Optimization of the pulsed electric field-assisted extraction of functional compounds from *Nepeta binaludensis*

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Abstract: Pulsed electric field (PEF) treatment was used for extracting effective components from Nepeta (*Nepeta binaludensis* Jamzad). A response surface method was used to investigate the effects of independent process variables (voltage of pulsed electric field (VPEF): 2, 4 and 6 kV cm^{-1} and number of pulsed electric field (NPEF): 20, 40 and 60 n) on the yield (*Y*) and antioxidant characteristics: total phenolic compounds (TPC), 1,1- diphenyl -2- picrylhydrazyl free radical scavenging ($DPPH_{sc}$), ferric reducing-antioxidant power (FRAP), half maximal of radical-scavenging activity (IC_{50}) of Nepeta extract (aerial parts). According to Derringer's desired function approach, the optimal conditions based on both individual and combinations of all process variables were VPEF 6 kV cm^{-1} and NPEF 60 n. At this optimum condition, the *Y*, TPC, $DPPH_{SC}$, FRAP, and IC_{50} of the extract were found to be 11.36%, 417.85 mg GA g^{-1}, 74.8%, 1688.53 μmol Fe^{2+} g^{-1}, and 0.32 mg mL^{-1}, respectively. The experimental values were in a good agreement with the predicted values. Also, the extract at optimal conditions of PEF (PEF_{opt-x}) had a higher quantity of chlorogenic acid, caffeic acid, rutin, para-coumaric acid, rosemarinic acid, kaempferol, and apigenin compared with solvent extract. The addition of PEF_{opt-x} to the purified soybean oil at the levels of 6% increased oxidative stability index (2.65 h) close to butylated hydroxy toluene (2.78 h).

Keywords: phenolic compounds, *Nepeta binaludensis*, response surface methodology, pulsed electric field, antimicrobial activity

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1 Introduction

Extraction plays a significant and crucial role in the final result and outcome of any medicinal plant. The qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of proper extraction method. Extraction of plant materials can be done by various extraction procedures. Conventional extraction methods with heating, boiling and refluxing are usually used for covering bioactive compounds. These conventional or more innovative extraction techniques may cause the degradation of the targeted compounds due to high temperature and long extraction times. Recently, improved methods have been developed to extract bioactive compounds from plants, for example, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction and pulsed electric field, etc. The use of these techniques has opened up some possibilities of commercialization due to promising results that they deliver.

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Pulsed electric field (PEF) has attracted particular attention due to their simplicity, environment friendliness, economy, consistency and high efficiency for the determination of bioactive compounds and microbial decontamination (Aadil et al., 2015). Use of PEF has been shown to be promising for intracellular extraction from plant food materials. The application of PEF treatment before extraction provokes cell membranes damage, thus facilitating subsequent extraction of nutritionally valuable compounds. Depending on electric conditions, such as electric field strength and number of pulses, PEF-treatment could also control the selectivity of extraction by regulating the degree of membrane destruction (Fincan et al., 2004). To the best of our knowledge, the influence of PEF for the recovery of valuable compounds from *Nepeta binaludensis* has not been reported yet.

Nepeta is a large genus belonging to the *Lamiaceae* family. This genus comprises about 280 species distributed in the central and southern parts of Europe, Asia, and the Middle East. *Nepeta binaludensis* Jamzad is an endemic and rare perennial medicinal plant which distribute in a limited area in Binalud Mountains in the northeast of Iran (Nadjafi et al., 2012). A lot of species of this genus are used in folk medicine as diuretic, diaphoretic, antitussive, antispasmodic, anti-asthmatic, febrifuge, emmenagogue, and sedative effects. Nepeta species contain bioactive phytochemicals such as phenolics, flavonoids, and terpenoids (Formisano et al., 2011).

Response surface methodology (RSM) is a useful tool for evaluating multiple parameters and their interactions using quantitative data; it is an efficacy mathematical and statistical technique for optimizing complex extraction procedures, thus reducing the number of experimental trials required (Bashi et al., 2012).

The main goal of this study was to evaluate and optimize the PEF extraction procedure for maximizing the yield of phenolic compounds and antioxidant activity from aerial parts of Nepeta.

2 Materials and methods

2.1 Plant material

The *N. binaludensis* were collected during the flowering stage of the plant, from Binaloud (Khorasan-Razavi province, Iran) in May. The aerial parts of *N. Binaludensis* were dried in the shade for one week and then were ground to a fine powder in a mill (Mulinex, Depose-Brevete S.G.C.G., France). The powders were sieved in order to maintain particle size unity (400 μm) and then were sealed under vacuum in dark plastic bags to protect from light and were kept at room temperature until used for further studies. Refined, bleached and deodorized soybean oil with no added antioxidants was supplied by Segol (Nishaboor, Iran).

2.2 Chemicals

2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ), Folin–Ciocalteu reagent, gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and all other chemicals and solvents used in this research were obtained from Sigma-Aldrich and Merck.

2.3 Experimental design

A face centered experimental design (FCED) response surface methodology was constructed using Design-Expert version 10.0.3 software (Minneapolis, USA). It was used to evaluate the effect of independent variables [voltage of pulsed electric field, VPEF (X_1) and number of pulsed electric field, NPEF (X_2)] on the extraction yield (Y; %), total phenolic content (TPC, mg gallic acid equivalents (mg GA) g^{-1}), ferric reducing-antioxidant power (FRAP, μmol Fe^{2+} g^{-1}), 1,1-diphenyl -2-picrylhydrazyl free radical scavenging ($DPPH_{SC}$, %) and the extract concentration providing half maximal (50%) of radical-scavenging activity (IC_{50}, mg mL^{-1}). The design comprises 13 sets of test conditions for each extraction method where three levels were attributed to each factor at high, central, and low levels, with additional five replicated center points. Each variable was coded at three levels of –1, 0 and +1 (Table 1). Maximum and minimum treatment levels were selected by carrying out preliminary screening tests and according to the literature reports and instrumental aspects.

2.4 Pulsed electric field extraction

Pulsed electric field (PEF) treatment was used for extracting effective components from *nepeta* according to Boussetta et al. (2014) procedure with some modifications (Boussetta et al., 2014). Pulsed electric

field treatment was applied using a Pure Pulse and a batch one-liter treatment chamber with two parallel stainless electrodes. A pulse frequency of 1 Hz was used. 10 g dried plant powder was introduced between the electrodes, and 100 mL of ethanol solvent (1:10 w/v) was added. Pulse generator with voltage of 6 kV and 60 pulses was applied at ambient temperature to the treatment chamber (in preliminary study, extraction conditions, i.e. voltage (4-6 kV cm^{-1}) and number of pulse electric field (20-60 n) were optimized for achieving the best extraction yield of antioxidant compounds). Then the mixture was agitated for 48 h in dark at ambient temperature. The solvent was evaporated in vacuum at 40°C. The dried extract (PEF_x) was stored at –18°C until use.

Table 1 Face-centered composite design of PEF with two variables with the resulting quality response parameters of ethanolic extract of Nepeta

Exp.no	Extraction conditions		Analytical results				
	VPEF (X_1, V cm^{-1})	NPEF (X_2, n)	Yield (%)	TPC (mg g^{-1})	$DPPH_{SC}$ (%)	IC_{50} (mg mL)	FRAP (μmol Fe^{2+} g^{-1})
1	2000(–1)	40(–1)	10.49±0.2	378.65±7.16	62.62±0.91	0.39±0.01	1290.26±41.22
2	6000(+1)	40(–1)	10.69±0.3	354.55±5.43	60.91±1.49	0.41±0.01	1976.66±71.39
3	4000(0)	40(–1)	10.51±0.15	395.99±3.32	57.73±0.19	0.44±0.08	1395/86±11.43
4	4000(0)	50(0)	11.12±0.1	314.17±4.74	62.61±0.76	0.39±0.01	1461.86±34.29
5	6000(+1)	50(0)	11.25±0.12	318.1±4.09	62.52±0.55	0.39±0.01	1529.86±80.02
6	4000(0)	50(0)	11.00±0.2	289.23±6.89	58.09±0.87	0.42±0.02	1151.66±11.43
7	4000(0)	50(0)	11.3±0.15	301.7±4.9	65.81±0.8	0.40±0.01	1245.40±15.09
8	4000(0)	50(0)	11.14±0.11	290.14±5.03	60.00±0.85	0.41±0.02	1339.98±17.11
9	2000(–1)	50(0)	11.15±0.1	338.11±3.06	66.10±0.03	0.37±0.01	1481.66±34.29
10	4000(0)	50(0)	11.12±0.12	311.90±4.85	59.90±0.79	0.42±0.01	1212.59±16.66
11	2000(–1)	60(+1)	11.36±0.23	374.34±0.74	76.57±0.14	0.32±0.01	1679.66±68.59
12	4000(0)	60(+1)	11.28±0.11	368.02±10.8	63.82±1.4	0.39±0.01	1437.46±69.54
13	6000(+1)	60(+1)	11.36±0.25	436.93±1.6	77.91±0.47	0.30±0.01	1757.66±30.24

Note: [a] Analytical results are the means ± SD (n = 3).

VPEF: Voltage of Pulsed Electric Field

NPEF: Number of Pulsed Electric Field

Y: Yield

TPC: Total Phenolic Compound.

FRAP: Ferric Reducing Antioxidant Power

$DPPH_{SC}$: scavenging activity of DPPH

IC_{50}: The Concentration of extract required to scavenge 50% of 2, 2-diphenyl-1-picrylhydrazyl free radical.

2.5 Solvent extraction

Ten grams of dried powder was extracted with ethanol (1:10 w/v) by agitation in a dark place at ambient temperature for 48 h. The solvent was evaporated in vacuum at 40°C. Then, the concentrate was dried in a freeze drier (Operon-Korea, –55°C, 0.15 mmHg) for 48 h. The dried samples (SO_x) were kept in the dark at –18°C for further analysis.

2.6 Determination of extraction yield

The mass ratio of freeze-dried extract and dried powder before extraction was taken as the extraction yield. Indeed, the yield of each extract was calculated from the following equation:

$$\text{Extraction yield } \% = \frac{w_2}{w_1} \times 100 \quad (1)$$

where, w_1 and w_2 are the weight of dried powder and weight of freeze-dried extract, respectively.

2.7 Total phenolic content (TPC)

Total phenolic content (TPC) was determined by Folin–Ciocalteau method (Singleton et al., 1999). Solution samples (100 mg extract in 10 mL of methanol) of 100 μL of the mixed with 6 mL of double distilled water and 500 μL of Folin-Ciocalteau reagent were added and allowed to remain at room temperature for 8.8 min, 1.5 mL of sodium carbonate (20% w/v) were then added. After standing for 30 min at room temperature, absorbance was measured at 765 nm. Results were expressed as mg gallic acid equivalents (GAE) per g sample. A mixture of water and reagents was used as a blank. A calibration curve of gallic acid (concentration range of 0.04-0.40 mg mL^{-1}) in methanol was prepared so that the TPC value could be obtained from the absorbance.

2.8 HPLC Analysis of phenolic compounds

The HPLC analyses of phenolic components were performed according to Zheng and Wang (2001). The plant extract was dissolved in 4 mL of methanol, and 20 µL aliquots an then were passed through a 0.45-µm filter (Millipore, MSI, Westboro, MA) before injection into a C_{18} reverse phase column (Spherisorb ODS-2, 4.6 mm × 25 cm, particle size 5 µm) at room temperature. A Waters 600E system controller coupled with a photodiode array detector (Waters 990 series) was used. The mobile phase was acetonitrile (A) and acidified water containing 2.5% formic acid (B). The gradient was as follows: 0 min, 5% A and 95% B; 10 min, 15% A and 85% B; 30 min, 25% A and 75% B; 35 min, 30% A and 70% B; 50 min, 55% A and 45% B; 55 min, 90% A and 10% B; 57 min, 100% A and then held for 10 min before returning to the initial conditions. The flow rate was 1.0 mL min^{-1} and scanning was carried out between 200 and 400 nm. The data were collected by the chromatography data system (Water 990 3-D). The phenolic components were identified according to their retention time in comparison to the commercial standard. Quantification was carried out using the external standard method and the final concentrations were expressed in mg per g of extract.

2.9 Evaluation of antioxidant activity

2.9.1 DPPH free radical scavenging assay

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity of the extracts (pre-diluted to 90 mg L^{-1} concentration) was measured by DPPH assay as described by Liu et al. (2009) with a slight modification. Aliquots of each extract (200 µL) were added to 3 mL of ethanolic DPPH solutions (0.1 mM). Discolorations were measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) after remaining for 30 min in the dark. The DPPH which was scavenged ($DPPH_{sc}$) was estimated using the Equation (2):

$$DPPH_{sc}\ \% = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \quad (2)$$

where, A_S is the absorbance of the solution when the sample 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}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This sample concentration corresponding to 50% of radical-scavenging activity was calculated by interpolation of the graph of radical-scavenging activity percentage against sample concentration.

2.9.2 Determination of ferric reducing/antioxidant power (FRAP assay)

FRAP assay was carried out by the method of Thaipong et al. (2006) with minor modifications. The principle of this procedure is based on the reduction of the ferrictripyridyl triazine complex to its ferrous, colored form in the presence of antioxidants. The stock solutions comprised 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working solution was made freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as FRAP solution. Sample (150 µL) was mixed with 3 mL of FRAP solution and kept for 30 min in dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Different concentrations of $FeSO_4 \cdot 7H_2O$ were used to obtain the calibration curve.

2.10 Oxidative stability index (OSI)

2.10.1 Purification and preparation of soybean oil

The soybean oil for eliminate natural antioxidant was purified with multilayer column (Aluminum oxide and silica gel) chromatography with modified method previously reported by Belhaj et al. (2010). Aluminum oxide and silica gel were used as absorbent which had been activated at 200°C for 3 h and 160°C for 3 h before use, respectively. 100 g of oil were added at the top of the multilayer chromatographic column. The bottom layer of chromatographic column (50×3 cm i.d.) was Aluminum oxide 60 (55 g, active, neutral) and the top layer was silica gel (85 g, activate, 60-200 mesh). Aluminum foil was used for wrapping chromatographic column and collection vessels, and the oil was drawn by suction without any solvent.

The purified soybean oil (PSO) was blended separately with 0.5%, 2%, 4% and 6% (w/w) of the optimized PEF_X of Nepeta ($PEF_{Opt\text{-}X}$), and 100 mg kg^{-1}

of BHT, and then exposed to the following stability test (Rancimat test).

2.10.2 Rancimat test

The oxidative stability index (OSI) was measured using a Metrohm Rancimat model 743 (Herisau, Switzerland) set for 3 g oil samples (in duplicate) at 120˚C, and at an airflow rate of 20 L h^{-1} (Méndez et al., 1996).

2.11 Statistical analysis

Since the various responses were the result of various interactions of independent variables, the following second order polynomial regression equation was fitted to the experimental data of all responses, Equation (3).

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{ij} X_j^2 + \sum_{i=1}^{j-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

where, Y represents predicted response; β_0 is a constant; β_j, β_{jj} and β_{ij} are linear, squared, and interaction coefficients, respectively; X_i and X_j are the independent variables and ε is noise or error (Azarpazhooh and Ramaswamy, 2012). The quality of the fitted polynomial models was expressed by regression coefficient (R^2), adjusted R^2, adequate precision (AP), and coefficient of variation (CV).

3 Results and discussion

3.1 Model fitting

Extraction variables were voltage of pulse electric field (VPEF; X_1) and number of pulse electric field (NPEF; X_2). The design for combined effects comprised of 13 experiments according to Table 1. Also, the levels of experimental variables used in this study are presented in Table 1. The extraction yields ranged from 10.51% to 11.36 %, TPC from 289.23 to 436.93 mg GA g^{-1} sample, FRAP from 1151.66 to 1976.66 μmol Fe^{2+} g^{-1}, $DPPH_{SC}$ from 57.73% to 77.91 %, and IC_{50} from 0.3 to 0.44 mg mL^{-1}.

Table 2 presents the analysis of variance (ANOVA) for the response surface quadratic model. Non-significant variables were omitted, and the remaining coefficients were used in final predictive equations. The data showed a good fit with Equation (1), which was statistically acceptable at $p<0.05$, $p<0.01$ and $p<0.001$ and high R^2 values (Myers et al., 2004). The lack of fit was not significant ($p>0.05$). The linear, quadratic were significant and used for the construction of three-dimensional response surface plots to assess the relationship between independent and dependent variables.

Table 2 Analysis of variance for predicted quadratic polynomial models for properties of extracted Nepeta polyphenols

Source	Df	Sum of squares				
		Yield (%)	$DPPH_{SC}$ (%)	IC_{50} (mg mL^{-1})	TPC (mg g^{-1})	FRAP (μmol Fe^{2+} g^{-1})
Model	5	1.07***	389.71*	0.017**	21554.37***	561700*
X_1-VPEF	1	0.015 ns	2.60 ns	0.00006667 ns	56.92 ns	110100**
X_2-NPEF	1	0.89***	228.66***	0.008817***	418.33 ns	7490.67 ns
X_1X_2	1	0.01 ns	2.33 ns	0.0004 ns	1878.79 ns	92537.64**
X_1^2	1	0.014 ns	92.25**	0.005214***	688.07 ns	160200**
X_2^2	1	0.15***	13.91 ns	0.0001971 ns	13411.34***	63568.95 ns
Residual	7	0.046	91.61	0.002053	2909.66	97566.38
Lack of Fit	3	0.00	55.60	0.001373	2361.35	38709.70
Pure Error	4	0.046	36.01	0.00068	548.31	58856.68
Cor Total	12	1.11	481.32	0.019	24464.03	659300
R-Squared		0.96	0.81	0.90	0.88	118.06
Adj R-Squared		0.93	0.68	0.81	0.80	1458.51
Pred R-Squared		0.94	-0.13	0.32	0.03	8.09
Adeq Precision		15.78	7.42	10.897	8.56	477100

Note: ns, Not significant ($p>0.05$).

* Significant at ($p<0.05$); ** Significant at ($p<0.01$); ***Significant at ($p<0.001$).

3.2 Effects of extraction conditions on the extraction yield

The extractions yield and the regression analysis of the data are presented in Tables 2 and 3, respectively. The regression analysis of the data showed that effects of linear and quadratic term coefficients of number of pulses (NPEF) on yield were significant ($p<0.05$). Equation (4) shows relationship between variables on yield:

$$Y = 11.15 + 0.38X_2 - 0.21 \times X_2^2 \quad (4)$$

The three-dimensional response surface plot (Figure 1a) illustrates the effects of experimental variables on the yield of extract. The maximum yield was achieved at a higher number and voltage pulse. They are synergic and therefore the combination of the two can be expected to enhance the extraction yield.

It has been well demonstrated that PEF increased the permeability of cell membrane (Wiktor et al., 2011). As the major effect to permeabilization, electroporation was still proposed to explain the extraction mechanisms of PEF treatment. Electroporation is the phenomenon in which a cell exposed to electric field pulses temporarily destabilizes the lipid bilayer and proteins of cell membranes. Therefore, the membrane molecules in the cell membrane separate according to their charge under their dipole nature. When the trans-membrane potential exceeds a critical value (typically 0.2-1.0 V for most cell membrane), pore occurs in weak areas of the membrane and causes a drastic increase of permeability. Pore formation is a dynamic process and can be reversible or irreversible depending on the treatment intensity. When the size and number of pores reach the critical value related to the total membrane surface, reversible breakdown turns into the irreversible breakdown, which causes the perpetual mechanical destruction of the cell membrane (Janositz and Knorr, 2010). Some research suggested that the expansion of the pores depend on the intensity of the electric field, pulse duration, and the ionic strength of the medium. Yi et al. (2006) found that when the PEF intensity was increased from 5 to 20 kV cm^{-1}, the extraction recovery of polysaccharide from *Rana temporaria chensinensis* was increased rapidly from 17.11% to 26.87%. Similar results were reported in the extraction of chondroitin sulfate from fish bone (He et al., 2014). When the electric field intensity exceeded 15 kV cm^{-1}, the growth rate of content of chondroitin sulfate leveled off, and when the electric field intensity reached 25 kV cm^{-1}, the content was maximized to 5.84 g L^{-1}.

Pulse duration is one of the important indicators that measure PEF treatment, defined as the product of the pulse numbers and pulse width. An increase in either of pulse numbers and pulse width enhances cell inactivation. As it can be shown in Figure 1a, the disruption of cell membranes increases gradually with increased number of pulses. The increase of disruption of cell membranes causes extraction of intracellular components from damaged cells. Parniakov et al. (2015) reported that increase PEF treatment number on papaya seeds caused by increasing extraction polyphenol yields.

3.3 Effects of extraction parameters on TPC

Total phenolic content data from PEF_x based on the Faced-central composite design is shown in Table 1. Multiple regression analysis was performed on the experimental data. Besides, the coefficients of the model were evaluated for significance. The values of the coefficients for TPC are presented in Table 2 were used for arriving at the final predictive model by neglecting the non-significant cross-terms as given below (Equation (5)):

$$TPC = 309.05 + 3.085X_1 - 8.35X_2 + 21.67X_1X_2 + 75.70X_1^2 \quad (5)$$

To illustrate the influence of variables on TPC, three-dimensional surface plots (Figure 1b) were constructed according to Equation (5). TPC increased slowly with the increase of VPEF and nearly reached a peak at 6 kV cm^{-1}, while it decreased by increasing NPEF from 40 to 50 n to approximately 290.14 mg g^{-1} and then the TPC increased with increasing number of pulses to 60 n.

It seems increasing number and voltage of pulses cause an increment in the number of permeated cells or in the permeabilization area of the cells by increasing the number or the size of pores, resulting in increasing of polyphenol extraction with release intracellular compounds into the solvent. Our results are in agreement with Álvarez et al. (2003) that reported an increment in the extraction efficiency by increasing the electric field strength applied.

3.4 Effect of process variables on antioxidant compounds

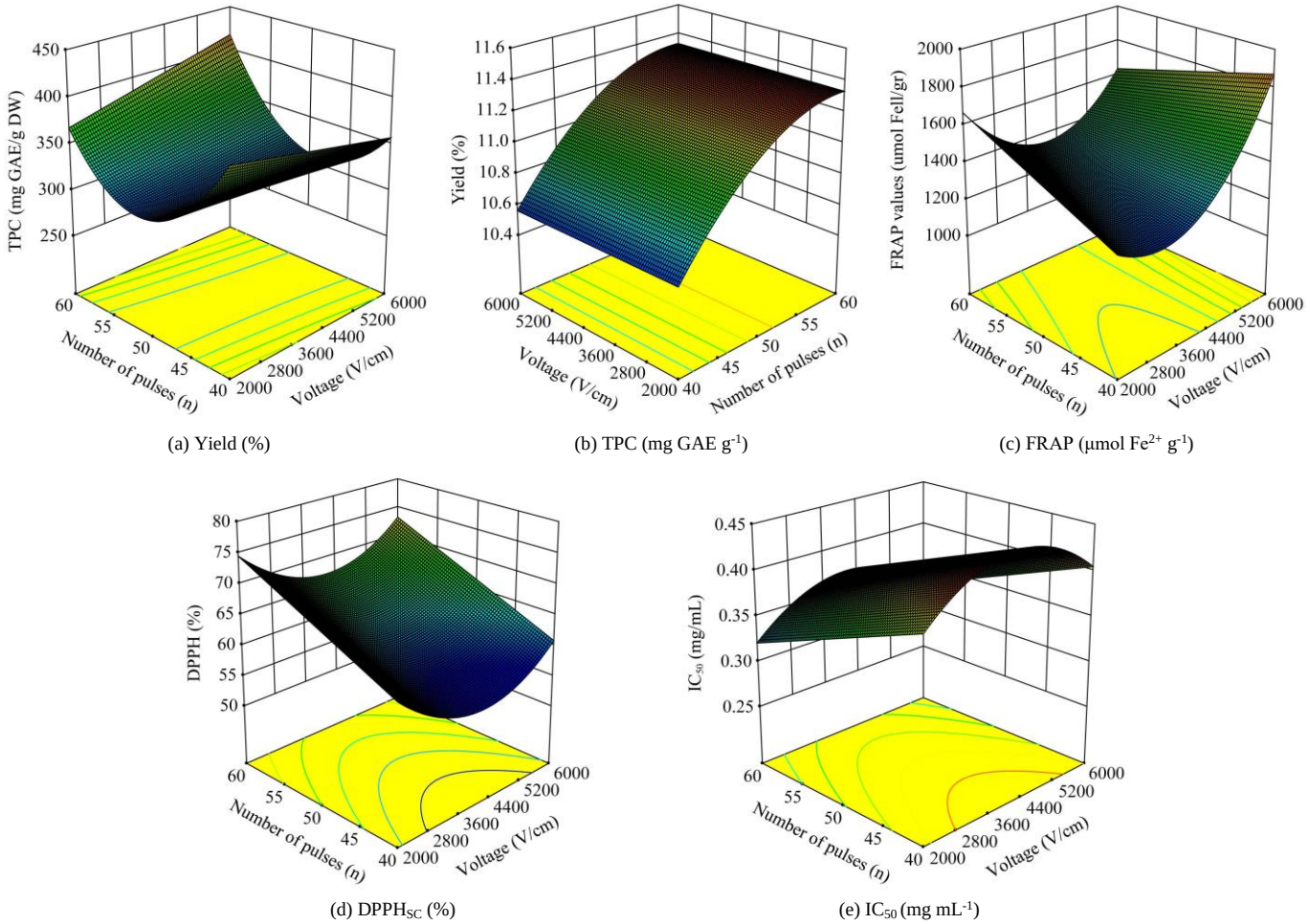
The analytical results of antioxidant activities (FRAP, $DPPH_{SC}$, and IC_{50}) of PEF_x are shown in Table 2. The regression equation for the response of antioxidant activities (*FRAP*, $DPPH_{SC}$, and IC_{50}) are given below (Equation (6), (7) and (8), respectively):

$$FRAP = 1320.69 - 135.43X_1 + 33.95X_2 - 152.10X_1X_2 + 298.61X_1^2 \quad (6)$$

$$DPPH_{SC} = 61.14 - 0.66X_1 + 6.17X_2 + 6.63X_1^2 \quad (7)$$

$$IC_{50} = 0.41 + 3.33X_1 - 0.038X_2 - 0.047X_1^2 \quad (8)$$

The response surface plots are shown in Figure 1, c, d, e. Both VPEF and NPEF had a positive effect (Figure 1c) on FRAP which decreased with increasing VPEF up to 4 kV cm^{-1} and followed increase while elevating NPEF resulted in increasing FRAP. FRAP assay is usually applied to study the antioxidant capacity of plant materials. The antioxidant capacity of PEF_x is determined by the ability of the antioxidants in these extracts to reduce ferric iron to ferrous. The reduction of ferric iron in FRAP reagent will cause the formation of a blue product (ferrous-TPTZ complex) (Jayaprakasha et al., 2001).



(a) Yield (%) (b) TPC (mg GAE g^{-1}) (c) FRAP (μmol Fe^{2+} g^{-1})

(d) $DPPH_{SC}$ (%) (e) IC_{50} (mg mL^{-1})

Figure 1 Response surface plots of the yield, total phenolic contents and antioxidant activity of PEFx as affected by voltage (V cm^{-1}) and number of pulses (n).

When PEF are applied, the electroporation creates pores in the membrane so helps to extract the phenolic compounds. As it was finally observed, the antioxidant activity is related to the phenolic content and is strongly dependent on the concentration of extract. This strong dependence in many studies has shown that phenolic compounds are responsible for the antioxidant activity (Craft et al., 2012; Moure et al., 2001). Proven by many studies, plant polyphenols have good antioxidant activities. Thanks to the antioxidant properties of phenolic compounds, they play an important role in removing free radicals, heavy metals, and preventing the formation of hydroperoxides in plants' cell (Nogala-Kalucka et al., 2005). As it mentions the extraction of polyphenols was improved by increasing the PEF voltage and number of pulses.

The DPPH free radical is commonly used for evaluation of antioxidant activities of compounds. Figure 1d shows the interactive effect of VPEF and NPEF on DPPH activity. Again, the DPPH values decreased by increasing VPEF up to 4 kV cm^{-1}, while it increased gradually by increasing NPEF from 40 to 60 n. Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective

antioxidants than vitamins E or C. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. The reduction activity of phenolic acids and their esters depends on the number of free hydroxyl groups in the molecule, which is manifested in high capacity to donate protons and thus stabilize DPPH radical (Rice-Evans et al., 1996). Therefore, the increment of the % $DPPH_{SC}$ of the orange peel extract by increasing the electric field strength intensity and number of pulse is probably correlated with the higher content of polyphenols in the extract. Moreover, Luengo et al. (2013) reported that the extraction of polyphenols of orange peel improved by pulsed electric fields.

There was an inverse relationship between IC_{50} and inhibition power. IC_{50} represents the concentration of the extract required for inhibiting 50% of DPPH free radicals. Response plots of VPEF and NPEF on the IC_{50} are illustrated in Figure 1e. Again, a decrease was observed in IC_{50} with the increase in VPEF until 4 kV cm^{-1}, and while further increase in VPEF increased.

3.5 Optimization of PEF and verification

After the development of the models for various responses (yield, TPC, FRAP, DPPH, and IC_{50}), optimization of the process variables for achieving targeted outputs was considered. The input parameters were constrained, for example, VPEF and NPEF within the experimental range, and the target was to obtain maximum yield, TPC, FRAP and DPPH, and minimum IC_{50} from PEF_x. The predicted values of responses have been summarized in Table 3. The suitability of the model equation for predicting the optimum response values as targeted was tested using the selected optimal conditions. The predicted optimum value for VPEF and NPEF were: 6 kV cm^{-1} and 60 n, respectively. Under this processing conditions, the experimental outputs of yield, TPC, FRAP, and $DPPH_{SC}$ were increased from 7.9% to 11.33%, from 101.93 to 413.36 mg GA g^{-1}, from 1613.32 to 1688.53 μmol Fe^{2+} g^{-1} and from 65.06% to 74.80% respectively, but also IC_{50} decreased from 0.41 to 0.32mg mL^{-1} the SOx (Table 3).

Table 3 Predicted and experimental values of the responses at optimum conditions

	Yield (%)	TPC (mg GA g^{-1})	$DPPH_{SC}$ (%)	IC_{50} (mg mL^{-1})	FRAP (μmol Fe^{2+} g^{-1})
Predicted values	11.36	417.85	74.80	0.32	1688.53
Experimental values	11.4 ± 0.03	425.93 ± 5	75.06 ± 0.05	0.31 ± 0.01	1697.06 ± 14.99
SO_X	7.9 ± 0.11	101.93 ± 2.52	65.06 ± 1.05	0.41± 0.01	1613.32 ± 14.99

Note: [a] Predicted using response surface quadratic model; [b]Mean ± standard deviation of triplicate determinations from experiments

Barba et al. (2015) found also that the TPC recovery of PEF on blackberries after supplementary extraction was 6-fold and 4-fold higher after hot water and ethanol extraction, respectively. It has been observed that PEF treatments improve the extraction of polyphenols from grape skin by-products (Vorobiev and Lebovka, 2010) or increase concentration of polyphenols in juices obtained from apple mash and whole grapes (Grimi et al., 2009, 2011; Jaeger et al., 2012). Moreover, Corrales et al. (2008) reported that the anthocyanin extraction yields up to 17% by applying electrical field at 3 kV cm^{-1} and ethanol concentration of 50% compared to the conventional extraction from the grape. Also, It has been observed that PEF treatment increased the antioxidant activity of grape byproduct extracts, Orange peel and apple juice extracted by PEF approximately two-fold higher than that to the control (press extraction) (Corrales et al., 2008; Grimi et al., 2011; Luengo et al., 2013).

3.6 Identification and quantification of TPC

The amounts of phenolic components in extracts (SO_x and PEF_{OPT-X}) determined by HPLC are shown in Table 4. The identification of phenolic compounds helps to explain the strong antioxidant properties of the extract. The chlorogenic acid, caffeic acid, rutin, vanillin, para-coumaric acid, rosemarinic acid, kaempferol and apigenin determined in the ethanolic extract of *Nepeta binaludensis*. The most abundant phenolic compounds in both treated and untreated samples were rosemarinic acid (235.70 mg g^{-1}) and apigenin (105.78 mg g^{-1}) (Table 4). There are reports indicating that rosemarinic acid have an anti-Warburg effect (glycolytic production of adenosine triphosphate (ATP) under aerobic conditions) (Janicsák et al., 1999), human immunodeficiency virus type 1 (HIV-1) (Mazumder et al., 1997) and gastric carcinoma (Janicsák

et al., 1999).

Table 4 The individual phenolic composition (mg/g extract) of the extracts

Phenolic composition	RT (s)	SO_x	PEF_{opt-x}
Total phenolic compound	-	117.80	449.25
Chlorogenic	17.48	7.51	16.30
Caffeic acid	19.68	0.77	22.24
Rutin	28.18	0.46	29.71
Vanillin	31.83	7.42	2.54
Para-coumaric acid	32.87	6.63	16.21
Rosemarinic acid	35.15	33.37	235.71
Kaempferol	36.08	20.68	20.78
Apigenin	41.13	40.97	105.78

Also, the result showed that in comparison with solvent extraction, PEF treatment increased the quantity of chlorogenic acid, caffeic acid, rutin, para-coumaric acid, rosemarinic acids, Kaempferol and apigenin in extract 117.04%, 788.31%, 6358.70%, 144.49%, 606.35%, 0.48% and 158.18%, respectively. However, the PEF decreased vanillin content from 7.42 to 2.54 mg g^{-1} (63.34% decreases) compared with solvent extraction.

3.8 Oxidative stability of PEF_{OPT-X}

The effects of adding 0.5%-6% of PEF_{OPT-X} and also 0.02% of BHT on the OSI (oxidative stability index) of the purified soybean oil (PSO) at 120˚C and air flow rate of 20 liters per hour are shown in Table 5. Rancimat test is one of the most widely-known accelerated methods for measuring the oxidative stability of oils and edible fats, which automatically measures the electrical conductivity of the changing of conductivity caused by volatile organic acids produced in oxidation (essentially formic acid). The moment when a rapid rise in conductivity occurs is the endpoint of Rancimat test and is known as OSI (Frankel, 2012).

Table 5 The oil stability index (OSI, h) of the PSO as affected by BHT (200 mg kg^{-1}), and different concentrations of PEF_{opt-x} (0.5%-6%) at 120°C

Sample		OSI (h)
PSO		0.77 ± 0.11 e
PSO+BHT		2.70 ± 0.1 a
PSO+PEF_{opt-x}	0.5	1.40 ± 0.03 d
	2	1.59 ± 0.03 c
	4	1.89 ± 0.04 b
	6	2.65 ± 0.01 a

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

The presence of high levels of unsaturated fatty acids (linoleic acids and linolenic acids), has made PSO very susceptible to oxidation (OSI=0.77 h). The PEF_{OPT-X} increased the OSI of the PSO from 0.77 to 2.65 h. The OSIs increased as the concentration of extract increased. The highest significant OSI was found in the PSO containing 6% PEF_{OPT-X} that improved 3.44 times the oxidative stability of PSO. The higher amounts of phenolic compounds especially rosemarinic acid caused to desirable antioxidative effects of PEF_{OPT-X} (Table 5). These antioxidant compounds with free radical scavenging effects prevent the formation of hydroperoxides and also delay the production of secondary oxidative compounds (Farhoosh et al., 2011). Pedro et al. (2018) showed that organic Goji berry extract had more effective antioxidative effects in soybean oil in comparison with BHT and BHA because of the presence of different antioxidant compounds.

4 Conclusions

The present study revealed that *Nepata binaludensis* extract has a potential source of active ingredients such as polyphenols that are well-known for their antioxidative properties. Pulse electric field extraction is an effective technique for extraction of these compounds. Optimization voltage of pulsed electric field and number of pulse electric field number strongly influence the number of bioactive components in terms of quantity, quality and antioxidative activity.

Abbreviates

PEF	Pulsed electric fields
FCD	Face-centered experimental design
RSM	Response surface methodology
Y	Yield (%)
TPC	Total phenolic compounds
$DPPH_{SC}$	Radical scavenging activity of DPPH
FRAP	Ferric reducing-antioxidant power
IC_{50}	50% of radical-scavenging activity
VPEF	The voltage of pulsed electric field (V cm^{-1})
NPEF	The number of pulsed electric field
PEF_{opt-x}	The extract at optimal conditions of PEF
PEF_x	The pulsed electric field extract
SO_x	The solvent extract

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