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Contributed Paper

Kinetics of Ultrasound-assisted Extraction of Flavonoids and Triterpenes and Structure Characterization of Chinese Northeast Black Bee Propolis

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ABSTRACT

Flavonoids and triterpenes from Chinese northeast black bee propolis were extracted using pulsed ultrasound-assisted extraction. The influences of extraction parameters such as ethanol concentration, liquid-solid ratio, temperature, time, and ultrasonic on-time/off-time on the extraction yield were investigated. The kinetics of the extraction process was studied. The second-order kinetic models were applied to fit the experimental data. Effects of ultrasound on microstructure of propolis were studied by Scanning Electron Microscope and Atomic Force Microscope. The results showed the great influence of extraction parameters on the extraction yield. Optimum extraction conditions were determined as: 80% ethanol concentration, 30:1 mL/g ratio, 53 °C, 16 min and 10s/5s on-time/off-time. Under these conditions, 60.34% of flavonoids and triterpenes extraction yield was achieved. Scanning electron microscope and atomic force microscope analysis indicated that the propolis matrix structure changed evidently during ultrasound. GC-MS and LC-MS analyses showed that terpenes and flavonoids content were consistent by both extraction methods, thus UAE resulted to be more efficient compared to conventional extraction by achieving extraction in shorter time. Kinetic predictions for yields distributions of ultrasound-assisted extraction are highly in agreement with the experimental data, which implies that the proposed model can be used to predict the mechanisms of the extraction process.

Keywords: propolis, ultrasound-assisted extraction, kinetics, flavonoids, triterpenes

1. INTRODUCTION

Propolis is a resinous substance gathered by bees *(Apis mellifera)* from tree buds, saps, resins, mucilage, lattices and other botanical sources [1]. Due to its waxy and mechanical properties, propolis is used as cement or glue for sealing cracks, and to keep moisture and temperature in the hive all year [2]. It has been used by humans as folk medicine since ancient times for its pharmaceutical properties [3]. So far, it is known to have antioxidant, antibacterial, antifungal, anti-inflammatory, anti-trypanosomal and anti-hepatotoxic properties [3-5]. Recently, propolis has gained popularity worldwide due to its potential contribution to human health [6]. Moreover, there is an increasing interest on its bioactivities [7]. Because of its low yield and high bioactive properties, propolis is very valuable, having an increasing price every year.

Propolis contains more than 300 compounds [8]. Although its composition depends on the vegetation at the collection site, it usually contains polyphenols, terpenoids and amino acids [9]. Bioactive compounds in propolis may be as high as 70%. Polyphenols constitute 58% of this amount. Out of this 58%, 20% are flavonoids [10]; while triterpenes are the main constituent of terpenoids [11]. These compounds are the main source of bioactivities [7, 12]. It is known that the best extraction method for compounds in propolis is solid-liquid extraction. The extraction yield is influenced by the solvent, solvent concentration, temperature, liquid-solid ratio and time [6, 11, 13].

Previous studies showed ethanol as the most efficient solvent for extraction of flavonoids and terpenoids [2, 11, 14], while different extraction methods have been used for extraction of bioactive compounds in propolis [11, 15]. Additionally, solid-liquid extraction of bioactive components from herbs was more amenable to ultrasound treatment [16]. Ultrasound-assisted extraction (UAE) is considered as a non-thermal physical-processing technology that offers advantages of simple operation, low energy requirement, and high efficiency [17]. Ultrasound generates bubbles in liquids causing the cavitation phenomena [18], enhancing the penetration of solvent into the material matrix and recovery rate of compounds from matrix to solvent [17, 19]. This method have shown higher extraction yield of albumin from rambutan seeds [20] and camptothecin from *Nothapodytes nimmoniana* [18] compared to that of conventional extraction respectively. However, there is still a gap in knowledge about the optimization of UAE parameters for the extraction of flavonoids and triterpenes from propolis. Mathematical modeling of kinetic parameters is an important engineering tool used to optimize the extraction conditions reducing energy, time and reagent consumption [21]. However, the kinetics of ultrasound-assisted solid-liquid extraction of flavonoids and triterpenes from propolis has not been reported.

Northeast black bee (NBB) (*Apis mellifera* ssp.) is a species of honeybee distributed in northeast China's Heilongjiang Province. It is generally accepted that this species was originated from the hybridization of the European black bee with Caucasian bees through natural selection and artificial cultivation in China [22]. NBB long-term production is adapted to the climate and nectar sources of Northeastern China. It has a strong ability to produce honey from bulk and sporadic nectar, and strong resistance to diseases and low temperatures (-35°C) during winter [23]. All physiological indexes of NBB are certainly superior to the

four famous bee species in the world. Therefore, NBB is an important species of bee for propolis production in the temperate region. However, few articles about the study and utilization of NBB propolis have been reported.

Therefore, the aims of this research were (1) to study systematically the influences of process factors such as ultrasonic on-time/off-time, ethanol concentration, temperature, liquid-solid ratio and time on the total extraction yield of flavonoids and triterpenes; (2) and to study the kinetic mechanisms of the extraction process of these compounds from northeast black bee propolis (NBBP). The kinetic model to be developed will attempt to predict the extraction process, and optimize the extraction parameters of flavonoids and triterpenes in propolis. It is expected that the results of this research will contribute in improving the efficiency of the overall extraction process.

2. MATERIALS AND METHODS

2.1 Materials

NBBP was obtained from Senhai black bee cooperatives, (Raohe, Heilongjiang, China). HPLC-grade acetonitrile (ACN), acetic acid, vanillin, rutin, ethanol, methanol, caffeic acid, ferulic acid, chrysin, benzaldehyde, isorhamnetin, p-coumaric acid, kaempferol, galangin, benzoic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of standard compounds was always higher than 98%. Water was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA). All other reagents were of analytical grade purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2 Preparation of NBBP Sample

NBBP sample was stored at -20°C once received. Then the frozen sample was rapidly ground in a mortar to obtain homogenous powder and passed through a sieve of 40 mesh, and stored again at -20 °C until analysis.

2.3 Conventional Ethanolic Extract of Propolis (CEEP)

The conventional extraction was carried out using a thermostat water bath with stirrer. NBBP was dispersed in 90% ethanol to obtain a liquid-solid ratio of 25:1 mL/g. Samples were stirred at 40 °C for 40 min. At the end of the extraction, the mixtures were centrifuged at 4000 rpm for 10 min (TGL-16M high speed desktop refrigerated centrifuge, Changsha Xiangyi Centrifuge Instrument Co., Ltd.). The supernatant was then vacuum-filtered and the solvent was added to final volume of 250 mL. Part of the extract was ready for detection and the rest was concentrated under vacuum at 40°C in a rotary evaporator (R-210 BUCHI Labortechnik AG, Flawil, Switzerland) to remove the solvent, and then dried in a vacuum drying oven (DZF-6050 Shanghai Yiheng Scientific Instrument Co., Ltd.). All measurements were conducted in triplicate.

2.4 Ultrasound-assisted Ethanolic Extract of Propolis (UAEEP)

Five extraction parameters (ultrasonic on-time/off-time, ethanol concentration, temperature, liquid-solid ratio and time) were studied. Parameters were chosen based on preliminary experiment (data not shown). A weighed amount of NBBP samples (9.60, 8.00, 6.86, 6.00 and 5.33 g) were dispersed in 240 mL of ethanol solutions (60, 70, 80, 90 and 100%) in order to obtain liquid-solid ratios (25:1, 30:1, 35:1, 40:1 and 45:1 mL/g) respectively. Then the suspensions were loaded individually to a countercurrent-type ultrasound equipment

operated at 220 W and 40 kHz (Figure 1) (Jiangsu Jiangda Wukesong Biological Technology Co., Ltd., Jiangsu, China). The sample was kept at 30°C in a water bath (HH-S, Changzhou Jintan Co., Ltd.) while circulating under constant flow using a peristaltic pump (BT300, Baoding Leifu Co., Ltd., Hebei, China). To reduce the experimental errors caused by uneven power transfer, the sonic probe was dipped 10 mm into the solution. At the end of each extraction procedures, the mixtures were centrifuged at 4000 rpm for 10 min. The supernatant was then vacuum-filtered and the solvent was added to final volume of 250 mL. Extractions were executed at 30, 40, 50, 60 and 70°C for 5, 10, 15, 20, 25, 30, 35 and 40 min, respectively. Ultrasonic-pulsed on-time/off-time was 10s/1s, 10s/3s, 10s/5s, 10s/10s and 10s/15s, respectively. Part of the extract was ready for detection and the rest was dried following the same procedures of section 2.3. All measurements were conducted in triplicate.

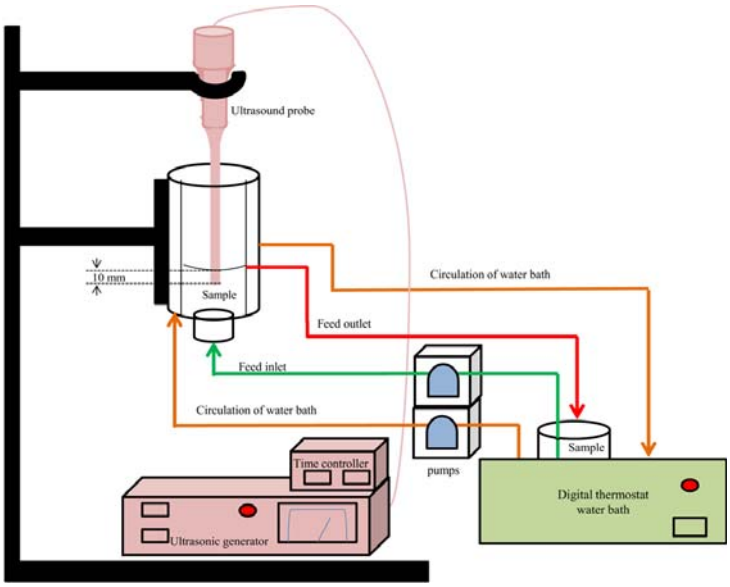

Figure 1. Schematic representation of the counter-current ultrasound equipment.

2.5 Determination of Flavonoids and Triterpenes Extraction Yield

Flavonoids were determined according to Al-Dhabi et al. [19] with some modifications. Briefly, 1 mL of sample was diluted with 4 mL of 80% (v/v) ethanol and 0.3 mL of 5% (w/v) sodium nitrite solution, and stirred for 30s. 1 mL aluminum nitrate solution (10% w/v) was added to the mixture and incubated for 6 min. 10 mL of 4.3% (w/v) sodium hydroxide solution was added and shaken for 15 min. Absorbance was measured at 510 nm in a spectrophotometer (Cary 100 UV-Vis, Agilent Technologies, Santa Clara CA, USA). Rutin was selected as standard; concentrations of 0-25 µg/mL in 80% (v/v) ethanol were prepared to obtain the standard curve. The extraction yield was determined according to equation 1. All measurements were performed in triplicate.

$$FEY\ (\%) = \frac{X \times 10 \times 25 \times 250}{m \times 10} \qquad (1)$$

Where FEY is flavonoid extraction yield, X is flavonoid concentration (mg/mL)

obtained from the regression equation, m is mass of sample (g), 10 is dilution factor and 250 is total volume of the extract (mL).

Determination of triterpenes was evaluated according to Oludemi et al. [24] with some modifications. Shortly, 1 mL of sample was dissolved in 5 mL of methanol and centrifuged (4000 rpm, 10 min). Then 40 µL of the supernatant was transferred to a test tube then to a 50°C water bath until completely evaporated the methanol. Then 0.3 mL of fresh diluted 5% vanillin-glacial acetic acid and 1 mL perchloric acid were added, and transferred to a water bath at 60°C for 20 min. After cooling at room temperature, 10 mL glacial acetic acid was added. The sample solutions were then stirred for 30s and their absorbance measured at 548 nm using a spectrophotometer. Quantification of triterpenes was done using standard curve in the concentration range of 0.02-0.18 mg/mL using oleanolic acid as standard. The extraction yield was determined according to equation 2. All measurements were performed in triplicate.

$$TEY\ (\%) = \frac{X \times 10 \times 25 \times 250}{m \times 10} \quad (2)$$

Where TEY is triterpenes extraction yield, X is triterpenes concentration (mg/mL) obtained from the regression equation, m is mass of sample (g), 5 is dilution factor and 250 is total volume of the extract (mL).

After calculating the extraction yield of both compounds, the total extraction yield of flavonoids and triterpenes was calculated according to equation 3.

$$\text{Total extraction yield } (\%) = FEY + TEY \quad (3)$$

2.6 Modeling of the Extraction Kinetics of Flavonoids and Triterpenes

According to previous reports [25, 26] and pretest data projections [27], the second-order kinetic model is the most suitable for ultrasound-assisted solid-liquid extraction. Two-stage kinetic models were used to simulate the extraction process of flavonoids and triterpenes. The second-order kinetic model can be written as follows:

$$\frac{dC_t}{dt} = k(C_e - C_t)^2 \quad (4)$$

Where k is the second-order extraction rate constant ($L \cdot g^{-1} \cdot min^{-1}$), C_e is the equilibrium concentration of flavonoids and triterpenes ($g \cdot L^{-1}$) and C_t is the concentration of flavonoids and triterpenes at a given extraction time t ($g \cdot L^{-1}$).

Under the conditions $t=0 \sim t$ and $C_t=0 \sim C_t$, the integrated rate law for a second-order extraction can be written as an equation (5) or linearized equation (6):

$$C_t = \frac{C_e kt}{1+C_e kt} \quad (5)$$

$$\frac{t}{C_t} = \frac{1}{kC_e^2} + \frac{t}{C_e} \quad (6)$$

When t approaches 0, the initial extraction rate, h ($g \cdot L^{-1}\ min^{-1}$), can be written as:

$$h = kC_e^2 \quad (7)$$

After readjusting equations 6 and 7, C_t can be expressed as:

$$C_t = \frac{t}{(1/h) + (t/C_e)} \quad (8)$$

The kinetic parameters of h and C_e were obtained experimentally from the slope and intercept by plotting t/C_t against t for each extraction parameter. All graphs were obtained by Microsoft Excel 2010.

2.7 Scanning Electron Microscope (SEM)

Dried CEEP and UAEEP were finely grinded in a mortar, then a thin layer of the dried sample was mounted on a copper

sample-holder, using a double sided carbon tape and coated with gold of 10 nm thickness to make the sample conductive. The surface morphology analysis was perform using scanning electron microscopy (JSM-7001F Thermal Field Emission SEM, JEOL Ltd, Tokyo, Japan) at 3 kV as described previously [28].

2.8 Atomic Force Microscope (AFM)

The liquid extracts of CEEP and UAEEP were diluted 100 times with ethanol (80% v/v) then 10 µL of suspension was dropped on a freshly cleaved mica plate and dried in a hood at room temperature (25±1 °C). The surface topography study of NBBP extracts was carried out using an atomic force microscope (MultiMode 8, Bruker Inc., Karlsruhe, Germany) in PeakForce^{QNM} mode as described previously [29]. Height map and surface morphology were obtained and images were analyzed from area of 20 µm^2 of each sample by an image processing software Nanoscope Analysis v 1.4.

2.9 GC-MS

The analysis of terpenes in CEEP and UAEEP was carried out by headspace solid-phase microextraction (HS-SPME) combined with GC-MS. HS-SPME was performed using a manual holder and fiber (050/30um DVB/CAR/PDMS, 24Ga). 0.5 g of powdered CEEP and UAEEP was placed in a 15 mL flat-bottom headspace vial sealed with a magnetic crimp cap and PTFE/silicone septa (Supelco). The sample was heated for 10 min in a thermostatic bath at 60 °C. The SPME device was then inserted into the sealed vial by manually penetrating the septum and the fiber was exposed to the headspace for 30 min during the extraction time. After sampling, the SPME fiber was immediately inserted into the GC injector and thermally desorbed at 270 °C for 5 min in splitless mode. The GC-MS analysis was carried out according to Wang et al. [30] with some modifications on an Agilent 6890 GC linked to 5973NMSD mass spectrometer system equipped with an HP5-MS capillary column (60 m × 0.25 mm i.d., 0.32 µm film thickness). Samples were analyzed with the column held initially at 40 °C for 2 min, ramped at 10 °C mL/min to 60 °C, holding for 5 min, and then linearly increased to 280 °C with a 3 °C/min heating ramp, holding at 280 °C for 10 min. Helium was used as a carrier gas with a flow rate of 1.0 mL/min.

Full-scan EI (Electron Impact) spectra were recorded from 30-450 m/z (Mass/charge) with 2 scans per second. The temperature was 230 °C in the ionization source and the ionization voltage was 70 eV for EI-MS in positive mode. The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with those published in the literature. NIST05a.L library sources were used to match the identified components.

2.10 LC-MS

Flavonoids in the CEEP and UAEEP were determined by LC-MS LXQ (Thermo Fisher Scientific; Bremen, Germany) system, including on-line DAD (200-600 nm) and UV at 280 nm analysis. The injection volume was 20 mL. The LC was run on a reversed phase ODX C18-column (250 mm × 4 mm i.d., 5 mm particle diameter) and its temperature was maintained at 30 °C. The mobile phase was composed of solvent (A) 1.5% (v/v) acetic acid in water, and solvent (B) methanol, which were previously filtered and degassed. The solvent gradient started with 90% (A) and 10% (B),

reaching 78% (A) at 25 min, 35% (A) at 45 min, 50% (A) at 53 min, 90% (A) at 60 min and followed by the return to the initial conditions. The dried extracts (10 mg of each) were dissolved in 1 mL of 50% of ethanol prior analysis. All samples were filtered through a 0.2 mm Nylon membrane (Agilent). The flow rate was 0.8 mL/min and split out 180 mL/min to MS.

The experimental parameters were set as follows: Nitrogen above 99.99% purity and the nebulizer (N_2) pressure was 32 psi, the drying gas (N_2) temperature was 350 °C, t flow was 9 L/min and the skimmer voltage was 40 V. Data were acquired by Thermo LXQ LC/MS system. The mass spectrometer was operated in the full-scan in the m/z range 50~1000 and automatically performed with helium as the collision gas by using the SmartFrag function. The temperature was 100 °C in the ionization source and the ionization voltage was -3.2kV for EI-MS in negative mode. Quantification was established based on spectra of the analytical standard solutions showing the precursor ion.

3. RESULTS AND DISCUSSION

3.1 Effect of Ultrasonic On-time/ Off-time on Extraction Yield of Flavonoids and Triterpenes

Sample (8 g) was extracted with ethanol solution with different on-time/off-time (section 2.4) by keeping other conditions fixed: 50 °C, 80% ethanol concentration and 30:1 (Figure 2A). It is known that pulsed sonication is generally more energy-efficient than continuous mode [18]. Therefore, optimal on-time/off-time was critical for reducing the processing time and energy consumption. The yield increased from 1 to 15 min and then slightly decreased from 20 until 40 min for all on-time/off-time. 10s/1s and 10s/10s showed the lowest extraction yield compared to the others. The reason maybe was that a long interval of off-time caused the sample to receive less sonication than short interval within the same processing time which restricted the time for completing the mass transfer [20]. While a short interval of off-time could cause the formation of hydroxyl free radical and chemical decomposition of flavonoids and triterpenes, resulting in a decrease in extraction yield. The highest extraction yield (43.68%) was achieved with 10s/5s at about 15 min. These conditions influenced a maximum disruption of material matrix due to an ideal exposure of acoustic cavitation.

3.2 Effect of Ethanol Concentration on Extraction Yield

Sample (8 g) was extracted with ethanol solutions (section 2.4) by keeping other conditions fixed: 10s/5s, 50 °C and 30:1. Vapor pressure, viscosity and polarity are properties of the solvent involved in the extraction. Accordingly, ethanol has higher vapor pressure than water, which generates more bubbles in the cavitation phenomena [18]. The solubility of flavonoids and triterpenes is dependent on the solvent polarity and degree of polymerization [6] as well as their interaction with other constituents and formation of insoluble complexes. Given this fact, flavonoids are commonly extracted from plant materials with methanol, ethanol, water or their combination [31]. In the present study, ethanol combined with water was chosen as the extraction solvent due to previous studies that have shown it to be better than the other solvents [14]. The extraction process could be assigned to two main stages during the extraction time: the first stage (within 15 min) is the penetration of solvent into the propolis matrix followed by dissolution of flavonoids and triterpenes and

the second stage (after 15min) is a decrease in extraction yield due to a loss of flavonoids and triterpenes [27]. The loss of these compounds may be caused by degradation and oxidation due to prolonged extraction time [32]. During the first stage, with the increase of ethanol concentration from 60 to 80%, the extraction yield increased gradually (Figure 2B). Then during the second stage, the extraction yield decreased when ethanol concentration was raised from 80% to 100%. The highest extraction yield (49.18%) was achieved with 80% ethanol concentration at 15 min. This result was similar to that reported by Mouhoubi-Tafinine et al. [6], in which the optimum conventional extraction of flavonoids in propolis was obtained with 85% ethanol; however that extraction was achieved in a longer time (15 h). The combination of 80% ethanol and 20% water favored the extraction yield because of the mixed characteristics of both. The low viscosity of water enhanced the cavitation and diffusion of solvent in the interior of the material matrix. At the same time, the high polarity of ethanol could dissolve polar solutes easily.

3.3 Effect of Temperature on Extraction Yield

Sample (8 g) with ethanol solution was extracted at temperatures (section 2.4) and keeping other conditions the same: 10s/5s, ethanol concentration 80% and 30:1. The extraction yield increased with the increase of temperature, and reached highest value (46.54%) at 50 °C in 15 min. After this, the yield declined gradually with the increase of temperature and time (Figure 2C). The optimum temperature here was similar to that reported by Al-Dhabi et al. [19] (45 °C) and Chen et al. [33] (53 °C). However in their study, the optimum extraction time was higher (36 and 38 min respectively). Cavitation and thermal effects are two physical events that play important roles in UAE. Cavitation enhances disruption of material matrix by providing high-intensity acoustic waves, due to the formation of cavitation nucleus above 30 °C. Cavitation bubbles are produced by high cavitation threshold; these bubbles explode with intense force which intensifies the disruption of propolis matrix and mass transfer. Magnitude and frequency of cavitation depends on the solvents' vapor pressure. Thermal effect could cause the breakdown of propolis matrix, increasing the mass transfer rate from matrix to solvent. Ethanol provides high sample soaking and penetration capacity at elevated temperatures. Whereas, above 50 °C, cavitation bubbles collapse, increase in solvent viscosity and surface tension interrupts cavitation [34]. Prolonging the exposure of extracted flavonoids and triterpenes to sonication caused degradation and structural destruction of them, thus the extraction yield decreased gradually after 15 min of extraction [19, 35].

3.4 Effect of Liquid-solid Ratio on Extraction Yield

Samples were extracted with 80% ethanol at different ratios (section 2.4) and other conditions kept same. The extraction yield increased gradually when the ratio increased from 25:1 to 30:1 (mL/g) reaching its highest value (47.15%) at 15 min of extraction (Figure 2D). Meanwhile the yield decreased on further increase of ratio and time beyond 15 min. Solvent volume is indirectly proportional to the viscosity and concentration [19]. In general, a higher solvent volume can dissolve compounds more effectively resulting in higher extraction yield. The concentration difference between material matrix and solvent was caused by

the higher ratio (from 25:1 to 30:1), which could dissolve the flavonoids and triterpenes more effectively causing enhanced mass transfer [36] and thus increasing the extraction yield. Whereas as the ratio increased beyond 30:1 (mL/g) the extraction yield decreased. This happened because of the reduction in dispersion of ultrasound energy in the solvent and increase of dissolved impurities which interfered with the dissolution of flavonoids and triterpenes. The same tendency was observed in phenolic compounds extraction from spent coffee grounds [19] and polysaccharides extraction from *Acanthopanax senticosus* [37] using UAE.

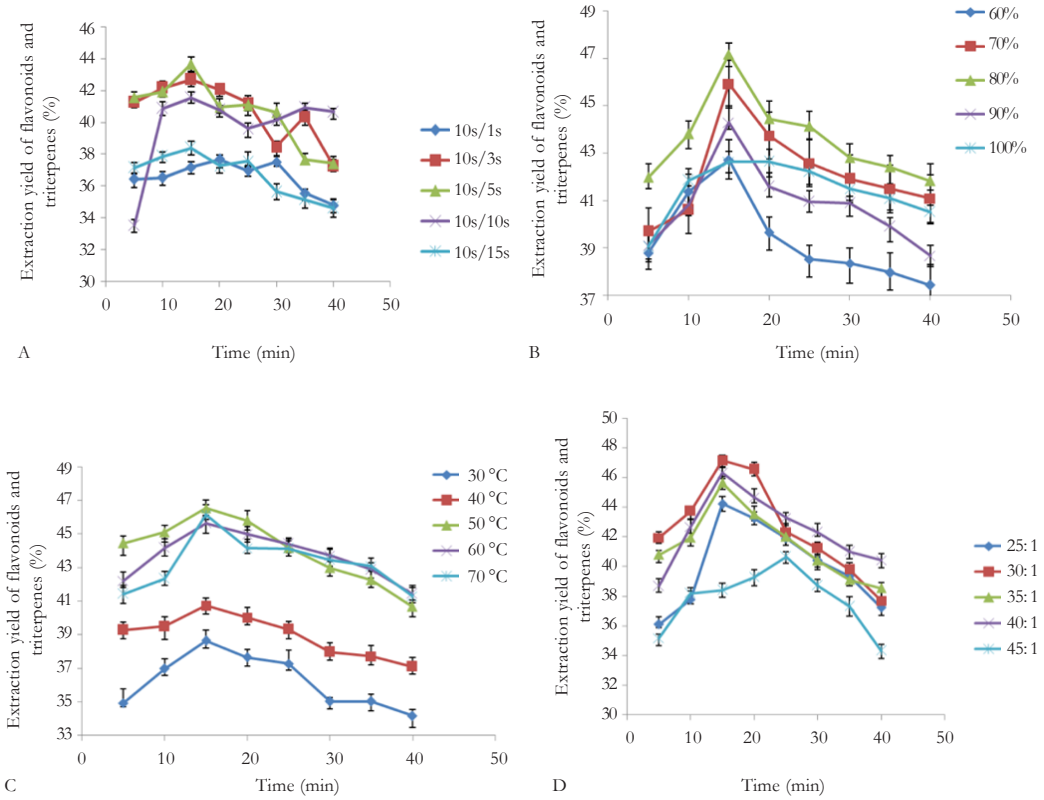

Figure 2. Effects of parameters on extraction yield of UAE of flavonoids and triterpenes. (A) ultrasonic on-time/off-time; (B) ethanol concentration; (C) temperature; (D) liquid/solid ratio.

3.5 Kinetics Parameters for UAE of Flavonoids and Triterpenes

The linearized forms of the second-order kinetic model for the UAE of flavonoids and triterpenes were shown in Figure 3A-D as a series of plots of t/Ct against time (t) of extraction. The linear equations from these graphs were used to calculate the equilibrium concentration of each extraction parameter. The initial extraction rate (h), extraction rate constant (k), equilibrium concentration (C_e) and coefficient of determination R^2 for different off-time (S), ethanol (L), temperature (T) and liquid-solid ratio (Z) are given for various temperatures in Table 1. The high coefficients of determination (0.9793-0.9977) showed that the model is suitable for the prediction of variables. The functions of kinetics parameters (h, k, C_e) for each extraction parameter (S, L, T, Z) are plotted

in Figure 4A-D and expressed as follows:

For *S*:

C_e= 0.0004 S^3 - 0.0077 S^2 + 0.0306 S + 1.0978, R^2 = 0.8676 (9)

k = 0.225 S^2 - 3.4119 S + 13.518, R□ = 0.9757 (10)

h = -0.0087 S^2 + 0.1362 S + 0.2089, R^2 = 0.9059 (11)

For *L*:

C_e= -5.6271L^2 + 8.6546L - 2.1046 R^2 = 0.9995 (12)

h = -56.878L^3 + 146.85L^2 - 124.08L + 34.969, R^2 = 0.9484 (13)

k = -75.325 L^3 - 183.91 L^2 + 147.9 L - 40.043, R^2 = 0.9162 (14)

For *T*:

C_e= -0.0002T^2 + 0.0255T + 0.2255 R^2 = 0.9854 (15)

h = 7E - 05T^4 - 0.0136T^3 + 1.0241T^2 - 33.302T + 39833R^2= 0.9999 (16)

k = -0.0005 T^3 + 0.087 T^2 - 4.7486 T + 87.356, R^2 = 0.9227 (17)

For *Z*:

C_e= -0.0271Z + 1.8887, R^2 = 0.9446 (18)

h = 0.0023Z^3 - 0.2423Z^2 + 8.4135Z - 93.888, R^2 = 0.909 (19)

k = 0.0031 Z^3 - 0.3269 Z^2 + 11.138 Z - 122.97, R^2 = 0.9974 (20)

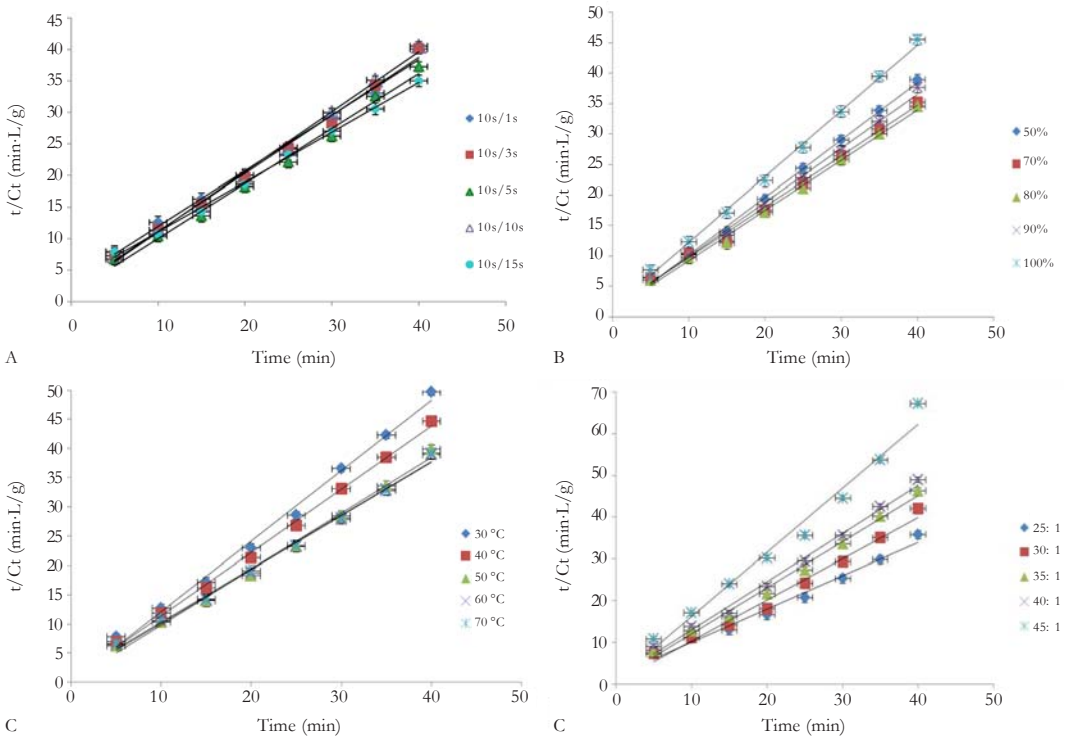

Figure 3. Change of reciprocal change rates of the total concentration of flavonoids and triterpenes with extraction time under different conditions. (A) ultrasonic on-time/off-time; (B) ethanol concentrations; (C) temperatures; (D) liquid/solid ratios

Table 1. Kinetic parameters of second-order kinetic model for UAE of flavonoids and triterpenes from propolis under different extraction conditions.

variables	Extraction conditions	h (Initial extraction rate) ($g \cdot L^{-1} \cdot min^{-1}$)	k (Extraction rate constant) ($L \cdot g^{-1} \cdot min^{-1}$)	C_e (Equilibrium concentration of flavonoids and triterpenes) (g/L)	R^2
Ultrasonic on-time/off-time (s/s)	10/1	0.3195±0.02	11.1328±0.25	1.1364±0.11	0.9912
	10/3	0.5097±0.02	4.1933±0.27	1.0893±0.07	0.9943
	10/5	0.7651±0.09	1.9635±0.14	1.1494±0.07	0.9918
	10/10	0.6349±0.09	2.6112±0.11	1.0526±0.12	0.9953
	10/15	0.3156±0.04	13.047±0.32	1.2660±0.14	0.9974
Ethanol concentration (%)	60	1.2544±0.11	1.1137±0.05	1.0613±0.03	0.9976
	70	0.7333±0.02	0.5096±0.03	1.1996±0.02	0.9949
	80	0.9464±0.03	0.7295±0.04	1.2140±0.03	0.9956
	90	0.9294±0.02	0.5611±0.02	1.1281±0.06	0.9955
	100	0.7427±0.03	2.5087±0.03	0.9226±0.04	0.9977
Temperature (°C)	30	6.3452±0.32	9.1708±0.41	0.8319±0.06	0.9931
	40	1.6636±0.05	1.9430±0.08	0.9259±0.08	0.9969
	50	3.2279±0.17	2.9323±0.13	1.0493±0.05	0.9937
	60	0.9936±0.03	0.8345±0.08	1.0917±0.06	0.9951
	70	0.7943±0.05	0.6606±0.05	1.0964±0.07	0.9945
Liquid-solid ratio (mL/g)	25:1	0.4961±0.02	0.3150±0.02	1.2550±0.18	0.9842
	30:1	2.0096±0.23	1.9526±0.17	1.0145±0.11	0.9850
	35:1	1.0064±0.14	1.2215±0.09	0.9076±0.08	0.9924
	40:1	0.6805±0.08	0.9065±0.10	0.8658±0.09	0.9917
	45:1	1.2439±0.12	2.9369±0.22	0.6507±0.05	0.9793

3.6 Kinetic Model of Flavonoids and Triterpenes Extraction

With the increase of off-time, h ascended firstly and then descended after 7s, k went down first and increased lately, and C_e changed little (Figure 4A). An ideal combination of this parameter was fundamental in the extraction process, which was related to the exposure time of ultrasound energy to material matrix. Because the kinetic parameters (h, C_e, k) were dependent on the on-time/off-time, the values for S were fitted by second-order and third-order polynomial functions (equations 9-11). Substituting these variables into the equation (5), the kinetic model is shown as follows:

$$C_{t,S} = \frac{t}{1/(-0.087S^2 - 1.1362S + 0.2089) + t/(0.0004S^3 - 0.0077S^2 + 0.0306S + 1.0978)} \quad (21)$$

The results showed that the equation can be used to predict the extraction of flavonoids and triterpenes under different on-time/off-time at a given time at 50 °C, 80% ethanol concentration and liquid-solid ratio 30:1.

The h and k decreased with the increase of ethanol concentration from 60 to 70%, and then increased. As ethanol concentration increased, C_e of flavonoids and triterpenes went up until reaching a peak at 80%, and

then tended to decrease until its lowest point at 100% (Figure 4B). It shows that lower concentrations of ethanol were not enough for reaching the equilibrium concentration to a maximum point and higher concentrations provoked saturation of solvent which negatively affected the equilibrium concentration. Because the kinetic parameters (h, C_e, k) were related to ethanol concentration, the relationship between kinetic parameters and L were fitted by second-order and third-order polynomial functions (equations 12-14). Substituting these variables into the equation (5), the kinetic model is shown as follows:

$$C_{t,L} = \frac{t}{1/(-75.325L^3 + 183.91L^2 - 147.9L + 40.043) + t/(-5.6271L^2 + 8.6546L - 2.1046)} \quad (22)$$

The results showed that the equation can be used to predict the extraction of flavonoids and triterpenes under different ethanol concentrations at a given time, 10s/5s, 50 °C and 30:1.

With the increase of temperature, the h and k tended to decrease. The temperature had a great influence on the kinetic parameters. With the increase of temperature, C_e increased gradually (Figure 4C). Temperature accelerates the extraction process as seen in the experimental results. Because the kinetic parameters (h, C_e, k) were related to the extraction temperature, the relationship between kinetic parameters and T were fitted by second-order, third-order and fourth-order polynomial functions (equations 15-17). Substituting these variables into the equation (5), the kinetic model is shown as follows:

$$C_{t,T} = \frac{t}{1/(7E-05T^4 - 0.0136T^3 + 1.0194T^2 - 33.149T + 396.48) + t/(-0.0002T^2 + 0.0254T + 0.2268)} \quad (23)$$

The results showed that the equation can be used to predict the extraction of flavonoids and triterpenes under different temperatures at a given time with 80% of ethanol concentration, 10s/5s, and 30:1.

With the increase of liquid-solid ratio, the kinetic parameters of h and k increased until 30:1 mL/g. They presented a first increase with 30:1, then decreased until 40:1, and at last increased with 45:1 mL/g. C_e showed a decreasing trend with the increase of liquid-solid ratio (Figure 4D) probably due to reduction in dispersion of ultrasound energy in the solvent and increase of dissolved impurities. Because the kinetic parameters (h, C_e, k) were related to the liquid-solid ratio, the relationship between kinetic parameters and Z were fitted by linear and third-order polynomial functions (equations 18-20). Substituting these variables into the equation (5), the kinetic model is shown as follows:

$$C_{t,Z} = \frac{t}{1/(0.0023Z^4 - 0.2423Z^2 + 8.41352Z - 93.888) + t/(-0.0271Z + 1.8887)} \quad (23)$$

The results showed that the equation can be used to predict the extraction of flavonoids and triterpenes under different liquid-solid ratios at a given time with 80% of ethanol concentration, 10s/5s and 50 °C.

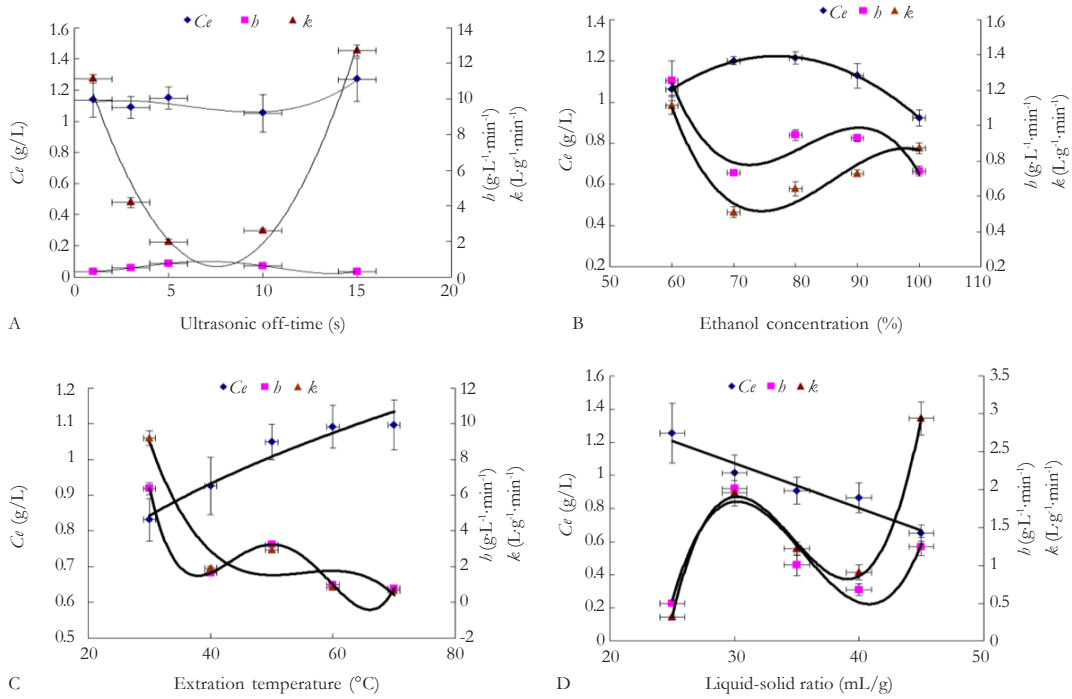

Figure 4. C_e, k and h of propolis with different conditions. (A) ultrasonic on-time/off-time; (B) ethanol concentrations; (C) extraction temperatures; (D) liquid/solid ratios.

3.7 Experimental and Predictive Fitting

To prove the effectiveness of the developed models, the equations of the second-order kinetic models for each extraction parameter were used to predict the total extraction yield and then the predictive values were compared to the experimental values (Table 2). At all process conditions, the predicted values showed satisfactory agreement with the values determined from the experimental data, which were in accordance to high coefficients of determination (R^2) shown in Table 1. Therefore the developed kinetic models are suitable to be used for the prediction of extraction yields with acceptable accuracy.

3.8 Surface Morphology of CEEP and UAEEP

Surface analysis was performed to the freeze-dried CEEP and UAEEP to compare the physical differences between both extraction methods (Figure 5A-B). In the case of CEEP the SEM image (Figure 5A) showed that the surface of extracts had a greater aggregation compared to that of UAEEP (Figure 5B). On the other hand, the surface of UAEEP showed dispersed particles and smaller aggregates compared to that of CEEP. These results showed that the internal material of extracts from CEEP was not uniform and that of UAEEP was comparatively more uniform. The surface characteristics are based on the content of flavonoids [14], so more uniform surface is related to higher contents. The material structures had hollow openings which could be caused by cavitation phenomena facilitating the extraction process. UAE has demonstrated its effects in extraction process, which in this study, intensified the process with 60.34% extraction yield in just 15 min

compared to CEEP requiring 40 min with only 48.55. The same tendency was resulted in camptothecin extraction from *Nothapodytes nimmoniana* using UAE [18].

Table 2. Experimental and predicted concentration of flavonoids and triterpenes under different extraction conditions. The developed models were used for predicting the values.

variables	Extraction conditions	Experimental concentration of flavonoids and triterpenes (g/L)	Predicted concentration of flavonoids and triterpenes (g/L)	Error (%)
Ultrasonic on-time/off-time (s/s)	10/1	1.1364	1.2183	7.1805
	10/3	1.0893	1.1577	6.2150
	10/5	1.1494	1.1228	2.2968
	10/10	1.0526	1.0397	1.2255
	10/15	1.2660	1.1792	6.8562
Ethanol concentration (%)	60	1.0613	1.0050	5.3048
	70	1.1996	1.0869	9.3947
	80	1.2140	1.1126	8.3525
	90	1.1281	1.0470	7.1890
	100	0.9226	0.8509	7.7715
Temperature (°C)	30	0.8319	0.8105	2.5724
	40	0.9259	0.9255	0.0432
	50	1.0493	1.0005	4.6507
	60	1.0917	1.0355	5.1479
	70	1.0964	1.0305	6.0105
Liquid-solid ratio (mL/g)	25:1	1.2550	1.1612	7.4741
	30:1	1.0145	1.0462	3.1246
	35:1	0.9076	0.916	0.9255
	40:1	0.8658	0.7853	9.2977
	45:1	0.6507	0.6611	1.5982

Experiments were performed in triplicate and average values are represented.
Error is the difference between experimental and predicted values and is expressed as percentage (%) of experimental value.

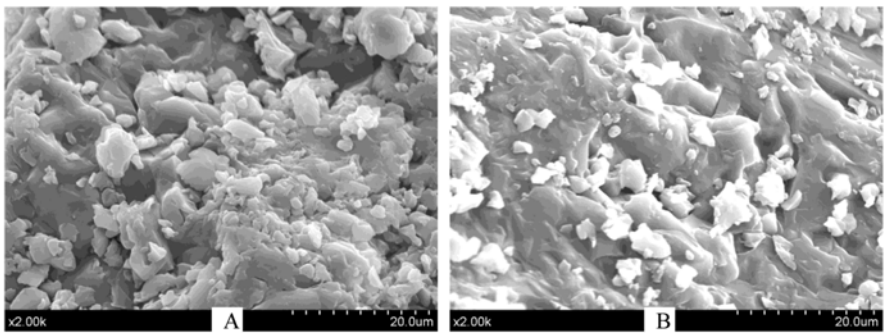

Figure 5. Scanning Electron micrographs of samples: (A) CEEP; (B) UAEEP. Magnification×2000.

3.9 Surface Topography of CEEP and UAEEP

In general, SEM analysis can only provide an overview of a specific zone, but AFM can provide more details of the material matrix such as three dimensional heights [29]. In order to investigate the effects of ultrasonic waves on the surface topography, AFM analysis was performed on CEEP and UAEEP respectively. CEEP had non-homogeneous surface and non-uniform height distribution (Figure 6A). The surface of CEEP had a disordered distribution of particles sizes and locations according to the two-dimensional diagram. However, UAEEP showed a fairly smooth surface of particles and more uniform distribution (Figure 6B) compared to that of CEEP. Diameters and heights of particles were remarkably enlarged in UAEEP. Ultrasonic waves caused molecules aggregations which are probably mainly flavonoids and triterpenes. Additionally, UAEEP showed homogeneous particles sizes which were uniformly distributed through the surface of the sample compared with those that did not receive ultrasound, as shown on the two-dimensional diagram. These results were in accordance with the results of Zhang et al. [28] on the surface morphology of wheat gluten pretreated with counter-flow ultrasound.

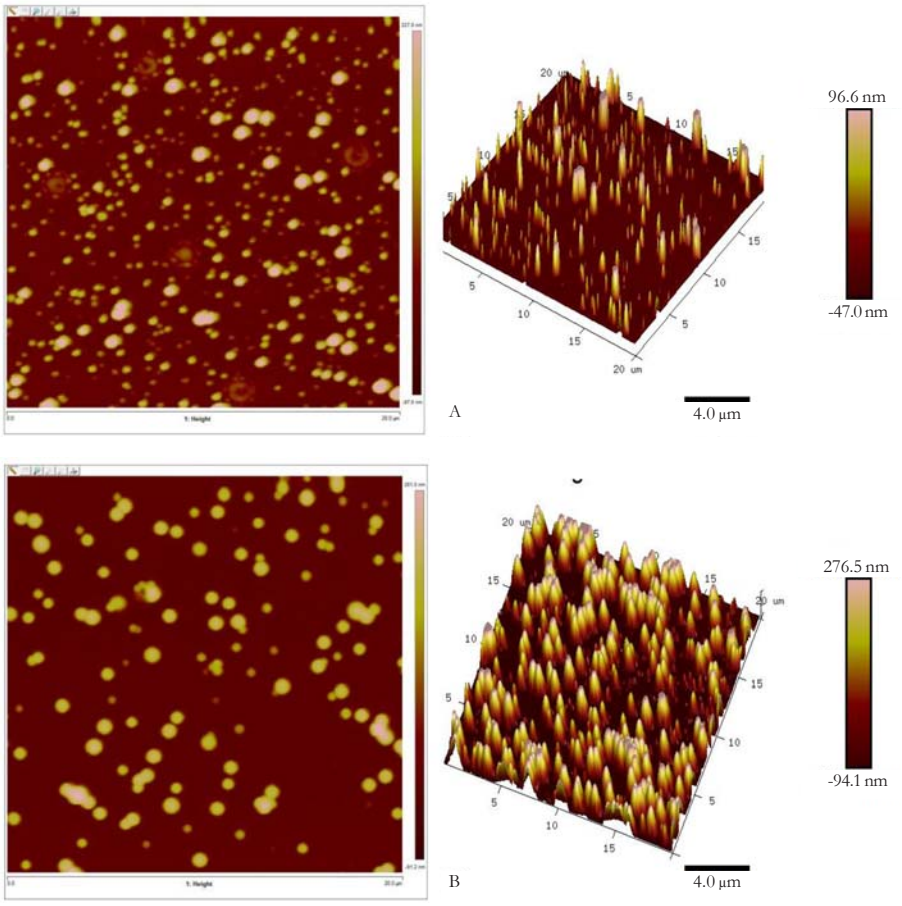

Figure 6. AFM morphology images of samples: (A) CEEP: left, two-dimensional; right, three-dimensional. (B) UAEEP: left, two-dimensional; right, three-dimensional

3.10 GC-MS Analysis of Terpenes

Propolis chemical composition depends on the characteristics of the site of collection, since bees collect propolis from different plants in different habitats. GC-MS is a suitable method for the identification of volatile compounds in propolis, which include but not limited to acids, esters, alcohols, terpenes, olefins and aromatics [38]. Among these groups, terpenes represent the highest proportion in NBBP volatile compounds [14]. Terpenes, which are biosynthetically derived from units of isoprene, are a large and diversified class of volatile compounds in propolis. These compounds are industrially used in perfumery and food additives as flavors, fragrances and spices. Moreover, they have been reported to have a broad range of bioactivities including analgesic, antiinflamatory, anticancer, antimicrobial, antiviral and antiparasitic activities [39]. The GC-MS analysis (Figure 7) identified 14 terpenes in the CEEP and UAEEP. Identification of these compounds was based in the peak area and retention time. A variety of terpenes were detected in the extracts (Table 3). The highest peak area (%) of 3.04±0.48 (CEEP) and 3.02±0.54 (UAEEP) was obtained for α-Selinene with a retention time of 26.73. By contrast, the lowest peak area (%) of 0.04±0.01 (CEEP) and 0.05±0.01 (UAEEP) was obtained for Limonene with a retention time of 15.65. UAE extracted the same compounds in a shorter time (16 min) compared to that of conventional extraction (40 min), which implies that UAE is more efficient than conventional extraction reducing time and energy consumption.

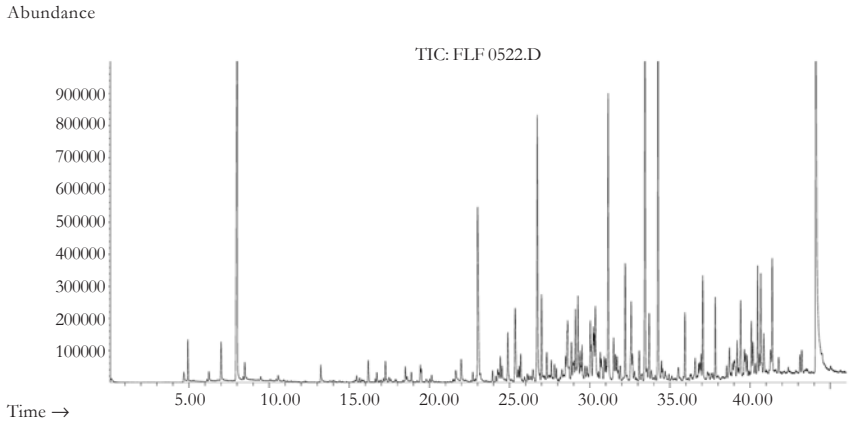

Figure 7. GC-MS total ion chromatogram of ethanolic extracts of northeast black bee propolis.

Table 3. Content of terpenes from CEEP and UAEEP identified by GC-MS.

			Content (%)	
RT (min)	Compounds	Formula	CEEP	UAEEP
10.08	α-Pinene	C10H16	0.42 ± 0.03	0.45 ± 0.02
15.65	Limonene	C10H16	0.04 ± 0.01	0.05 ± 0.01
15.83	D-Limonene	C10H16	0.07 ± 0.01	0.08 ± 0.02
18.56	Germacrene	C15H24	0.27 ± 0.05	0.25 ± 0.06
21.98	Cis-β(Z)-β-methylstyrene	C10H16	0.18 ± 0.03	0.17 ± 0.02
22.36	δ-Cadinene	C15H24	0.34 ± 0.05	0.36 ± 0.05
24.53	Cedrene	C15H24	0.17 ± 0.04	0.16 ± 0.03
25.00	α-Cedrene	C15H24	0.13 ± 0.05	0.13 ± 0.04
25.70	Aristolene	C15H24	0.21 ± 0.08	0.23 ± 0.09
26.73	α-Selinene	C15H24	3.04 ± 0.48	3.02 ± 0.54
27.32	α-Caryophyllene	C15H24	0.35 ± 0.07	0.36 ± 0.05
30.56	α-Guaiene	C15H24	0.42 ± 0.12	0.45 ± 0.09
31.15	α-Curcumene	C15H22	2.62 ± 0.57	2.63 ± 0.58
34.48	α-Calacorene	C15H20	0.09 ± 0.01	0.08 ± 0.01
	Total		8.35	8.42

3.11 LC-MS Analysis of Flavonoids

Flavonoids are a big group of polyphenols, which vary widely with respect to structure and properties. They are the most widespread substances of plant origin. Flavonoids are characterized by powerful antioxidant properties, which is one of their most appreciated properties [10]. Additionally, these are the most active compounds in propolis, which not only perform antioxidant functions but also others biological activities including antimicrobial such as quercetin and chrysin. Formononetin, an isoflavonoid, has been reported to have estrogenic, antiradical, cytotoxic and antifungal activities. Biochanin A has inhibitory effects on cancer cells, antiinflamatory effects and others [39]. Galangin, ferulic acid, quercetin and rutin were found to poses cytotoxic activity against cancer cells. Also, chrysin has been reported to have cytotoxic and apoptosis effects against cancer cells and antiinflamatory and immunomodulatory properties [40]. LC-MS analysis (Figure 8) revealed the presence of caffeic acid (29.68 min), p-coumaric acid (37.82 min), ferulic acid (39.95 min), galangin (44.57 min), benzoic acid (40.8 min), rutin (48.57 min), quercetin (49.73 min), isorhamnetin (53.65 min), benzaldehyde (17.26 min), chrysin (32.67 min) and kaempferol (57.9 min) (Table 4). The total concentration of flavonoids were consistent in both CEEP (18.27%) and UAEEP (18.37%), especially on important compounds such as ferulic acid, chrysin, kaempferol, galangin, quercetin and rutin, which as previously stated, are the most bioactive flavonoids in these group.

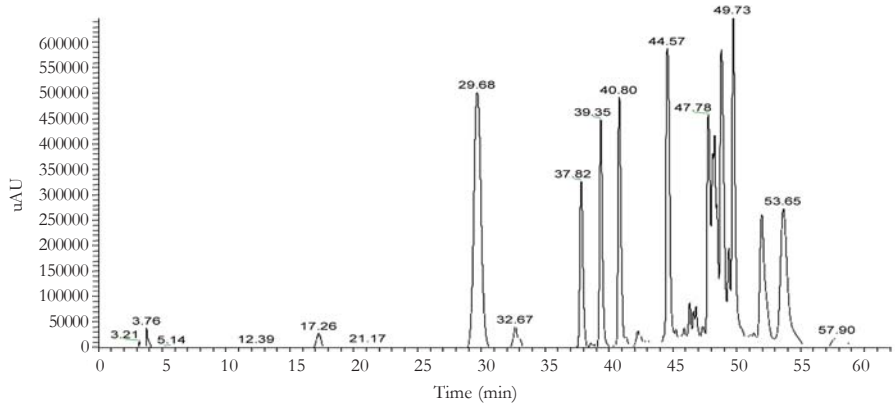

Figure 8. LC-MS total ion chromatogram of ethanolic extracts of northeast black bee propolis.

Table 4. Content of flavonoids from CEEP and UAEEP identified by LC-MS.

			Content (%)	
RT (min)	Compounds	Formula	CEEP	UAEEP
17.26	Benzaldehyde	C7H6O	0.42 ± 0.05	0.41 ± 0.05
29.68	Caffeic acid	C9H8O4	3.07 ± 0.23	3.03 ± 0.21
32.67	Chrysin	C15H10O4	0.88 ± 0.09	0.87 ± 0.08
37.82	p-coumaric acid	C9H8O3	2.08 ± 0.28	2.11 ± 0.27
39.95	Ferulic acid	C10H10O4	1.92 ± 0.17	1.94 ± 0.18
40.80	Benzoic acid	C7H6O2	1.89 ± 0.21	1.88 ± 0.17
44.57	Galangin	C15H10O5	2.05 ± 0.26	2.04 ± 0.23
48.57	Rutin	C27H30O16	1.66 ± 0.21	1.69 ± 0.19
49.73	Quercetin	C15H10O7	1.88 ± 0.13	1.92 ± 0.15
53.65	Isorhamnetin	C16H12O7	1.44 ± 0.14	1.52 ± 0.13
57.90	Kaempferol	C15H10O6	0.98 ± 0.11	0.96 ± 0.12
	Total		18.27	18.37

4. CONCLUDING REMARKS

In summary, this study reported about the extraction kinetics of flavonoids and triterpenes from NBBP using ultrasound assisted solid-liquid extraction and the results were compared with conventional extraction. The kinetic models were successfully developed for describing the extraction processes under different extraction parameters, including ethanol concentration, liquid-solid ratio, extraction temperature, extraction time and on-time/off-time. These results demonstrated that the kinetic models of the process parameters are highly significant, reliable and accurate in predicting the extraction yield. The optimum extraction conditions were 80% ethanol concentration, 30:1 mL/g ratio, 53 □C , 16 min and 10s/5s on-time/off-time. Extraction yield obtained under the optimum conditions is 60.34% of flavonoids and triterpenes. These results support the fact that process parameters have significant effect on extraction efficiency. Furthermore UAE showed higher extraction yield compared to conventional extraction

(48.55%), which was achieved in short time. UAE changed the microstructure of propolis favoring the penetration of solvent and intensification of extraction process. Also, the terpenes and flavonoids composition were consistent as shown in the GC-MS and LC-MS analyses. Therefore, ultrasound-assisted extraction may be a desirable method, which is able to extract of bioactive compounds in propolis in a shorter time compared to that of conventional extraction. Thus the results of this study may be a very useful basis for further industrial applications.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

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