Application of Mechanochemistry-Assisted Treatment to Aqueous Extraction of Isofraxidin from *Acanthopanax senticosus*

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Abstract

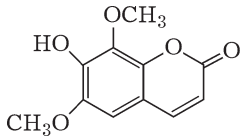
Mechanochemistry-assisted treatment (MT) was applied for the first time to extract isofraxidin from *Acanthopanax senticosus* using water as solvent. Six extraction variables, *i.e.* solvent, Na_2CO_3 content, sample granularity, extraction time, liquid/solid ratio (ml/g), extraction temperature were investigated with respect to the yield of isofraxidin. The most favourable conditions were obtained by using superfine powered plant material (D_{95} of 47 μm) with Na_2CO_3 content of 0.5 mass %, extracted with water (liquid/solid ratio of 20 : 1, ml/g) for 3 min at 25 °C. The results obtained using the optimized conditions were compared to those with heat reflux extraction. MT extraction was found to increase the yield of isofraxidin considerably while excluding organic solvents and reducing both extraction time and temperature.

INTRODUCTION

Secondary metabolites constitute biologically and chemically interesting group of substances extracted from the plant kingdom. Their utilization is widespread, ranging from perfumes and fragrances, to spices and medicines. Extraction is the first essential step for the isolation and purification of many secondary metabolites from plants. Time and solvent consuming multistage treatments required in traditional extraction techniques make their commercialization problematical. In the last decade, there has been an increasing demand for new extraction techniques enabling shortening extraction time and reduction of organic solvent consumption [1–3].

Accordingly, a primary goal of mechanochemistry-assisted treatment (MT) extraction research is the development of more efficient and eco-friendly alternative methods. Early experimental mechanochemistry was based on investigations by Carey Lea [4, 5], who, toward the end of the 19th century, described the decomposition of silver and mercury halides during attrition in a mortar. Several applications utilizing MT for the synthesis of organic or inorganic compounds [6–8], denaturalization of cellulose [9], discomposing of organic wastes [10] *etc.* were published during the last few years, but very few applications have been published in the phytochemical and pharmaceutical areas.

The development of mechanical devices plays an especially important role in MT application. Most applications of MT described so far have employed ball mills of various kinds, planetary and vibration devices, Spex mills. In these devices, mechanical action occurs due to pressure and shear. The technique of MT extraction includes mechanochemical processing of powered mixtures of plant material and a specially selected solid phase. Grinding is accompanied by an increase in the total contact surface area, as well as the destruction of cell covering and walls, which ensures that complete chemical transformations will occur between chemical reagents and substances in the plant cell [11]. Therefore, the role of MT is not only to increase the effective surface area of the mixture components but also to transform the target substances chemically into forms that



Scheme 1. Molecular structure of isofraxidin (7-hydroxy-6,8-dimethoxycoumarin).

are more soluble in water or other solvents. Korolev *et al.* (2003) presented the first use of MT for the preparation of water-soluble forms of triterpene acids from fir needles. The aqueous extraction yield of triterpene acids increased substantially from 2.9 to 3.9 % by using preliminary mechanochemical treatment [11]. O. I. Lomovsky *et al.* (2003) reported the application of MT for the extraction of phytoecdysteroids from *Serratula coronata L.* using saccharose as "water-soluble collectors". The yield of phytoecdysteroids increased to 20–25 % [12]. With the wide availability of appropriate mechanical devices, MT extraction of plant bioactive compounds seems as promising directions because of its possibility of drastic decrease in technological costs and organic solvents exclusion.

Acanthopanax senticosus (*Eleutherococcus senticosus* or Siberian Ginseng), is well known to be prophylactic for various diseases such as chronic bronchitis, hypertension and ischemia [13] and is also effective for reducing many kinds of stress [14] or fatigue [15]. Isofraxidin – 7-hydroxy-6, 8-dimethoxycoumarin (Scheme 1) known as an important bioactive components of *Acanthopanax senticosus* is reported to have good anticancer, anti-inflammatory and cholagogic effects [16]. Heat reflux extraction is usually employed for the extraction of isofraxidin, which is time consuming and requires a large quantity of organic solvent [17]. According to conventional extraction practice, isofraxidin, is a coumarin, the acidic site (hydroxyl group) of which could be neutralized by alkali, yielding a salt form of isofraxidin that is soluble in water. By regulating the pH value of aqueous extract, salt form of isofraxidin could return to its original structure [18]. According to the conventional theory, isofraxidin could potentially be transformed by MT into a water-soluble form.

Although mechanochemical process is used in many fields, little information is available about the application of MT to the extraction of Chinese traditional herbs. This paper offers a new extraction method based on MT, the main purpose of which is to convert isofraxidin to a water-soluble form. Various parameters affecting the yield of isofraxidin were investigated. The method is compared with heat reflux extraction with respect to their effect on extraction efficiency.

EXPERIMENTAL

Reagents and materials

Analytical grade ethanol, Na_2CO_3 were purchased from Tianjin Siyou Biomedical Technology Ltd. (Tianjin, China). HPLC grade acetonitrile and methanol were obtained from Tedia (USA). HPLC grade H_3PO_4 and acetic acid were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ultrapure water was purified using a MILLI-Q RG (Millipore, Bedford, MA, USA).

Authentic roots and caudexes of *Acanthopanax senticosus* were purchased from local medicine market (Dalian, China), moisture content, 9.26 %, and stored at room temperature ((20±3) °C) until used. Standard isofraxidin (98 % purity) was purchased from Chinese Medical and Biological Products Institute (Beijing, China).

Apparatus

Mechanical treatment grinding activation was carried out in WZJ(BFM)-6J vibrational mill (Billionpower Tech&Engineering Ltd., China) with water-cooled drums (drum volume, 1.2 l; grinding bodies, steel rods; diameter, 18 mm; length, 174 mm; the ratio of mass of milling bodies to the load mass, 100 : 1). Sample granularity was determined with laser diffraction particle analyzer (LS100 Q, Beckman-Coulter Corp., USA).

A conventional heat reflux extraction system was employed in order to compare with the results obtained by MT extraction.

Sample preparation

The plant raw material of *Acanthopanax senticosus* was performed under various conditions: no mechanical treatment; mechanical activation, but no chemical reagents and mechanical activation in the presence of chemical reagents. Accordingly, sample No. 1 (see Fig. 2) consisting of plant material only was crushed by a laboratory pulverizer and sieved with 60 mesh screen. Sample No. 2 (consisting of plant material only) and sample No. 3 (consisting of plant material and Na_2CO_3) were prepared by mechanical activation in WZJ(BFM)-6J mill.

MT extraction

1.0 g of prepared sample was accurately weighted out and mixed with the respective volumes of solution according to a pre-designed experimental trial. The liquid/solid ratio (ml/g) (10 : 1–100 : 1) and extraction time (2–20 min) with different temperature (15–45 °C) were investigated for the extraction of *Acanthopanax senticosus* with different Na_2CO_3 content and granularity. Sample extracts were further clarified by centrifugation at 8000 rpm for 10 min to separate out the fine particulates. Supernatant was collected, acidified to pH 4.5, and evaporated in a rotary evaporator under vacuum at 40 °C. The residue was rinsed twice with 5 ml water and evaporated to dryness, then dissolved in 20 ml 80 vol. % aqueous ethanol, filtrated through 0.45 μm microporous membrane and directly used for HPLC analysis.

Heat reflux extraction

Heat reflux extraction was performed between 80 and 100 °C at azeotropic temperature of the solvents (liquid/solid ratio was 50 : 1, ml/g) in a water or oil bath with reflux apparatus using the same amount of sample in MT extraction. The extracts were filtered through 0.45 μm microporous membrane.

Quantitative analysis

HPLC quantitative analysis was performed using an Agilent 1100 HPLC system with UV detection at 344 nm (25 °C). The UV spectra of HPLC peaks were obtained using the online diode array detector. One analytical HPLC method (Method I) and one semipreparative HPLC method (Method II) were used. For Method I, eluent was mobile phase A (prepared by adding 1 ml H_3PO_4 into 1 l of water): acetonitrile (20 : 80, v/v) at 1 ml/min. The injection volume was 10 μl. A Kromasil C18 (250 mm × 4.6 mm, *i.d.* 5 μm) was used. The yield was determined as μg (isofraxidin)/g (dry material) in triplicate. For Method II, the conditions were the same as for Method I except that a column of the same type with a larger diameter and larger particle size (15 cm × 1 cm, 5 μm) was used; accordingly, the flow rate was scaled up to 4.8 ml/min and the injection volume was 100–300 μl. Column temperature was 25 °C for both Methods I and II. The HPLC peaks of samples were identified by comparing their retention time with that of standard, which was determined under the same chromatographic conditions (Fig. 1). Calibration curve for isofraxidin was strictly linear (r^2 = 0.9992) in the concentration range from 40 to 320 ng per injection. The yield of isofraxidin was calculated using the linear equation.

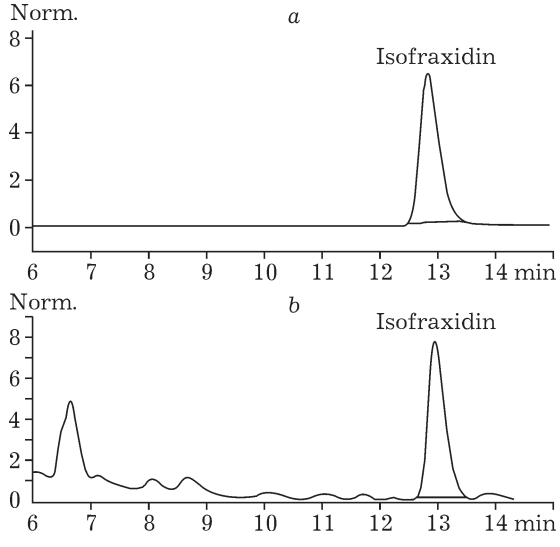


Fig. 1. Chromatograms of isofraxidin: *a* – standard, *b* – sample.

MS analysis

In order to maintain good bioactivity, the procedure requires extracting without changing the chemical form (molecular structure) of the target compounds in herbs. MS was employed to analyze the mass of target compound prepared from sample No. 3. MS analysis was performed on a Hewlett Packard (HP, USA) Series 1100 MSD mass spectrometer with an API-ES source and MSD mass analyzer in the positive ion mode. The extract of isofraxidin was isolated by semipreparative HPLC.

RESULTS AND DISCUSSION

Effect of extracting solvent

Solvent choice is dictated by the solubility of analytes of interest and by the interaction between solvent and matrix. Solvents of different polarity were tested, ranging from ethanol to water with different samples (Fig. 2). Water gave sample No. 3 the highest yield of isofraxidin, while ethanol presented the lowest. As used for the conventional extraction of isofraxidin – 60 vol. % aqueous ethanol and 40 vol. % aqueous ethanol gave the best yield for samples Nos. 1 and 2, respectively. Both samples showed the lowest yield with pure water. These results confirmed that Na_2CO_3, preliminary treated with *Acanthopanax senticosus* by MT, could neutralize the acidic parts of isofraxidin, converting isofraxidin into a water-soluble form. Without MT, pure water was not sufficient to extract isofraxidin from *Acanthopanax senticosus*. Water was selected as the extracting solvent for further investigations of MT samples (sample No. 3).

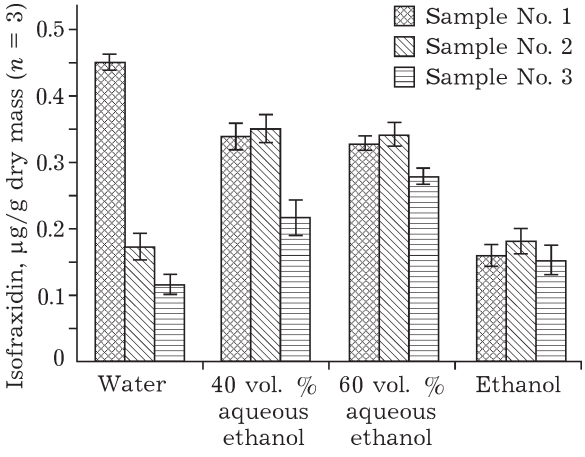


Fig. 2. Effect of different solvents on the extraction of isofraxidin by MT and heat reflux extraction. The experimental conditions were: samples Nos. 2 and 3 preliminarily treated by WZJ(BFM)-6J mill with a D_{95} of 47 μm and extracted according to MT extraction procedure; extraction time, 3 min; temperature, 25 °C; liquid/solid ratio, 20 : 1(ml/g). Na_2CO_3 content of sample No. 3, 1 mass %. Sample No. 1, extracted according to heat reflux extraction for 3 h.

Effect of Na_2CO_3 content

The minimal content of alkali depends on the kinetics of the mechanochemical process, as well on the content of isofraxidin in plant material. In order to investigate the effects of reagent content in *Acanthopanax senticosus* raw material, MT extraction of sample No. 3 was carried out with Na_2CO_3 content of 0.5, 1, 2, 3, 5, 6, 7 and 8 mass %, respectively. As shown in Fig. 3, the yield of isofraxidin kept steady with an increase in Na_2CO_3 content from 0.5 to 3 mass % and started to decrease slightly when that was higher than 5 mass %. The highest yield was obtained with the Na_2CO_3 content of 0.5 and 1.0 mass %, conforming that in the process of MT extraction of isofraxidin from *Acanthopanax senticosus*, a Na_2CO_3 content of 0.5 mass % was sufficient to extract quantitatively the investigated analytes.

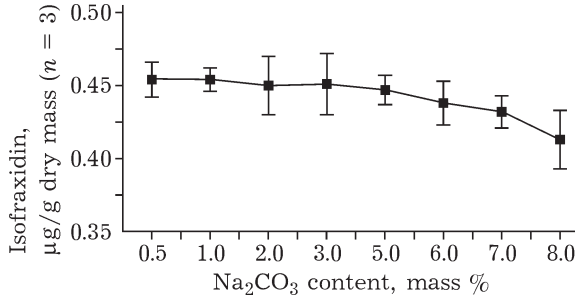


Fig. 3. Effect of Na_2CO_3 content on the extraction of isofraxidin by MT. The experimental conditions were: sample No. 3, preliminarily treated by WZJ(BFM)-6J mill with a D_{95} of 47 μm, and extracted according to MT extraction procedure; extracting solvent, water; extraction time, 3 min; temperature, 25 °C; liquid/solid ratio, 20 : 1 (ml/g).

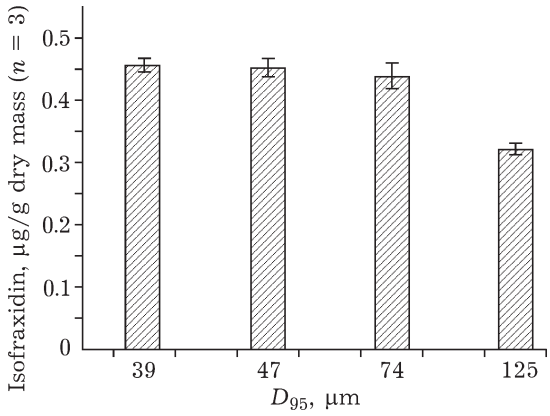


Fig. 4. Effect of granularity on the extraction of isofraxidin by MT. The experimental conditions were: sample No. 3, preliminarily treated by WZJ(BFM)-6J mill, extracted according to MT extraction procedure; Na_2CO_3 content, 0.5 mass %; extracting solvent, water; extraction time, 3 min; temperature, 25 °C; liquid/solid ratio, 20 : 1 (ml/g).

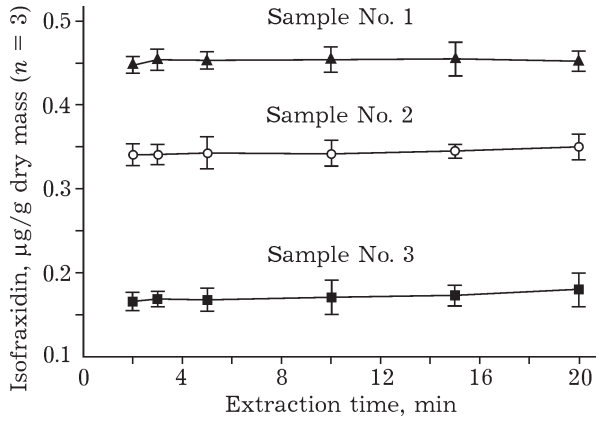


Fig. 5. Effect of extraction time on the extraction of isofraxidin by MT and heat reflux extraction. The experimental conditions were: samples Nos. 2 and 3, preliminarily treated by WZJ(BFM)-6J mill with a D_{95} of 47 μm and extracted according to MT extraction procedure; extracting solvent, water (for sample No. 3), 60 vol. % aqueous ethanol (for sample No. 2); temperature, 25 °C; liquid/solid ratio, 20 : 1(ml/g). Na_2CO_3 content of sample No. 3, 0.5 mass %. Sample No. 1, extracted with 60 vol. % aqueous ethanol according to heat reflux extraction.

Effect of sample granularity

Investigations were carried out to study the effect of sample grinding. During the procedure of MT, finely grinded powder required longer treating period that provided more chances for chemical transformation of target compounds. Besides, samples with smaller particle size were more easily extracted because of the larger surface area available to provide contact between the sample and the solvent. Figure 4 shows that the granularity (D_{95}) had a strong influence on the efficiency of extraction, with enhanced extraction being obtained with samples of smaller D_{95} values. When D_{95} was smaller than 47 μm, the yield had no obvious increase, confirming that a D_{95} of 47 μm was sufficient to extract isofraxidin from *Acanthopanax senticosus*. The D_{95} of 47 μm was therefore chosen for all further investigations.

Effect of extraction time

The influence of extraction time on the extraction of three different samples was studied. Figure 5 shows that 3 min was sufficient to bring about the quantitative extraction of isofraxidin from samples treated by mechanical procedure (samples Nos. 2 and 3). For sample No. 1 (sieved with 60 mesh screen and treated by heat reflux extraction method), increasing the extraction time to 20 min did not improve the yield of isofraxidin significantly. Compared with conventional method, MT extraction of isofraxidin from *Acanthopanax senticosus* is a very fast process. An extraction time of 3 min was chosen for further investigations of MT samples (sample No. 3).

Effect of liquid/solid ratio

The effect of altering the liquid/solid ratio (ml/g) in the procedure of MT extraction of sample No. 3 was also investigated. When the liquid/solid ratio (ml/g) was varied from 10 : 1 to 100 : 1, no significant increase in yield could be observed, confirming that solubility is not a limiting factor in the investigated interval. Considering the difficulties of evaporating solvent with higher liquid/solid ratio, 20 : 1 was chosen to extract isofraxidin by MT extraction.

Effect of extraction temperature

Similar experiments were carried out in order to investigate the effect of extraction temperature during the procedure of MT extraction. The temperature was varied from 15 to 60 °C, no significant influence on the yield of

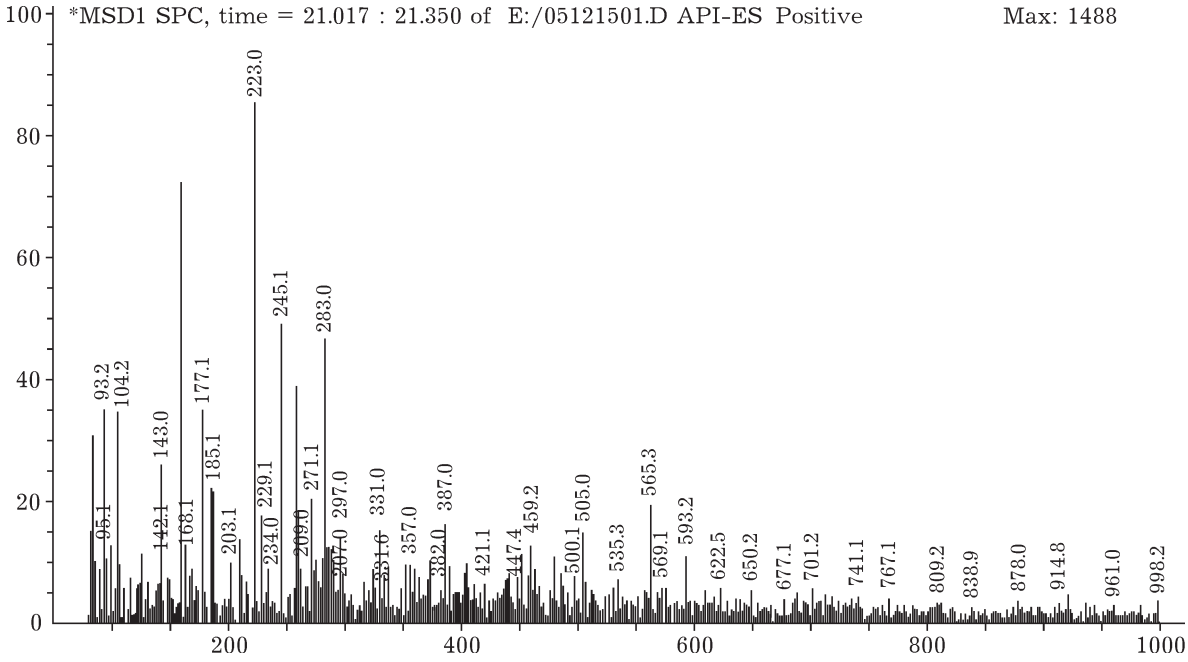


Fig. 6. Mass spectrum analysis of isofraxidin extract from *Acanthopanax senticosus* treated by MT (sample No. 3). The analysis was performed on a Hewlett Packard (HP, USA) Series 1100 MSD mass spectrometer with an API-ES source and MSD mass analyzer in the positive ion mode.

iso-fraxidin was observed. The results confirm that extraction temperature had no influence on extraction; room temperature (25 °C) was sufficient to extract the investigated analytes.

MS analysis

The extract of sample No. 3 prepared by MT procedure and semipreparative HPLC was analysed by MS. The ion chromatogram is presented in Fig. 6. The main ion for most of these peaks was the ion with m/z = 223, which corresponds to the pseudomolecular ion $[M + H]^+$. The ion with m/z = 245.1 corresponds to $[M + Na]^+$. These results show that the molecular mass of the compound separated by semipreparative HPLC was identical to that of isofraxidin (M = 222), suggesting that MT extraction could not change the molecular structure of isofraxidin.

Chemical stability of isofraxidin under the applied conditions

To assure that the yield of isofraxidin was not interfered by matrix effect, MT extraction was carried out adding known amounts of standard solution of isofraxidin at the beginning of extraction under the optimized extraction procedure outlined above: no degradation occurred and the average recovery was 93.6 % (n = 3).

Comparing MT extraction with heat reflux extraction

Heat reflux extraction was conducted according to the conventional method: both samples Nos. 1 and 2 were extracted by 60 vol. % aqueous ethanol with the liquid/solid ratio (ml/g) of 50 : 1, extracted for 12 and 3 h, respectively. Increasing the extraction time to 24 h did not improve the yield of both samples.

The present study showed the main advantages of MT over heat reflux extraction. They were mainly associated with the improved yield of isofraxidin (0.454 μg/g *vs.* 0.348 or 0.341 μg/g), organic solvent exclusion (pure water *vs.* aqueous ethanol) and extraction time (3 min *vs.* 3 or 12 h) as shown in Table 1.

CONCLUSIONS

Mechanochemistry-assisted treatment on the extraction of plant bioactive compounds, as a new extraction method, is used to extract iso-

TABLE 1

General comparison between MT and heat reflux for the extraction of isofraxidin from *Acanthopanax senticosus*

Parameter	Heat reflux extraction		MT extraction,
	Sample No. 1	Sample No. 2[a]	sample No. 3[b]
Sample mass, g	1	1	1
Extraction time, h	12	3	0.05
Total handing time, h	14	4.5	1
Solvent used	60 vol. % aqueous ethanol	60 vol. % aqueous ethanol	Water
Solvent volume, ml	50	50	20
Yield of isofraxidin[c], μg/g dry mass	0.341±0.011	0.348±0.010	0.454±0.021

[a] D_{95} = 47 μm.

[b] D_{95} = 47 μm, Na_2CO_3 content of 0.5 mass %.

[c] n = 3; significant effects ($P < 0.01$) of yield of isofraxidin by MT and heat reflux extraction.

fraxidin from *Acanthopanax senticosus*. The optimized extraction conditions are: extracting solvent, water; Na_2CO_3 content, 0.5 mass %; D_{95} = 47 μm; extraction time, 3 min; temperature, 25 °C; liquid/solid ratio, 20 : 1 (ml/g). The extraction parameters determined result in distinct advantages of MT extraction over conventional heat reflux extraction. Ethanol used in the conventional method was replaced with less hazardous and less expensive water; the yield of isofraxidin increased substantially – from 0.341 or 0.348 μg/g to 0.454 μg/g ($P < 0.01$). The proposed MT extraction employed low temperature and shorter extraction time which helped in minimizing the cost and improving the extracting efficiency. Based on our results, it can be stated that the proposed MT technique is a simple, eco-friendly, efficient and reliable extraction method that can be used in phytochemical and pharmaceutical fields.

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