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Optimization of biologically active substances extraction process from *Potentilla reptans* L. aerial parts

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ABSTRACT

European cinquefoil (*Potentilla reptans* L.) (Rosaceae) is very common in the Central Europe and Balkan Peninsula. Since ancient times the European cinquefoil have been traditionally used in herbal medicine for treatment of tooth ache, ulcers and inflammation of the throat, as well as certain forms of cancer, infections due to bacteria, fungi and viruses, diarrhea, diabetes mellitus and other ailments. Most species of genus *Potentilla* contain hydrolysable tannins, proanthocyanidins, flavonoids and triterpenes as bioactive substances. The aim of this study was to establish the most suitable condition for extraction of biologically active substances from *Potentilla reptans* aerial parts. The influence of the time of the ultrasonic extraction and solvent concentration (ethanol-water) in different ratio over the extraction process was studied. The optimal conditions for the extraction of biologically active substances from European cinquefoil were as follow: 40% ethanol-water as solvent system and extraction time 45 min in ultrasonic bath with frequency 35 kHz. Under these conditions the maximum values of total polyphenols content, total proanthocyanidins and total hydrolysable tannins (60.2 mg GAE/ g dw, 93.1 mg LE/100g dw, 101.3 mg TAE /g dw) were obtained.

INTRODUCTION

The *Potentilla reptans* L. is a member of the family Rosaceae, distributed in the Northern hemisphere. The aerial parts of this plant have been applied in traditional medicine for the treatment of uterine fibroids, tumors, hemorrhoids, inflammation of the stomach and intestines, diarrhea, liver disease, inflammation of the eyes (Tomczyka and Lattéb, 2009).

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The water and 70% ethanol extracts from *Potentilla* species aerial part and roots possessed antidiarrhoic, anti-ulcerogenic, anti-neoplastic, antiviral, antimicrobial, antihyperglycemic, anti- inflammatory, spasmolytic, hepatoprotective and antioxidative activities (Tomovic *et al.*, 2015, Tomczyk *et al.*, 2010, Watkins *et al.*, 2012). However, the medicinal plants are the important source of natural antioxidants (Irulandi *et al.*, 2016). The high content of tannins, phenolic acids, flavonoids and triterpenes, presenting in the different parts of the plant could explain most of the observed biological effects (Tomovic *et al.*, 2015 Tomczyk *et al.*, 2010, Mari *et al.*, 2013 Hoffmann *et al.*, 2016).

The aim of current study was to establish the best condition for extraction of biologically active substances from *Potentilla reptans* aerial parts. The influence of the duration of the ultrasound-assisted extraction and solvent system (ethanol-water) in different concentration ratio over the extraction process has been studied.

MATERIALS AND METHODS

Plant material

Aerial parts (leaves) *Potentilla reptans* (ALIN, Bach number L56. 02.2015) was purchased from a local herbal pharmacy in May 2016, Plovdiv. The samples were finely ground by laboratory homogenizer. The powder was used for extraction of biologically active compounds.

Extraction procedure

Half gram of dry ground leaves were placed in a plastic tube and 50 mL solvent in different ratio mixture ethanol (from 20% to 60%) and water was added. Ultrasound-assisted extraction was performed in ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) with frequency 35 kHz and power 240 W at temperature 30 °C for different time (from 15 to 45 min.). The ratio between ethanol-water and extraction time were varied in order to obtain the highest yield of biologically active substances.

Total proanthocyanidins assay

Acid butanol assay for proanthocyanidins, described by Porter *et al.*, (1986) was used. Briefly, six milliliter of the acid butanol reagent (950 mL of n-butanol with 50 mL concentrated HCl), 0.5 mL aliquot of the sample, and 0.1 mL of the iron reagent (2 % ferric ammonium sulfate in 2 mol/L HCl) were mixed to 10 mL screw cap tube and then vortexed. The tube was capped loosely and put in a boiling water bath for 50 min. The absorbance of formed colored complex was read at 550 nm. Condensed tannins were expressed as leucosyanidin equivalent (LE) per 100 grams herb (Hagerman, 2011).

Total phenolics

The total phenolic contents were measured using a Folin-Ciocalteu assay. Folin- Ciocalteu reagent (1 mL) (Sigma) diluted five times was mixed with 0.2 mL of sample and 0.8 mL 7.5% Na_2CO_3. The reaction was 20 min at room temperature in darkness. After reaction time, the absorption of sample was recorded at 765 nm against blank sample, developed the same way but without extract. The results were expressed as mg equivalent of gallic acid (GAE) per g dry weight (DW), according to calibration curve, built in range of 0.02 - 0.10 mg gallic acid (Sigma) used as a standard.

Total tannins assay

Phenolic Browning Assay

A spectrophotometric assay of the rate of browning of low-molecular-weight phenolic compounds was adapted to measure the browning of tannins. The samples 0.3 mL (diluted 1:2 with 70% ethanol) was dissolved in 7.7 mL pH 10 buffer (5 mM Na_2CO_3: 5 mM $NaHCO_3$ in ratio 6:4). Absorbance was measured at 415 nm, beginning at 15 sec after the addition of the sample. Subsequent measurements were made with a kinetic protocol every 60 sec over a period of 8 min. The initial, linear rate of browning (Abs/min) was measured within the first 6 min of the reaction. A pentagalloyl glucose standard was run on each day to confirm that measurements were consistent through time. Plots of browning rate vs. sample concentration (mg) were made for each sample to confirm that rate was a linear function of concentration for each compound. The slopes of these plots were used as the browning rates (normalized for sample concentration as Abs/min/mg phenolics) (Moilanen, 2015).

Antioxidant activities

DPPH assay

Each analyzed extract (0.15 mL) was mixed with 2.85 mL freshly prepared 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, Sigma) in methanol (Merck). The reaction was performed at 37 °C at darkness and the absorptions at 517 nm were recorded after 15 min against methanol. The antioxidant activity was expressed as mM Trolox equivalents (TE) per g dry weight (DW) by using calibration curve, built by 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Fluka) dissolved in methanol (Sigma).

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to method, as follows: the FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3.6H_2O$ (Merck) in d. H_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. Blank sample, prepared with methanol instead of extract was developed as well. The reaction time was 10 min at 37 °C in darkness and the absorbance at 593 nm of sample against blank was recorded. Antioxidant activity was expressed as mM TE/g DW by using calibration curve, build in range of 0.05- 0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

Response surface optimization method

In order to evaluate the effects of extraction parameters and optimize conditions for various responses response surface methodology (RSM) optimization method was applied. Independent variables used in experimental design were solvent concentration (20, 40, 60 %) and extraction time (15, 30, 45 min). The coded and uncoded independent variables used in the RSM design are listed in Table 1. Ranges of ethanol (X_1) and time (X_2) and the central points were selected based on literature data. Statistical analysis of experiment was performed using Statistical Software MINITAB 16.

Table 1: Coded and uncoded levels of independent variables used in the response surface methodology.

Independent variable	Symbol	Level Low (-1)	Middle (0)	High (+1)
Solvent concentration, %	X_1	20	40	60
Time, min	X_2	15	30	45

The response variables were fitted to the following second-order polynomial model (Eq. 1), which was able to describe the relationship between the dependent output variable and the independent variables:

$$Y = b_0 + \sum b_i \, x_i + \sum b_{ii} \, x_i^2 + \sum b_{ij} \, x_i x_j \quad (1)$$

where Y represents response variable (total phenolic, total tanins, total proanthocyanidines and antioxidant activity – DPPH and FRAP methods); Xi and Xj are the independent variables (Table 1); β0, βi, βii and βij are the regression coefficients for the intercept, linear, quadratic and interaction coefficient, respectively.

RESULTS AND DISCUSSION

There is an increasing interest for using of phytochemicals from natural plants. The extracts obtained from different plant origin had different effects for human health. Extraction efficiency depends on large number of parameters - extraction method, solvents, temperature, extraction time. In this reason it is very important to find optimal extraction parameters for obtaining extracts with the highest content of biologically active compounds (Cvetanovic *et al.*, 2015, Mašković *et al.*, 2016). Response surface methodology is an effective technique for optimization of complex process, because it allows efficient and easier interpretation of experiments (Bezerra *et al.*, 2008, Zekovic *et al.*, 2014). Several researchers already employed RSM for the optimization of extraction process in order to maximize yield of various polyphenolic compounds from various sources (Radojković *et al.*, 2012, Kim *et al.*, 2014, Claus *et al.*, 2015, Mašković *et al.*, 2016). In the presented research, RSM was used to optimize the solid–liquid extraction of compounds with improved antioxidant ability from *Potentilla reptans* L. The influence of solvent composition and time on the extraction yield of total polyphenols, total hydrolyzed tannins, total proanthocyanidines and antioxidant activity was investigated. The experiments were designed according to RSM design, and results are presented in Table 2. The effect of linear, quadratic or interaction coefficients on the response was tested for significance by analysis of variance. Experimental results from Table 3 were processed with multiple linear regressions using the second-order polynomial model – Eq. (1). The regression coefficients of the intercept, linear, cross product and quadratic terms are presented in the Table 3.

Suitability of the model was also analysed by the MINITAB 16. Calculated statistical parameters are presented in Table 3. According to the *p*-values of the F-value for suggested model was suitable for the investigated extraction system. Model equations for relationship between total phenol content, total hydrolyzed tannins, total proanthocyanidines content and antioxidant activity and independent variables were obtained by applying multiple regression analysis (Table 3). By applying these equations, it is possible to predict the values of each response. The values of R^2 for total polyphenol content, total hydrolyzed tannins content, total proanthocyanidines and antioxidant activity were 0.74, 0.94, 0.79 and 0.96 (FRAP method) and 0.95 (DPPH method), respectively (Table 3). Therefore, it was suggested that quadratic model fitted well with the experimental data.

Table 2: Experimental matrix and values of the observed responses of total polyphenolics, total tannins, total proanthocyanidines, and antioxidant activities (FRAP and DPPH methods).

Independent variable values		Corresponding values				
X_1 (Solvent ratio) ethanol, %	X_2 Extraction time, min	TPC, mg GAE /g	Tannins, mg TAE /g	Proanthocyanidines, mg LE/ 100g	FRAP, mM TE /g	DPPH, mM TE /g
20	15	45.2	40.8	30.4	244.0	270.4
40	15	53.1	74.1	65.2	283.2	319.4
60	15	54.9	74.9	57.9	374.2	302.6
20	30	51.5	47.3	29.4	325.3	289.4
40	30	52.8	84.1	60.7	328.9	355.3
60	30	54.8	86.4	81.9	248.0	314.9
20	45	47.9	53.4	34.8	434.4	326.8
40	45	60.2	101.3	70.5	329.4	353.1
60	45	54.1	78.9	93.1	329.0	329.7

Table 3: Regression equation coefficients for the selected responses.

Variable	Regression coefficient	F-value	*p*-value
Total polyphenols concentration			
Intercept b_0	25.10		
Linear		2.91	0.198
b_i	1.04	4.78	0.117
b_j	0.33	1.04	0.383
Square (quadratic)		1.24	0.405
b_{ii}	-9.93 x10^{-3}	2.45	0.216
b_{jj}	-1.92 x10^{-3}	0.03	0.876
Interaction		0.24	0.658
b_{ij}	-2.92 x10^{-3}	0.24	0.658
R^{2a}	0.740		
Total hydrolyzed tannins			
Intercept b_0	-67.76		
Linear		16.28	0.025
b_i	5.61	27.20	0.014
b_j	1.31	5.35	0.104
Square (quadratic)		8.84	0.055
b_{ii}	-57.17 x10^{-3}	17.54	0.025
b_{jj}	-9.00 x10^{-3}	0.14	0.736
Interaction		0.31	0.619
b_{ij}	-7.12 x10^{-3}	0.31	0.619
R^{2a}	0.944		
Total proanthocyanidines			
Intercept b_0	-3.93		
Linear		1.68	0.323
b_i	2.56	0.24	0.660
b_j	-0.88	3.13	0.175
Square (quadratic)		0.59	0.610
b_{ii}	-0.03	0.15	0.728
b_{jj}	-5.96x10^{-3}	1.03	0.386
Interaction		7.11	0.076
b_{ij}	0.03	7.11	0.076
R^{2a}	0.795		
Antioxidant activity			
FRAP method			
Intercept b_0	185.10		
Linear		39.51	0.007
b_i	3.06	71.46	0.003
b_j	1.55	7.56	0.071
Square (quadratic)		2.70	0.213
b_{ii}	0.03	5.32	0.104
b_{jj}	0.14	0.08	0.795
Interaction		5.30	0.105
b_{ij}	-0.19	5.30	0.105
R^{2a}	0.967		
DPPH method			
Intercept b_0	96.72		
Linear		14.32	0.029
b_i	8.62	6.03	0.091
b_j	3.05	22.61	0.018
Square (quadratic)		13.53	0.032
b_{ii}	-0.10	26.90	0.014
b_{jj}	-0.01	0.16	0.713
Interaction		2.11	0.242
b_{ij}	-0.02	2.11	0.242
R^{2a}	0.950		

[a] - Coefficient of multiple determinations.

Table 4: Comparison between theoretically calculated and experimentally obtained yields of total polyphenolics, total tannins, total proanthocyanidines, and antioxidant activities (FRAP and DPPH methods).

	Theoretically calculated			**Experimentally obtained**			**Deviation between $\bar{Y}$ and Y**
	X_1^1	X_2^1	$\bar{Y}$	X_1	X_2	Y	
TPC	45.8	45.0	57.0	40	45	60.2	-5.6 %
Tannins	46.3	45.0	95.4	40	45	101.3	-6.2 %
Proanthocyanidines	60.0	40.0	93.3	60	45	93.1	0.2 %
FRAP	20.0	45.0	435.9	20	45	434.4	0.3 %
DPPH	40.8	45.0	361.2	40	45	353.1	2.2 %

Correlation between antioxidant assays, total phenolic, total proanthocyanidines and total taninns contents.

Table 5: Correlation between antioxidant assays, total phenolic, total proanthocyanidines and total tannins contents

	TPC	**PRO**	**Tannins**	**FRAP**	**DPPH**
TPC	-				
PRO	0.7003	-			
Taninns	0.9006	0.8256	-		
FRAP	0.0256	-0.2056	-0.0405	-	
DPPH	0.6179	0.5579	0.7884	0.34578	-

The optimization procedures carried out using “Response optimizer” of MINITAB 16 software gave the following values of variable X_1 and X_2 for maximum yield of total polyphenolics, total tannins, total proanthocyanidines, and antioxidant activities (FRAP and DPPH methods) (Y) by *Potentilla reptans* L. (Table 4). The deviation between the theoretically studied maximal amounts of total polyphenolics and experimentally obtained (at 45.8% ethanol and 40 min time of extraction) was only 3.2 mg/g DW; total tannins and experimentally obtained (at 40% ethanol and 45 min extraction time) was only 5.9 mg/g DW; total proanthocyanidines and experimentally obtained (at 60% ethanol and 45 min time of extraction) was only 0.2 mg/100g DW; and antioxidant activities (FRAP and DPPH methods) (at 20% ethanol and 45 min time of extraction) (at 40% ethanol and 45 min time of extraction) under ultrasonic influence (Table 4). On this basis we propose 40 % ethanol in water and 45 min time of extraction as the optimal for yield of biologically actives substances from *Potentilla reptans* L leaves. The similar results for amount of total proanthocyanidins and total polyphenols content of *P. reptans* aerial part have been obtained from Tomovic *et al.*, (2015).

Antioxidant activities measured by DPPH and FRAP assays on the one hand and content of total phenolic (TPC), total proanthocyanidines, total tannins on the other hand, were correlated in the different ways (Table 5). Antioxidant assays were moderate to strongly correlate between each other. Antioxidant assays were more strongly correlated to total phenolic and total tannins than to total proanthocyanidines.

CONCLUSION

The optimal conditions for the extraction of biologically active substances from European cinquefoil were as follow 40% ethanol-water as solvent system and time of ultrasonic-assisted extraction 45 min in ultrasound bath with frequency 35 kHz. Under these condition the maximum amount of total polyphenols content, total proanthocyanidins and total hydrolysable tannins (60.2 mg GAE/ g dw, 93.1 mg LE/100g dw, 101.3 mg TAE /g dw) were obtained.

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