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Extraction of bioactive compounds from *nepeta* (*Nepeta binaludensis* Jamzad) by novel technologies

Afsaneh Azimi Mahalleh[1], Seyed Ali Mortazavi[2], Parvin Sharayei[3]*, Elham Azarpazhooh[4], Razieh Niazmand[5]

1,2 Department of Food Science & Technology, Sabzevar Branch, Islamic Azad University, Sabzevar, Iran.
3,4 Agricultural Engineering Research Department, KhorasanRazavi Agricultural and Natural Resources Research and Education Center, AREEO Mashhad, Iran.
5 Department of Food chemistry, Research Institute of Food Science and Technology, Mashhad, Iran.

E-mail : parvin_sharayei@yahoo.com Contact No. : +98 - 5133822373

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Abstract

The assisted extraction by ultrasound (UAE) and pulsed electric field (PAE) technologies were used for extraction of bioactive compounds from aerial parts of nepeta (*Nepeta binaludensis*Jamzad). The effect of independent variables in UAE (ultrasonic extraction time, UET: 5 and 15 min and ultrasound amplitude, UA: 20 and 100%) and PAE (voltage: 2000 V and number of pulses: 60) on yield (Y), total phenolic compounds (TPC), 50% of radical-scavenging activity (IC_{50}) and ferric reducing-antioxidant power (FRAP) of crude ethanolic extract (CEx) of aerial parts of*Nepetabinaludensis*were investigated.Results showed that the best conditions for CEx with UAE were UET and UA, 15 min and 100%, respectively. At this optimum condition, the Y, TPC, IC_{50} and FRAP of the CEx were found to 11.6%, 383.98 mg GA/g, 0.31mg/mL and 2795.06µmol Fe^{2+}/mass, respectively. Also results showed that the CEx extracted by UAE and PAE had the higher phenolic compounds, antioxidants andantifungi activities than maceration extraction.These results indicate that novel extraction techniques such as ultrasonic and pulse electric field have been used to maintain more antioxidant compounds and improve the efficiency of CEx.

Key words : Phenolic compounds, Ultrasound,Pulse electric field, *Nepetabinaludensis*

INTRODUCTION

Today, a wide range of additives are used for various purposes in the preparation of different kinds of food products. The importance of these additives is such that without the use of them, the production and consumption of many food products is almost impossible. One of the most important food additives is antioxidant and antimicrobial synthetic compounds that play an important role in prolonging the life of food storage and reducing waste. Hundreds of synthetic additives have been introduced by responsible organizations as authorized and usable ingredients in food, but their use in foods is limited due to safety [1]. Natural additives are extracted from various plant (such as medicinal plants) and animal tissues whose levels of active compounds vary depending on the source.

Some researchers showed that two-thirds of the plant species are of medicinal value. In fact, medicinal plants used in folk medicine got to be further studied and used in the pharmaceutical and nutrition. In addition, the so-called phytomedicinesare playing many important roles in human health care system[2, 3].

Nepeta, one of the medicinal plants, is a large genus belonging to the Lamiaceae families and comprised of about 280 species distributed mainly in Southwest and Central Asia, Europe,North Africa and North America. Some of these species are wellknown folk medicines. The pharmacological and antimicrobialproperties such as feline attractant, canine attractant, insect repellant, arthropod defense, antibacterial, antifungi, antiviral, etc., are usually due to their terpenoid constituents [4, 5].*Nepetabinaludensis* Jamzad,is a rare perennial herb native which distribute in limited area in Binalud mountains in the North East of Iran[6].

Conventional techniques such as soxhlet extraction, maceration and hydro-distillation have long been used to extract effective compounds from plant materials [7]. The limitations associated with these methods are long extraction time, requirement of pure and expensive solvent, and damage to thermolabile compounds encouraging.For these reasons, researchers to look for more favorable extraction techniques [8, 9].

Up to now, several new techniques for the extraction of bioactive compounds from plants ,including ultrasound) [10] and pulsed electric field [11]have been reported. Ultra sound assisted extraction (UAE) is one of the useful extraction techniques which is more efficient in comparison to the conventional extraction [12].The advantages claimed for UAE include shorter operation time, easier operation, reduced solvent consumption and temperature, saved energy and increased yield[13].Pulsed electric field (PEF) technology consists in the application of short duration pulses of high electric field strengths. Short-duration high-intensity field strengths caus epermeabilization of cell membranes and electrophoretic movement of charged species between cellular compartments [14, 15].The use of PEF can facilitate the selective recovery of valuable compounds without deteriorating the treated matrix, thus favoring the subsequent separation and purification steps[15].

The main goal of this study was to evaluate the UAE and PAE extraction procedure for maximizing the yield of phenolic compounds and antioxidant activity from *Nepetabinaludensis.*

MATERIALS AND METHODS

Plant material

The aerial parts of nepeta (*Nepata binaludensis* Jamzad) were collected during the flowering stage of plant, from Binaloud, Khorasan-Razavi province, Iran, in August 2016 .The aerial parts of nepeta (ANB) were dried in the shade for one week and then were ground to a fine powder in a grinder. The powders was passed through a sieve in order to maintain particle size unity (300µm) and then were packed in plastic bags, vacuumed, sealed,

protected from light, and were kept at room temperature until used for further studies.

Chemicals

All chemicals (DPPH (1,1-Diphenyl-2-picryl-hydrazyl), TPTZ(2,4,6-tripyridyl-S-triazine), sodium carbonate, gallic acid, ferricchloride, ferrous sulphate, sodium acetate, glacial acetic acid, HCl,methanol, ethanol, folin-ciocalteu) and solventsused in this study were of analytical reagent grade and supplied by Merck and Sigma -Aldrich companies.

Physicochemical characteristics of *Nepeta binaludensis*

The protein, fat, moisture, and ash of dried ANB were measured by the methods of association of official analytical chemists'society [16]. Soluble dietary fiber was also determined with some modifications by the procedure of Zhang et al[17]. The carbohydrate content was calculated by subtracting the amount of other ingredients from 100.

Extraction procedure

UAE was conducted in an ultrasonic apparatus (Heilscher, Germany Ultrasonic Electronic Equipment Co. Ltd.,). 10 g of dried ANB powder were placed into 250 mL flask, and 100 ml of ethanol solvent (1:10 w/v) was added and then placed in ultrasonic bath at different sonication amplitude (UA: 20 and 100%) and extraction time (UET: 5 and15 min).Then the mixtures were agitated for 48h in the dark at ambient temperature. When the extraction finished, the mixtures were filtered through filter paper. Then solvents were removed from the vacuum rotary evaporator (Laborota 4000 efficient, Germany). At last, the solution was dried in a freeze drier (Operon-Korea)and kept sealed in the dark and -18°C.

A pulsed electric field (PEF) was applied using a pure pulse (PurePulse Technologies, San Diego, USA) and a batch one-liter treatment chamber with stainless electrodes. The electrodes of the treatment chamber were two parallel disks. Thecircuit configuration and the electrode shape generated exponential decay pulses. A pulse frequency of 1 Hz was used. 10 g ANB powder were introduced between the electrodes, and 100 ml of ethanol solvent (1:10 w/v) was added. pulse generator with voltage of 2000V and 60 pulses was applied at ambient temperature to the treatment chamber. The next stages similar to the UAE extraction.

Yield determination

The determination of yield of each extract was performedas follows: Mass ratio of ANB before and after completion of extraction (fresh and freeze-dried extract) was taken as the extraction yield. Indeed, extraction yield was calculated using Eq. (1)

$$\text{Extraction yield } (\%) = \frac{w_1}{w_2} \times 100$$

Where, w_1 and w_2 were the quantity of ANB and freeze-dried ANB extract (CEx), respectively.

Total phenolic content (TPC)

FolinCiocalteau method was used to measure the total phenolic content (TPC) of the extracts [18]and was performed as follows: 100 µl of the sample solutions (100 mg in 10 mL of Methanol), 6 ml of twice distilled water and 500 µl of Folin-Ciocalteau reagent were added, after waiting 8.8 min at room temperature, 1.5 ml of sodium carbonate (20% w/v) were added.The extracts were mixed and stored for 30 min at room temperature and the spectrophotometric analysis was performed at 765 nm. A mixture of water and reagents was used as blank. A calibration curve of gallic acid in methanol was performed in concentration range 0.04±0.7 mg/ml.TPC was presented as gallic acid equivalents in mg per g dried weight (mg GA/g).

Ferric Reducing-Antioxidant Power (FRAP) assay

Ferric reducing-antioxidant power (FRAP) was assayedthe use of 2, 4, 6-Tripyridyl-S-triazine (TPTZ) according to(Benzie and Strain) [19]. The results were reported in µmol Fe^{2+} per mass at 595 nm, against the control solution.

DPPH radical-scavenging assay

The determination method of radical-scavenging activity consisted of spectrophotometric measurement of the intensity of the color change in solution depending on the amount of 2,2-diphenyl-1-picrylhydrazyl (DPPH)DPPH radical-scavenging assay was carried out according to Ramadanet al[20]method and using the Eq. (2):

$$DPPH\% = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

Where A_S is the absorbance of the solution when thesample has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution.

The sample concentration providing half maximal (50%) of radical-scavenging activity(IC_{50})was calculated by interpolation from the graph of radical-scavenging activity percentage against sample concentration.

Antifungi activity

The antifungiactivity against*Aspergillus niger* (ATCC6404) was determined with the Broth Macro dilution susceptibility assay[21].To prepare fungi suspensions, a few days before the experiment, some of the main culture was added to the Total Plate Count culture. After 24 hours of incubation in the logarithmic phase of microorganism growth, using the spectrophotometer, concentration of the fungi was equalized with the standard McFarland tube No. 0.5 (1.5×10^8). This suspension was considered as storage and was diluted 1: 100 in a normal saline environment at the same day (1.5×10^6).At first ethanolic extract in the optimum extraction condition by UAE (UA: 100 and UET: 15 min) and PAE (voltage:2000V and number of pulses:60) and maceration was extracted. To determine the Minimum Inhibitory Concentration of extracts (MIC), a series of 6 sterilized tubes were used. Three tubes were tested for different dilutions (dilution of 2, 4 and 6% of extract) along with a culture medium, normal saline and a fungi suspension) and a tube as a negative control (containing Total Plate Count and normal saline), a tube as a positive control (containing fungi suspension, culture medium and normal saline) and one another tube (containing potassium sorbate, fungi suspension, culture medium and normal saline) were used. After culturing, the tubes containing*Aspergillusniger* were incubated for 3 to 5 days at 25 ° C. Following the incubation of the tubes, they were examined for turbidity caused by the growth of inoculated bacteria. To determine the turbidity of the specimens, a spectrophotometer model A-UV-1600 and a wavelength of 600 nm were used.

After examining the MIC results, in order to determine the

Minimum fungicidial Concentration (MFC), 100 µl of the existing solution, in tubes without turbidity was separately cultured on a Total Plate Count medium and after 3 to 5 days at 25°C, the lowest concentration of treatments was considered as the concentration of MFC where the fungi had not grown.

Statistical analysis

All experiments and measurements were carried out in triplicate, and data were subjected to analysis of variance (ANOVA). ANOVA and regression analyses were performed according to MStatC and Slide Write software. Significant differences between means were determined byDuncan's multiple range tests.P values less than 0.05 were considered statistically significant.

RESULTS

The chemical composition of dried aerial part of *Nepatabinaludensis* plant used in this study is shown in Table 1. The values chemical composition for ANB was moisture (8.6%), Ash (58%), protein (15.43%), fat (2.6%),dietary fiber (18.89%) and carbohydrate (15.37%).

Table 2 summarizes the effects of UA on Y, TPC, IC_{50} and FRAP of CEx. As it can be seen the effects of UA on Y, TPC and FRAP were significant ($p<0.05$).

The effect of UET on Y, TPC, IC_{50} and FRAP of CEx is shown in Table 3. The results showed that the effects of UET on extraction yield was significant ($p<0.05$) and extraction yield increased with increasing ultrasound time.

The interaction effect ofUA and UET on yield, TPC, IC_{50} and FRAPof CEx is shown in Table 4. The result showed that the interaction effect of UA and UET on values of FRAP was significant ($P<0.05$). With the increase in UA and UET, the values of FRAP increased. The maximum FRAP value was obtained atUA and UET 100 % and 15 min, respectively.

According to the results, the best condition for UAE in terms of the UET and UA was15 min and 100%, respectively. In this condition maximum values of extraction yield, TPC, FRAP and minimum values of IC_{50} were obtained.

The effects of UAE, PAE and maceration on extraction yield, TPC, IC_{50} and FRAPare shown in Table 5.By comparing the optimum extraction condition by UAE(UA: 100 and UET: 15 min)andPAE (voltage: 2000V and number of pulses:60)with maceration extraction showedthat theUAE and PEF had no significant difference in values of Y, TPC, IC_{50} ($P>0.05$). However, the maceration extraction had significant lower Y, TPC and FRAP andhigher IC_{50} value ($P<0.05$).

The results of the minimum inhibitory concentration (MIC) of different concentration of CEx are shown in Fig 1. The results showed that the MIC of CEx for *Aspergillus niger* (ATCC 6404) were equal to 4% extract. MIC in concentration 4% of maceration, PEF and UAE extracts were 34, 40 and 39%, respectively. Additionally, the result of CEx for 1000 (mg/kg) concentration of potassium sorbate was 32%. Therefore, CEx at 4% has a greater antifungi effect compared to potassium sorbate (at 0.1% concentration).

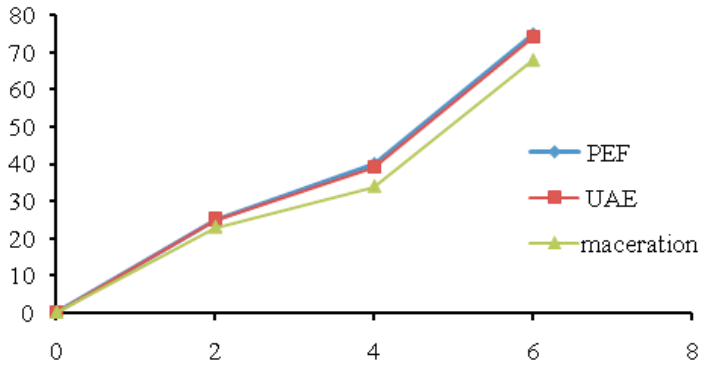

Fig 1 : Minimum inhibitory concentrations (MIC s) of CEx (2, 4 and 6%) on *Aspergillus niger* by PAE, UAE and maceration technology.

Table 1: Chemical properties of dried aerial part of *Nepatabinaludensis* plant

Compounds	(%)
Moisture	8.6 ± 0.67[a]
Lipid	2.6 ± 0.31
Protein	15.43 ± 1.82
Ash	58 ± 2.59
Total dietary fiber	18.89 ± 1.94
Carbohydrates	15.37 ± 0.78

[a] : Mean ± standard deviation of triplicate determinations from experiments

The results show that the MFC of CEx for *Aspergillus niger* was equal to a 6% concentration of the extract and its amount in concentration 6% of maceration, PEF and UAE extracts were was 55, 68 and 65%,respectively.

DISCUSSION

As can be seen in Table 1, the moisture content of ANB was 8.6%. Determination of moisture is one of the most important and most used analytical amounts in food processing and testing. The moisture content is often the criterion of sustainability and product quality. The purpose of measuring moisture is to determine the amount of water used to control the growth of microorganisms (in packaging and during storage) [22].Our findings showed that *Nepata binaludensis* had valuable fiber of

Table 2: The effect of ultrasonic amplitude (UA, %) on Y, TPC, FRAP and IC_{50} of CEx

UA (%)	Yield (%)	TPC (mg GA /g)	IC_{50} (mg/ml)	FRAP (µmol Fe^{2+}/mass)
20	10.35 ± 0.08b	370.55 ± 2.18b	0.34 ± 0.02a	1788.56 ± 35.62b
100	11.30 ± 0.08a	378.11 ± 2.18a	0.32 ± 0.02a	2610.26 ± 35.62a

Means ± SD (standard deviation) with the same lowercase letters are not significantly different at $p < 0.05$.

Table 3: The effect of ultrasonic exposure time (UET, min) on Y, TPC, FRAP and IC_{50} of CEx

UET (min)	Yield (%)	TPC (mg GA/g)	IC_{50} (mg/ml)	FRAP (µmol Fe^{2+}/mass)
5	10.50 ± 0.08b	371.44 ± 2.18a	0.32 ± 0.17a	2230.76 ± 35.62a
15	11.15 ± 0.08a	377.22 ± 2.18a	0.33 ± 0.17a	2168.06 ± 35.62a

Means ± SD (standard deviation) with the same lowercase letters are not significantly different at $p < 0.05$.

Table 4: Interaction effects of UA (%) and UET (min) on Y, TPC, FRAP and IC_{50} of CEx

UET (min)	UA (%)	Yield (%)	TPC (mgGA /g)	IC_{50} (mg/ml)	FRAP (µmol Fe^{2+}/mass)
5	20	10 ± 0.1	370.46 ± 2.49	0.31 ± 0.03	1541.06 ± 57.16d
	100	10.7 ± 0.19	370.64 ± 0.72	0.32 ± 0.07	2036.06 ± 68.59c
15	20	11 ± 0.3	372.23 ± 4.07	0.32 ± 0.02	2425.46 ± 85.94b
	100	11.6 ± 0.1	383.98 ± 9.5	0.35 ± 0.01	2795.06 ± 30.24a

Means ± SD (standard deviation) with the same lowercase letters are not significantly different at $p < 0.05$.

Table 4: Interaction effects of UA (%) and UET (min) on Y, TPC, FRAP and IC_{50} of CEx

	Yield (%)	TPC (mgGA /g)	IC_{50} (mg/ml)	FRAP (µmol Fe^{2+}/mass)
UAE	11.6 ± 0.1a	383.98 ± 9.5a	0.31 ± 0.01b	2795.06 ± 30.24a
PAE	11.36 ± 0.23a	374.34 ± 0.74ab	0.32 ± 0.01b	1679.66 ± 68.59b
maceration	7.9 ± 0.1b	365.86 ± 4.39b	0.4 ± 0.01a	1613.32 ± 34.82b

Means ± SD (standard deviation) with the same lowercase letters are not significantly different at $p < 0.05$.

approximately 18.89%.Indigestible ingredients such as cellulose, hemicelluloses, oligosaccharides, and pectin as well as lignin and waxes are altogether considered as dietary fibers. The dietary fibers play an important role in a healthy food and diet.

As can be seen in Table 2. The value of Y, TPC and FRAP increased as concentration of UA increased that it is related to the physical effect of ultrasound, such as cavitations, which results in a significant increase in the mass transfer rate. Ultrasound can be used effectively to increase the efficiency and mass transfer rates in many solid-liquid extraction processes. Kadam et al[23] reported that ultrasound could increase the recycling of bioactive compounds from *Ascophyllum nodosum*.

The results showed that the extraction yield increased with increasing ultrasound time. Adding ultrasound time causes more solvent contact with the sample and increases the permeability of the compounds into the solvent. Also, Ghafoor et al[9] and Dahmoune et al[24] reported that extraction of bioactive compounds from *P. lentiscus* after ten minutes.

As can be seen in table 4, with the increase in UA and UET, the values of FRAP increased.Due to cavitation with UAE, cracking in the cell wall increases the permeability of plant tissues to enter the solvent into the interior of the wall material and also cause the extract of the bioactive coumpound. Rodrigues et al [25] reported that as well as the amount of effective compounds increased also the trapped of Fe III increased. Our results are also in agreement with studies on UAE extraction bioactive compounds from arecanut (*Areca catechu* L.) [26].

By comparing the optimum extraction condition by UAE and PAEwith maceration extraction showedthat the maceration extraction had significant lower Y, TPC and FRAP and higher IC_{50} value.

Extraction improvement by PEF treatment is usually attributed to cell membrane damage, which facilitates the release of intracellular compounds [27, 28]. Increasing the number of pulses causes an increase in disruption of cell membranes and the extraction of intracellular compounds from damaged cells. Parniakov et al [29] also showed that pulse electric field increases the efficiency of bioactive compounds from papaya seeds.

The presence of antimicrobial compounds in medicinal plants has multiplied the importance of these plants for the production of natural and new antibiotics in medical sciences. Mohammadpour et al[21]reported that the essential oil of *N. binaludensis* showed

moderate antimicrobial activity against *S. aureus* and *B. cereus* as gram positive bacteria and *E.coli* as gram negative bacteria and *C. albicans*as fungi Strain with MIC equal to 6.25 mg/ml, 3.125 mg/ml, 3.125 mg/ml and 12.5 mg/ml, respectively. They found 1, 8-cineol and α-terpineol as major compounds were in essential oil of *N. binaludensis*. Kumar et al [30]were found *N. leucophylla* and *N. ciliaris*oils more effective, in the terms of the MIC against most of the plant pathogens tested, with their respective MIC values ranging from 1000 to 3000 µg per ml.

The inhibitory properties of microbial growth are related to the phenolic compounds of the extracts that are soluble in polar solvents. Similar amounts of MIC and MFC extract on selected fungi indicates their strong fungicidal and fungistatic effects for PEF and UAE than maceration. Moreover, a lower amount of MIC than MFC in cultured extracts indicates that these extracts have fungistatic properties at lower concentrations and have fungicidal properties at higher. Finally, according to the results obtained from this part of the research, it can be stated that CEx has significant antifungi activity under PEF and UAE rather than maceration extract.

CONCLUSION

The present study revealed that aerial parts of nepeta (*Nepeta binaludensis* Jamzad) have a potential source of active ingredients like polyphenols that are well-known for their antioxidant and antimicrobial properties. Ultrasound and pulse electric field extraction are effective techniques for extraction of these compounds. Optimization of ultrasonic amplitude (UA) and ultrasonic exposure time (UET) and PEF may be necessary to produce high quality and high performance nepeta extract.

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