Journal of Chromatography A, 1217 (2010) 2441–2446

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Improved extraction and identification by ultra performance liquid chromatography tandem mass spectrometry of phenolic compounds in burdock leaves

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ARTICLE INFO

Article history:
Available online 21 December 2009

Keywords:
Burdock leaves
Phenolic compounds
Simultaneous ultrasonic and microwave assisted extraction
UPLC–MS/MS
Identification

ABSTRACT

The simultaneous ultrasonic and microwave assisted extraction (UMAE) technique was first employed to obtain phenolics. The effects of UMAE variables including extraction time, microwave power, and solvent to solid radio on the yield of phenolics were investigated. The optimized conditions were as follows: solvent to solid ratio was 20:1 (ml/g), extraction time was 30 s, microwave power was 500 W and two times of extraction. Moreover, the phenolic yield of UMAE was higher than that by maceration, indicating a significant reduction of extraction time and an improvement of efficiency. The phenomenon is related to the strong disruption of leaf tissue structure by microwave induced expansion and ultrasonic shaking, which had been observed with the scanning electron microscopy. The phenolic compositions of the extract was then identified by ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), 10 compounds had been characterized, providing a more complete identification of phenolic compounds in burdock leaves than previously reported. The occurrence of benzoic acid and p-coumaric acid is reported for the first time. This study suggests that UMAE is a good alternative for the extraction of phenolics, with a great potential for industrial application. Also, UMAE provides a new sample preparation technique for characterization of the phenolic compounds from plants.

1. Introduction

In vitro and epidemiological studies strongly suggest that phenolic compounds have protective effects against many diseases. These compounds could be used as antimutagenic, antibacterial and anti-inflammatory agents [1]. Increasing evidence indicates that consumption of phenolic compounds can lower the risk of serious health disorders for the bioactivity of phenolics [2,3]. Presently, increasing attention is being paid on these compounds.

Burdock, *Arctium lappa* L. which is a popular vegetable in China and Japan, has been extensively studied for its phenolic compounds and other components in root and seed due to their various biochemical activities [4,5]. However, there is less study about phenolic-rich burdock leaves, which are used in Chinese traditional medicine for heat-clearing and detoxifying. Previous work resulted in the discovery of arctiin [6] and several phenolic compounds including caffeic acid, chlorogenic acid, rutin, meletin, cynarin, quercitrin, and luteolin [7]. However, information on other phenolic compounds in burdock leaves is not available.

Extraction is one of the key steps in the investigation and utilization of phenolic components from various plants. The ability of a variety of extraction techniques has been evaluated, such as solvent extraction [8], enzyme-assisted extraction [9] and heat extraction [10]. However, these extraction methods have drawbacks to some degree. For instance, conventional solvent extraction is time consuming; heat treatment results in thermal decomposition, and enzyme in enzyme-assisted extraction is easy to denature.

Ultrasonic is one of the most popular methods used to enhance mass transfer phenomena [11–13]. The increasing interests on applying sonochemistry to product extraction lie in its advantage on reducing extraction time, saving energy, increasing yield, etc. Meanwhile, microwave heats the extracts quickly and accelerates the extraction process for adsorption and desorption of the targeted compounds from matrix [14]. Hence, coupling microwave with ultrasonic extraction is a complementary technique and may present many advantages. However, simultaneous ultrasonic and microwave assisted extraction of phenolics is not reported.

The aim of this study was to investigate the effect of simultaneous ultrasonic/microwave treatment on the extraction of phenolics and the microstructure of the burdock leaves. Also the major phenolic compounds of burdock leaves are identified.

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doi:10.1016/j.chroma.2009.12.022

2. Materials and methods

2.1. Materials

Burdock leaves were provided by Xuzhou Wangda Farm and Sideline Products Co., Ltd. Caffeic acid (≥99%), chlorogenic acid (≥99%), quercetin-rhamnoglucoside (rutin) (≥98%), vanilic acid (≥98%), ferulic acid (≥99%), benzoic acid (≥99%) quercetin (≥98%), cynarin (≥96%), quercitrin (≥96%), luteolin (≥98%), p-coumaric acid (≥98%), arctiin (≥98%) were obtained from Sigma (Shanghai, China). Methanol, acetonitrile and formic acid, all MS grade, were purchased from Fisher Scientific Co., Ltd. (Shanghai, China).

2.2. Maceration

The dried powder of burdock leaves (20 g) was mixed with 400 ml of 70% ethanol. The extraction was carried out at 50 °C, 250 rpm. Multiple extractions were also performed according to the method of Zuo et al. [15]. Burdock leaves powder (3 g) was extracted three times with 60 ml 70% ethanol for 3 h and then two times with 60 ml 70% ethanol containing 0.15% HCl for 3 h. The extracts were combined, filtered and then concentrated using a rotary evaporator at 40 °C under vacuum and lyophilized using a freeze-dryer (LGJ-10D, Four-Ring Science Instrument Beijing Co., Ltd., China) to obtain extracts.

2.3. Simultaneous ultrasonic/microwave assisted extraction

Simultaneous ultrasonic/microwave assisted extraction (UMAE) experiment was carried out in a simultaneous ultrasonic and microwave extracting apparatus (CW-2000, Shanghai Xintuo Microwave Instrument Co. Ltd., China). The schematic diagram of the apparatus was shown in Fig. 1. The dried powder (20 g) of burdock leaves was mixed with 70% ethanol (400 ml). Extraction process was performed in the apparatus chamber with simultaneous different microwave power and a fixed ultrasonic power of 50 W.

Multiple extractions were also performed [15]. Burdock leaves powder (3 g) was extracted three times with 60 ml 70% ethanol for 30 s and then two times with 60 ml 70% ethanol containing 0.15% HCl for 30 s. The extracts were combined. The post-treatment of the extracts was the same as that mentioned above in maceration.

2.4. Determination of phenolics

The phenols contents were determined using the Folin–Ciocalteu method as described by Yoo et al. [16]. The total phenols were expressed as gallic acid equivalents. The yield of phenolics was expressed as mg per gram of burdock leaves on dry weight basis.

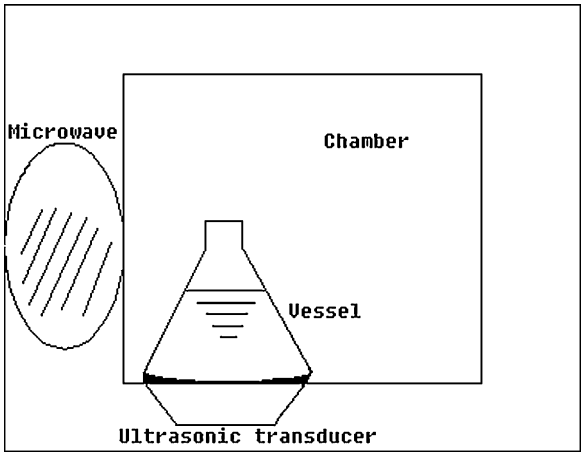

Fig. 1. Schematic diagram of simultaneous ultrasonic and microwave extracting apparatus.

2.5. Identification of phenolic compounds

UPLC–MS/MS analyses were carried out using an Ultra Performance Liquid Chromatography apparatus equipped with a Waters Acquity PDA detector (Waters, USA) and a Acquity UPLC™ BEH C_{18} column (150 mm × 2.1 mm, particle size 1.7 μm) (Waters, USA). The eluents were: A, water 0.1% formic acid; B, acetonitrile/methanol (20:80, v/v). The gradient program was as follows: 10–30% B (10 min), 30–50% B (10 min), 50–70% B (3 min), 70–10% B (2 min) at a constant flow of 0.28 ml/min. The peaks of the phenolic compounds were monitored at 280 nm. UV–vis absorption spectra were recorded on-line from 200 nm to 700 nm during the UPLC analysis.

Mass spectroscopic analysis of phenolic compounds in the sample was performed using a SYNAPT Mass Spectrometer (Waters), equipped with an electrospray ionization source operating in negative mode. The effluent was introduced into an electrospray source (source block temperature 100 °C, desolvation temperature 400 °C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was used as collision gas (collision energy 16 eV) and nitrogen as desolvation gas (500 l/h). Identification of the phenolic compounds from burdock leaves was achieved by comparison with retention times of standards and their UV–vis absorption spectra and MS spectra comparisons with reference standards or literature reports.

2.6. Behaviour of phenolic compounds under the UMAE conditions

Under the ultrasonic and microwave extraction conditions used, stability tests for individual phenolics were carried out according to the method of Liazid et al. [17]. Stock standard solutions of phenolic compounds were prepared in methanol and water 70:30 (v/v) and stored in a freezer (−20 °C). A two times extraction (30 s of each extraction) was performed at 500 W of microwave power and 50 W of fixed ultrasonic power, which were the extraction conditions for burdock leaves. Another two times extraction was also performed under the same conditions with longer extraction time (120 s of each extraction). The extraction protocol used was the following: 1 ml of solution of each phenolic compound, 9 ml of the extraction solvent (70% ethanol). After first extraction, the extract was made up to 10 ml with extraction solvent and extracted again. After two times extraction, the volume of extract was made up to 25 ml with the 70% ethanol. All extractions were performed in triplicate.

2.7. Scanning electron microscopy (SEM) analysis

In order to investigate the influence of ultrasound and microwave on the microstructure of the samples and to understand the mechanism of extraction, the residue after extraction of phenolic compounds was collected and dried for the scanning electron microscopy (SEM) analysis [14,16,18]. Sample particles were fixed on the silicon wafer and sputtered with gold to a thickness of about 100 nm. The shape and the surface characters of the samples were observed and recorded on the scanning electron microscope (Quanta-200, FEI Ltd., The Netherlands).

3. Results and discussion

3.1. Effect of simultaneous ultrasonic and microwave treatment on the yield of phenolics

In simultaneous ultrasonic/microwave assisted extraction, burdock leaves was added with a ratio of solid(g):solvent(ml) of

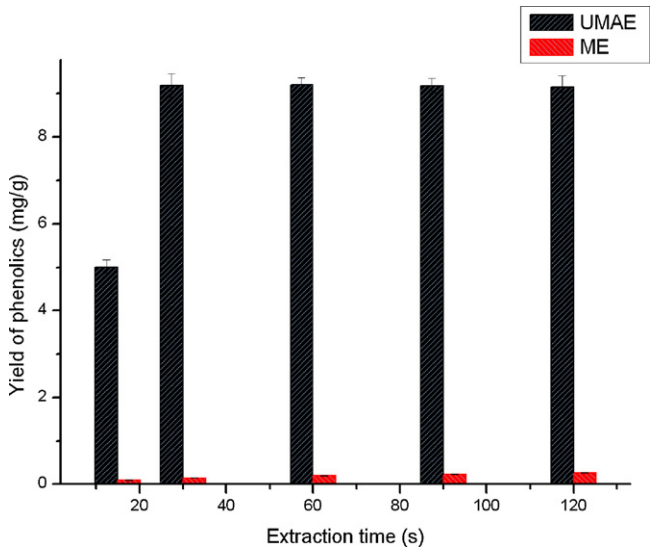

Fig. 2. Effect of UMAE and time on the yield of phenolics. Values expressed as means ± standard deviation ($n = 3$).

1:20, in a beaker and treated in an ultrasonic and microwave extracting apparatus with microwave power of 500 W and fixed ultrasonic power of 50 W. Conventional maceration extraction at 50 °C under the same condition of other factors was also performed. The effect of simultaneous ultrasonic/microwave treatment and its extraction time on the extraction of phenolic compounds was shown in Fig. 2. The yield of phenolics by UMAE was much higher than that by ME. The extraction yield of phenolics increased very fast during the first 30 s of the UMAE and then the yield leveled off. For example, the yield of phenolics increased from 5.01 ± 0.15 mg/g to 9.19 ± 0.27 mg/g when the extraction time increased from 15 s to 30 s. The results suggest that the simultaneous ultrasonic/microwave assisted extraction procedure is rapid, highly efficient.

3.2. Effect of microwave power

The microwave power exhibited significant effects on the extraction of phenolics from burdock leaves (Fig. 3). By 30 s simultaneous ultrasonic/microwave assisted extraction, when the microwave power increased from 300 W to 500 W coupled with a fixed ultrasonic power of 50 W, the yield of phenolics increased very fast. For example, with an microwave power increased from 300 W to 500 W for 30 s, the yield increased from 7.90 ± 0.21 mg/g to 9.18 ± 0.15 mg/g. Beyond 500 W, the recoveries of the phenolic components did not increased, but exhibited a slightly decrease.

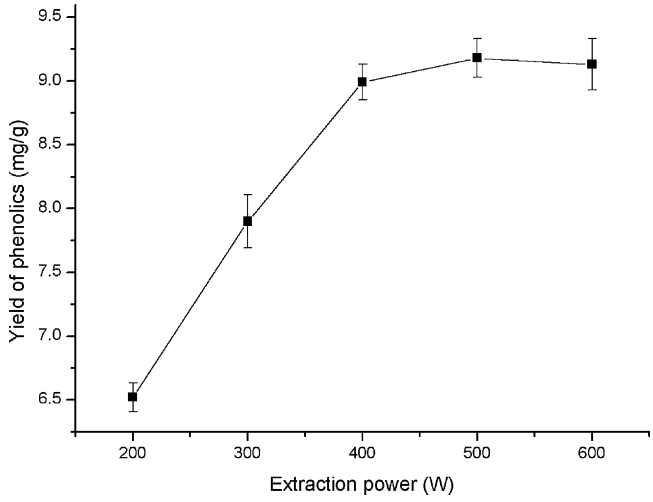

Fig. 3. Effect of microwave power on phenolics yield. Values expressed as means ± standard deviation ($n = 3$).

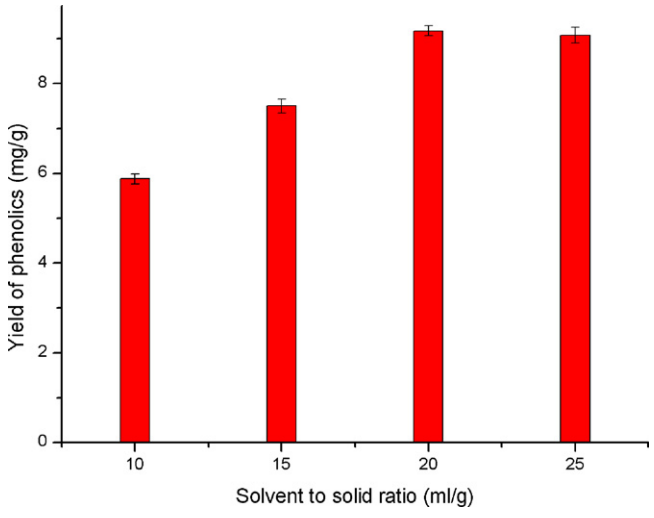

Fig. 4. Effect of solvent to solid ratio on the extraction of phenolics. Values expressed as means ± standard deviation ($n = 3$).

The increase of phenolic yield in UMAE is probably due to the high pressure gradient formed inside the plant material. Microwave absorption causes fast internal heating thus creating significantly high internal pressures which enhance phenolics extraction. The cell walls can swell and burst because of internal heating and thus further promotes the release of target components into the solvent. Therefore an appropriate increase in microwave power led to a rise in yield. Thus, the microwave power of 500 W was chosen as the output microwave power in UMAE.

3.3. Effect of solvent to solid ratio

As shown in Fig. 4, the yield of phenolics was found to increase with the increase of solvent to solid ratio and then fall down at the high ratios. The larger liquid (solvent) to solid ratio means a larger concentration difference which favors mass transfer. But in microwave assisted extraction, a higher solvent volume may give lower yield [19]. As found in Fig. 4, the yield of phenolic compounds increased with the increase of solvent before the solvent to solid ratio reached 20 ml/g, and then they fell down. The phenomena were probably due to the inadequate stiring of the solvent when the microwave was applied at larger volumes. Hence, the solvent to solid ratio of 20:1 (ml/g) seems to be appropriate for UMAE.

3.4. Effect of multiple extractions

A five times extraction was carried out to examine the efficiency of UMAE. The results were shown in Fig. 5. It was found that the phenolics yield of UMAE gradually increased with the increase of extraction times. When the extraction times were more than twice, the increase of yield was no longer significant. There were less phenolics in the third and fourth extractions (UMAE). The phenolics yield of a two times UMAE was 10.28 mg/g, which was a bit higher than the yield of a five times maceration extraction (10.21 mg/g).

3.5. Effect of simultaneous ultrasonic and microwave assisted extraction on the microstructure of samples

Burdock leaves samples, which were the same plant materials and pretreated in the same conditions as aforementioned, were

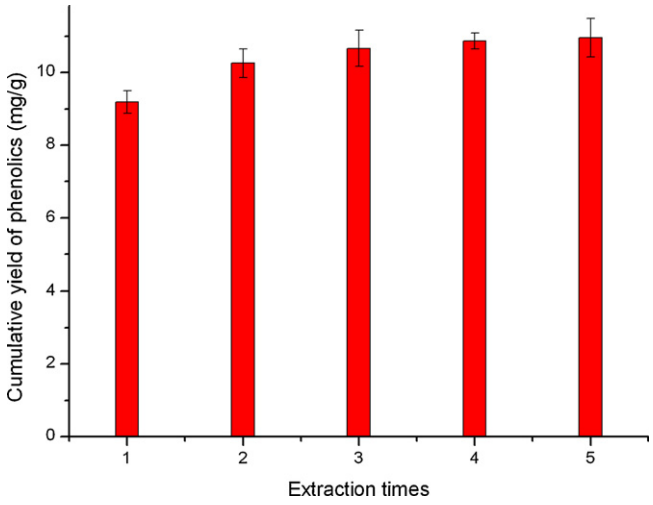

Fig. 5. Effect of multiple extractions on the yield of phenolics. Values expressed as means ± standard deviation ($n = 3$).

extracted by UMAE and maceration, respectively. In order to investigate the effect of simultaneous ultrasonic/microwave assisted extraction on the structure of burdock leaves, the microstructure of simultaneous ultrasonic/microwave assisted extraction samples and maceration extraction samples were examined by scanning electron microscopy (Fig. 6).

After 30 s of UMAE, the cells of burdock leaves sample could not be distinguished and the microstructure of the sample was destroyed. There is more destruction to the leaves microstructure in Fig. 6b than that in Fig. 6a. It was the result of intense shaking and violent collapse of microbubbles of ultrasound coupled with the heating and expansion of microwave. On one hand, large instantaneous energy generated by the ultrasound system leads to breaking of burdock leaf cells and faster access for the solvent to remove solutes from these cells, which renders the components more accessible to extracting solvent so that the external and internal mass diffusivities are significantly increased. Meanwhile, water molecules in the cells, absorb microwave energy efficiently, causing efficient heating of the sample. The selective interaction between the internal free water molecules and microwave results in rapid increasing of temperature and causes expansion with subsequent rupture of the cell walls. Electric induced movements of dissolved ions increase solvent penetration into the hydrophobic components contained within. Such systems undergo a continuous expansion, and subsequent breaking of the cell walls, allowing fast release of the compounds into the solvent [20]. Therefore the rupture of cell walls and migration of compounds into extraction

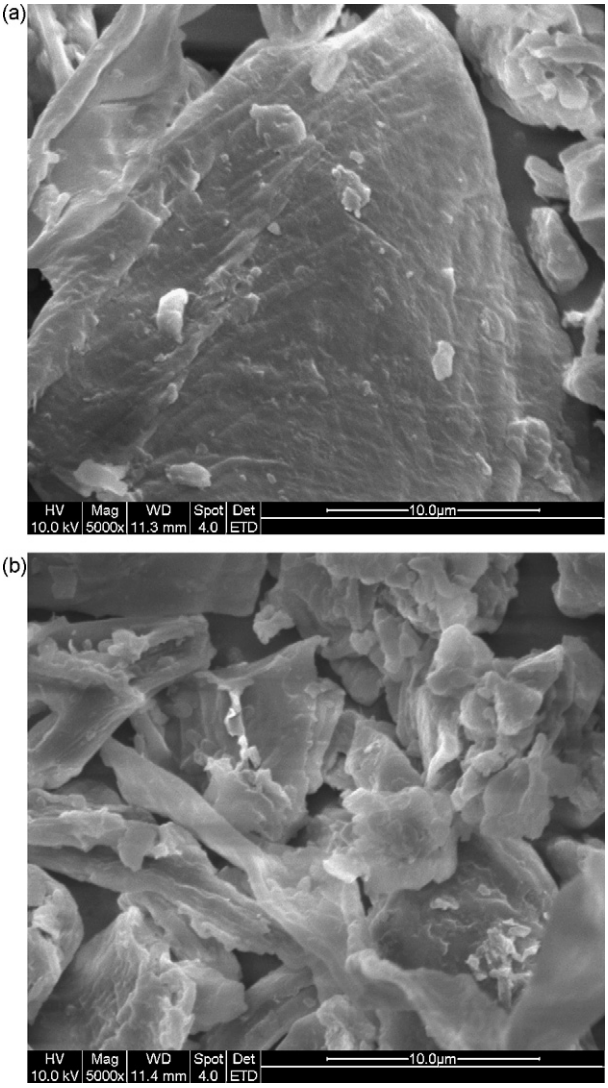

Fig. 6. Scanning electron microscope images of burdock leaves after extraction by: (a) ME (b) UMAE

solvent in UMAE were both easier than that in ME. UMAE causes more cell wall damages than ME (Fig. 6a,b). In ME process, a heated solvent slowly diffuses through the material, dissolving and carrying away target compounds, therefore little destruction of sample microstructure occurs (Fig. 6a).

Table 1
Characterization of phenolic compounds in the extract of burdock leaves using UPLC with PDA and electrospray ionization MS^n detection.

Peak number	t_R	Precursor ion $[M-H]^-$ (m/z)	Product ions (m/z)	MW	λ_{max} (nm)	Identification
1	3.51	301	179 245	302	255	Quercetin
2	6.33	515	191 349	516	295	Cynarin
3	6.50	121	105	122	236	Benzoic Acid
4	7.44	447	147	448	355	Quercitrin
5	7.62	179	135	180	243 322	Caffeic acid
6	7.80	285	133	286	255	Luteolin
7	8.08	353	191	354	325	Chlorogenic acid
8	10.63	163	119	164	310	p-Coumaric acid
9	10.90	533	465	534	278	Arctiin
10	18.50	609	301	610	256 354	Rutin

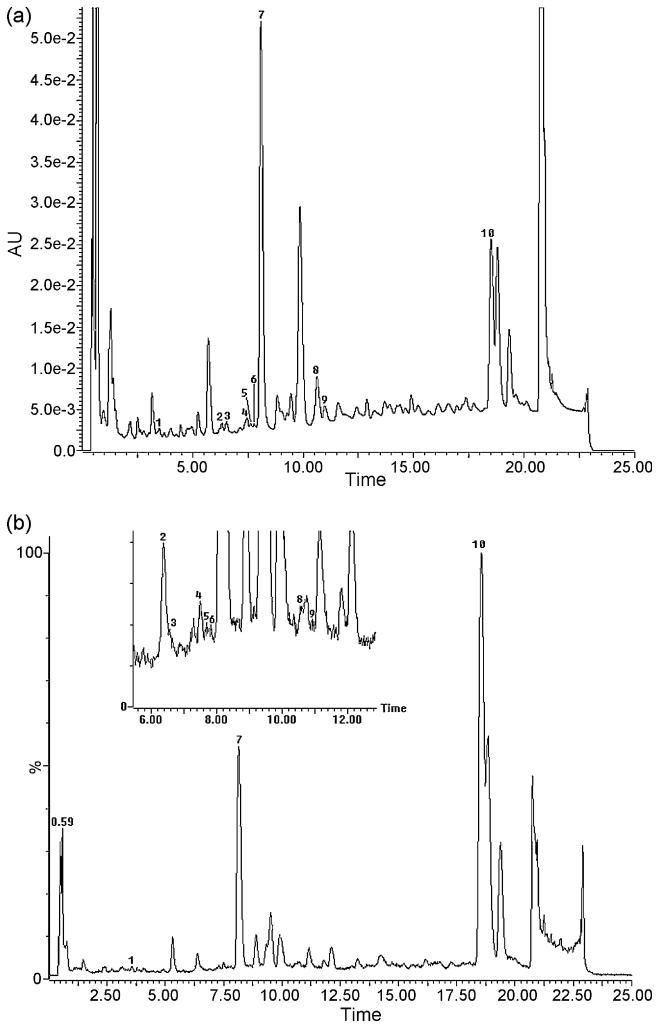

Fig. 7. Chromatograms of burdock leaves extract. (a) UPLC–PDA chromatogram at 280 nm. (b) TIC chromatogram in negative ion mode.

3.6. Identification of phenolic compounds

The chromatogram TIC and UV of an extract of burdock leaves was shown in Fig. 7. Peak identification was performed by comparing retention times (t_R), UV–vis spectra and mass spectra (Table 1) with those of reference standards and literature data. Peak 1 exhibiting an $[M-H]^-$ ion at *m/z* 301 with the λ_{max} of 255 and a t_R of 3.51 (same as those of standard quercetin), was identified as quercetin. It also showed the release of the predominant fragment ion at *m/z* 179 and 245. These results are in agreement with those reported in the literature [21]. Peak 2, with the t_R of 6.33 and λ_{max} of 295, was identified as cynarin and the $[M-H]^-$ peak of it was observed at *m/z* 515. Its characteristic fragment ions, such as *m/z* 191 and 349, were in consistent with those reported in the literature [22,23]. Similarly, Peak 4 was identified as quercitrin [24,25]. Peak 3 yielded $[M-H]^-$ at *m/z* 121, with λ_{max} of 236 and t_R of 6.50. Compared with the standard benzoic acid, it was identified as benzoic acid [26]. Peak 5, with the t_R of 7.62, was identified as caffeic acid (λ_{max} 243, 322) and the $[M-H]^-$ peak of caffeic acid was observed at *m/z* 179. Its characteristic fragment ions, such as *m/z* 135, were also identical with those of the standard and those reported in the literature [27,28]. Peak 6 yielding $[M-H]^-$ at *m/z* 285 was identified as luteolin with the t_R of 7.80 and λ_{max} of 255. Its fragment ions at 133 were found, similar to those reported elsewhere [29]. The seventh peak was identified as chlorogenic acid with λ_{max} of 325 and t_R of 8.08. The $[M-H]^-$ peak of 353 (along with the fragment ions at 191) was similar to those reported by Weisz [27]. The eighth peak yielding $[M-H]^-$ at *m/z* 163 was identified as p-coumaric acid with the t_R of 10.63 and λ_{max} of 310 which are same as those of the standard p-coumaric acid. Its fragment ions at 119 were found, similar to those reported elsewhere [28]. In agreement with Liu et al. [6], the fragmentation patterns of peak 9 could be assigned to arctiin. It exhibited an $[M-H]^-$ parent ion at *m/z* 533. CID of that component led to the formation of a predominant fragment at *m/z* 465. Identification of compound 10 (rutin) was based on the comparison of its retention time and UV spectra with those of reference substance (standard rutin). Peak assignment was confirmed by their mass spectrometric behaviour exhibiting $[M-H]^-$ ions at *m/z* 609, with fragment ions at 301 [30]. Quercetin, caffeic acid, chlorogenic acid, arctiin and rutin were previously reported from burdock leaves [6,7]. The occurrence of benzoic acid and p-coumaric acid in burdock leaves was never reported.

Table 2
Recoveries for UMAE of phenolic standards[a].

Phenolic compound	Recovery	
	30 s	120 s
Quercetin	97.6 ± 2.1	97.7 ± 4.6
Cynarin	102.8 ± 3.5	102.4 ± 2.4
Benzoic acid	99.7 ± 1.6	100.7 ± 1.4
Quercitrin	103.1 ± 3.7	102.6 ± 3.2
Caffeic acid	97.9 ± 4.2	97.1 ± 2.2
Luteolin	98.2 ± 1.9	99.3 ± 2.1
Chlorogenic acid	102.8 ± 2.5	103.2 ± 3.0
p-Coumaric acid	101.0 ± 1.8	98.0 ± 2.1
Arctiin	103.7 ± 4.7	96.2 ± 3.8
Rutin	96.9 ± 3.6	97.1 ± 4.3

[a] Mean ± SD for recoveries relative to the reference.

The stability of phenolic compounds was evaluated [17] under the optimized UMAE conditions (500 W of microwave power, 50 W of ultrasonic power, twice of extraction and 30 s of each extraction) and under the conditions with longer extraction time (120 s of each extraction). The solvent was also 70% ethanol. The results were presented in Table 2. The mean recoveries (relative to the reference) of the extracted phenolic compounds were around 100.3% and 99.5% when the extraction time was 30 s and 120 s. All the phenolic compounds were found stable under the conditions used.

After checking the behaviour of the phenolic compounds under the UMAE conditions, the recovery of these compounds from real samples was determined under the same conditions as those applied to the standards. Burdock leaves were spiked with different amounts of the standards. The sample was spiked with phenolics for 1.5 h before extraction. Recovery was obtained by dividing the difference between the phenolics amount in the spiked sample and the original amount in sample by the amount of added standards. The results for each phenolic compound were shown in Table 3. The mean recoveries of the phenolic compounds were around 99.1% and

Table 3
Recoveries for UMAE of phenolics from spiked burdock leaves[a].

Phenolic compound	Recovery	
	30 s	120 s
Quercetin	96.3 ± 3.8	103.0 ± 4.1
Cynarin	102.1 ± 4.2	98.1 ± 5.8
Benzoic acid	98.3 ± 2.3	97.8 ± 2.7
Quercitrin	101.2 ± 3.6	96.7 ± 3.4
Caffeic acid	97.8 ± 4.7	96.3 ± 3.9
Luteolin	96.6 ± 1.9	102.2 ± 2.6
Chlorogenic acid	96.1 ± 2.7	104.3 ± 4.3
p-Coumaric acid	98.0 ± 2.6	97.6 ± 2.5
Arctiin	103.4 ± 5.1	97.2 ± 2.2
Rutin	101.8 ± 5.2	96.3 ± 3.6

[a] Mean ± SD for recoveries relative to the amount spiked ($n = 3$).

98.9% when the samples were extracted for 30 s and 120 s twice. Therefore the technique of UMAE is considered to be viable for the extraction of these compounds.

4. Conclusion

The simultaneous ultrasonic and microwave assisted extraction of phenolic compounds was first evaluated. The results showed that UMAE has an obvious predominance for the extraction of phenolic components. The destruction of sample microstructure was more obvious in the process of UMAE, as seen in the SEM images. It is believed that UMAE has a great potential for the industrial extraction of phenolics, and the results obtained in this study would have implications for the natural phenolic industry. Due to the high efficiency and the dramatically short extraction time, UMAE also provides a new sample preparation alternative for characterization and determination of the phenolic compounds from plants.

The phenolic composition of the extract from burdock leaves was identified by UPLC–MS/MS based on retention time, UV and MS spectra compared with those of authentic compounds or literature data. The compounds were quercetin, cynarin, benzoic acid, quercitrin, caffeic acid, luteolin, chlorogenic acid, p-coumaric acid, arctiin, rutin. Chlorogenic acid, rutin and benzoic acid were found to be the main components. The high complexity of the phenolic compounds present indicates that burdock leaf is interesting for bioactivity studies.

Acknowledgments

We thank Xuzhou Wangda Farm and Sideline Products Co., Ltd. for providing the burdock leaves for this study. This research was partly supported by PCSIRT0627.

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