-rib}]^+$
			487	$[2M + H]^+$		
3	Tyrosine	181	182	$[M + H]^+$	165	$[M + H\text{-}NH_3]^+$
					136	$[M + H\text{-HCOOH}]^+$
4	Uridine	244	243	$[M - H]^-$	200	$[M\text{-H-NHCO}]^-$
					111	$[M\text{-H-rib}]^-$
5	Phenylalanine	165	166	$[M + H]^+$	149	$[M + H\text{-}NH_3]^+$
					120	$[M + H\text{-HCOOH}]^+$
6	Guanosine	283	284	$[M + H]^+$	152	$[M\text{-H-rib}]^+$
			567	$[2M + H]^+$		
7	Goitrin	129	130	$[M + H]^+$	70	$[M + H\text{-OCS}]^+$
			152	$[M + Na]^+$	96	$[M + H\text{-}H_2S]^+$
8	Adenosine	267	268	$[M + H]^+$	136	$[M + H\text{-rib}]^+$
					107	$[M + H\text{-rib-}NHCH_2]^+$
9	Isaindigodione	326	327	$[M + H]^+$	201	$[M + H\text{-A}^*]^+$
			349	$[M + Na]^+$	223	$[M + Na\text{-A}^*]^+$
10	Salicylic acid	138	137	$[M - H]^-$	93	$[M\text{-H-}CO_2]^-$
11	Indigoticalig-nanoside A	522	521	$[M - H]^-$	329	$[M\text{-H-glc-}CH_2O]^-$
					359	$[M\text{-H-glc}]^-$
12	Hydroxy-lindirubin	278	279	$[M + H]^+$	245	$[M + Na\text{-2CO}]^+$
			301	$[M + Na]^+$		
			579	$[2M + H]^+$		

A^*: $CH_3CHC(COOH)COCH_2$.

method. The samples were regurgitated for 2 h after soaking with 25%, 50%, 75%, and 95% ethanol for 12 h, respectively. The extract was centrifuged at 3500 g for 10 min. Supernatant fluid was extracted and filtered by a membrane filter (0.2 μm) with collection following, for HPLC analysis. The

Table 2. MS and MS/MS regulation of amino acids in ESI/MS/MS

Scan mode	MS	MS/MS
Positive	$[M + H]^+$	$[M + H\text{-}NH_3]^+$
	$[M + Na]^+$	$[M + H\text{-}H_2O]^+$
		$[M + H\text{-HCOOH}]^+$
Negative	$[M - H]^-$	$[M\text{-H-}NH_3]^-$
		$[M\text{-H-}CO_2]^-$

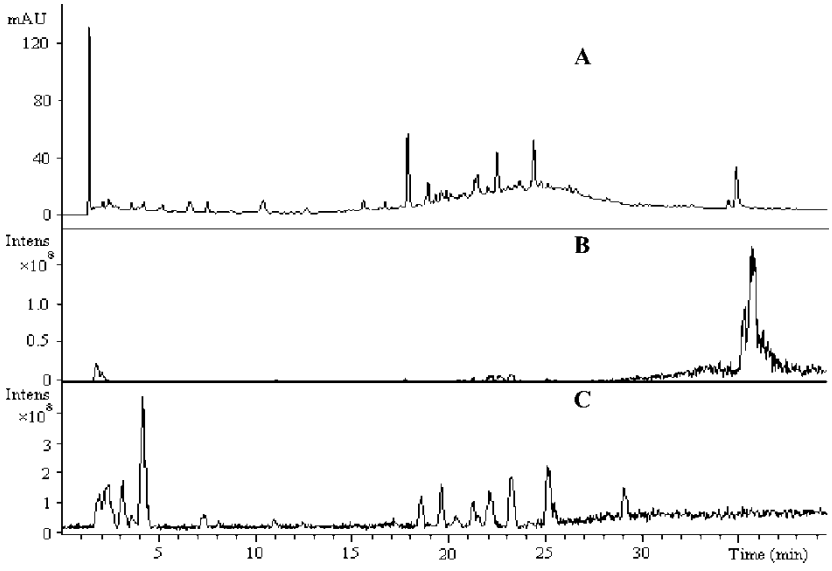


Figure 5. LC/MS chromatograms of the extraction of ROBCB. (A) UV chromatogram; (B) positive TIC; (C) negative TIC. Chromatographic conditions were the same as Figure 2.

UV chromatograms showed that there were less polar constituents than the extracts of water. The higher the concentration of ethanol, the less the peaks in front of the chromatograms would be, while the peak responses behind did not change. So water was chosen as the solvent for extracts.

CONCLUSION

For the first time, 12 compounds extracted from ROCIIT and ROBCB were identified by the HPLC/DAD/ESI/MS/MS technique. The constituents in ROBCB are much different from ROCIIT in species and contents, both the UV chromatograms and the TICs reveal the differences. The differences suggest that the pharmacological effects of ROCIIT and ROBCB would differ. So, it is concluded that ROCIIT should not be substituted by ROBCB for medical use.

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