Radical scavenger and Antioxidant activities of extracts and fractions from Bulgarian Ononis spinosa L. and GC-MS analysis of Ethanol extract

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Citation

M Valyova, V Hadjimitova, S Stoyanov, Y Ganeva, T Traykov, I Petkov. *Radical scavenger and Antioxidant activities of extracts and fractions from Bulgarian Ononis spinosa L. and GC-MS analysis of Ethanol extract*. The Internet Journal of Alternative Medicine. 2008 Volume 7 Number 2.

Abstract

Extracts and fractions from Ononis spinosa roots were investigated for their radical scavenging activity using two different in vitro assays, 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The antioxidant potential measurements were expressed as Trolox equivalent antioxidant capacity (TEAC). The chloroform fraction possessed the highest radical scavenging activity (RSA) in both assays, DPPH (0.235 mmol g^{-1}) and ABTS (0.264 mmol g^{-1}). Total phenolic content was also determined using Folin–Ciocalteu reagent. The greatest total phenolic content was detected in the EtOAc and $CHCl_3$ fractions from EtOH extract (108.7 mg/g GAE and 102.1 mg/g GAE, respectively). A correlation between radical scavenging capacities of samples with total phenolic compound content was not observed. GC-MS analysis of $CHCl_3$ fraction showed that the triterpene 9, 19-cyclo-27-lanostan-25-on was the major constituent (13.17%), followed by ß-sitosterol (9.61%), medicarpin (9.4%), maackiain (8.01%) and linolic acid (7.98%).

INTRODUCTION

In recent years there has been increasing interest in the presence and availability of compounds in plant materials that may possess bioactive properties, in particular, antioxidant activity. Plant antioxidants are composed of a broad variety of different substances like polyphenolic compounds, tocopherols or terpenoids. Most antioxidants isolated from higher plants are phenolic compounds (e.g. phenolic acids, tannins, coumarins, anthraquinones, flavonoids) ([1]).

The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides ([2] , [3]).

One of the best approaches for discovering new antioxidants is the screening of plant extracts. The goal is to use phytochemicals in foods and pharmaceutical preparations to replace synthetic antioxidants, which are being restricted due to their carcinogenicity ([4] , [5] , [6]).

Ononis is a Fabaceae genus that comprises some 75 species, occurring in the Canaries, the Mediterranean region, Europe to Central Asia ([7]). Several plants belonging to the genus are known to be used in the treatment of jaundice, urinary tract inflammations and kidney stones ([8]). The major phytochemicals detected in Ononis species are flavonoids, phenylpropanoids and resorcinol derivatives ([9] , [10] , [11]). Some members of the genus Ononis have been previously examined for their antioxidant potential ([12] , q [13] , [14]). The species O.spinosa is used in traditional Bulgarian medicines, owing to its various ethnopharmacological properties ([15]). No report available in the literature on the screening of different solvent extracts and fractions of O. spinosa roots for their antioxidant properties. Therefore, the aim of this study is to investigate total phenolic content and the antioxidant property of Bulgarian O. spinosa as a potential source of natural antioxidants. The relationship between phenolic content and antioxidant activity was also statistically investigated. In addition, we report our results on the GC-MS analysis of chloroform fraction of ethanol extract of this species for the first time.

MATERIALS AND METHODS

PLANT MATERIAL

The roots of O. spinosa, which grows wild in Bulgaria were collected in Sevlievo region, Bulgaria. The voucher

specimen SO 105292 has been deposited in the herbarium of faculty of Biology, Sofia University "St. Kliment Ohridski".

PREPARATION OF PLANT EXTRACTS

The air-dried roots of the plant O. spinosa (100 g) were extracted twice with ethanol at 50˚-55˚C for 3 h. The ethanol solutions were evaporated under vacuum to give ethanol extract (yield: 6.68 g). In the same conditions was obtained methanol extract (yield: 15.96 g). The ethanol extract of O. spinosa (6.68 g) was subsequently partitioned between petroleum ether and water. Petroleum ether was separated and evaporated to dryness to get petroleum ether soluble fraction (0.41 g). The aqueous layer was further partitioned by chloroform and ethyl acetate. These were finally evaporated to dryness under reduced pressure to get chloroform (0.31 g) and ethyl acetate (1.16) soluble fractions.

DPPH RADICAL SCAVENGING ASSAY

The antioxidant activity using the DPPH assay was assessed by modifying the method of Blois ([16]). One ml of 0.1 mM DPPH $^{\bullet}$ methanol solution was added to 3 ml solution of the extracts and fractions or 3 ml pure methanol for the blank sample. The absorbance was read at 517 nm after 30 min incubation. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as a reference compound. The Trolox equivalent antioxidant capacity (TEAC) was expressed as mmol Trolox g $^{-1}$ of sample. Unicam UV 500 Spectrophotometer (Thermo Spectronic, UK) and 1 cm disposable cuvettes (Brandt, Germany) were used for all the absorption measurements reported. All the analyses were done in 3 replicates.

ABTS RADICAL CATION SCAVENGING ASSAY

The ABTS $^{\bullet+}$ scavenging test was used to determine the antioxidant activity. ABTS $^{\bullet+}$ radical was obtained by reaction between ABTS and potassium persulfate ([17] , [18]). Blank sample was prepared from the daily solution by adding 1 ml ethanol, which gives an absorbance of 0.7 ± 0.01. The radical scavenging activity was assessed by mixing 2 ml of ABTS $^{\bullet+}$ solution with 1 ml ethanol solutions of the investigated plants with different concentrations. The reactive mixture was allowed to stand at room temperature for 10 min and the absorbance was recorded at 734 nm. The Trolox was used as a standard. The TEAC values were calculated like DPPH assay.

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

Total phenolic content (TPC) in the investigated extracts and fractions was determined by the Folin-Ciocalteu colorimetric method, based on the procedure of Singleton and Rossi ([19] , [20]), using gallic acid as a standard phenolic compound. Briefly, 0.5 ml (three replicates) of the samples was mixed with 3 ml of distilled water and 0.25 ml Folin–Ciocalteu reagent. After 2 min, 0.75 ml of 20% sodium carbonate were added and the volume made up to 5 ml with distilled water. The absorbance of the resulting blue-colored solution was measured at 765 nm after 2 h with intermittent shaking. Quantitative measurements were performed, based on a standard calibration curve of seven points from 0.01 to 0.2 mg/ml of gallic acid in methanol. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg g $^{-1}$ of sample.

GAS CHROMATOGRAPHY AND GC-MS ANALYSIS

The chloroform soluble fraction of ethanol extract was analyzed by GC and GC-MS. GC analysis was carried out on a PERKIN-ELMER Auto System GC, equipped with FID and split/splitless injector and a glass capillary column – HP-5 MS (5 % phenyl dimethylsiloxane) with a length of 30 m, an inside diameter of 0.25 mm and a film thickness of 0.25 μm was used; carrier gas He with linear velocity 42 cm.min $^{-1}$, temperature programmed – from 60°C to 310°C, with 10°C/min. GC/MS analysis was performed on Hewlett-Packard GCD System G 1800A. The optimum conditions of analysis were employed: ionization type: EI; ionization energy: 70 eV; temperature of ion source: 200˚C. The column and temperature program were the same as in GC analyses. The GC–MS peaks were identified by comparison with data from the literature and the profiles from the Wiley 275 and NIST 05 libraries.

RESULTS AND DISCUSSION

EFFECT OF DPPH RADICAL SCAVENGING ACTIVITY

Free radical scavenging activity of methanol extract, ethanol extract and its sub-fractions (petroleum ether, chloroform and ethyl acetate), measured by DPPH assay was reported for the first time (Table 1). The effect of the investigated samples on DPPH radicals has been checked at various concentrations: from 15 to 120 μg/ml for chloroform fraction and from 30 to 240 μg/ml for the total extracts and ethyl acetate fraction. The DPPH scavenging activities expressed

as TEAC values were presented in Table 1. According to these data chloroform fraction was the most efficient free radical scavenger by the highest TEAC value of 0.235 mmol g^{-1} among all the samples. Since TEAC is a quantification of the effective antioxidant activity of the samples, a higher TEAC would imply greater protective action. The methanol extract was the least active of all samples.

EFFECT OF ABTS RADICAL CATION SCAVENGING ACTIVITY

The results of ABTS experiment expressed as TEAC values are presented in Table 1. The TEAC values ranged from 0.095 to 0.264 mmol g^{-1} sample. Various concentrations were used: from 10 to 250 g/ml. The highest scavenging potential (0.264 mmol g^{-1}) was found for chloroform fraction like DPPH method. As can be seen from the results ethanol extract showed the weakest antioxidant activity, which is in contrast to DPPH method. Petroleum ether fraction was inactive in both antioxidant assays.

Figure 1

Table 1. Antioxidant potential of , investigated by DPPH and ABTS methods.

Sample	*DPPH method* *TEAC (mmol g^{-1} extract)*	*ABTS method* *TEAC (mmol g^{-1} extract)*
methanol extract	0.059±0.003	0.110±0.005
ethanol extract	0.075±0.003	0.095±0.001
chloroform fraction	0.235±0.032	0.264±0.049
ethyl acetate fraction	0.084±0.008	0.166±0.021

The values are mean of three replicates ± standard deviation

TOTAL PHENOLIC CONTENT

The total phenolic content in the evaluated samples was determined from a regression equation for the calibration curve (y=8.18181x, R^2 =0.998) and expressed in GAE. The obtained values are summarized in Table 2. The ethyl acetate fraction contained the highest amount, 108.70±0.68 mg GAE/g of sample, which was not in agreement with its lower antioxidant activity in relation to chloroform fraction. The methanol extract showed the lowest total phenolic content, which correspond to its weakest antioxidant potential.

Figure 2

Table 2. Total phenolic content of L.

Samples	*Concentration* *(g/ml)*	*TPC* *(mg GAE g^{-1} sample)*
methanol extract	1 10^{-3}	25.03±1.63
ethanol extract	1 10^{-3}	33.93±1.11
chloroform fraction	2.5 10^{-4}	102.14±5.40
ethyl acetate fraction	2.5 10^{-4}	108.70±0.68

*The values are mean of three replicates ± standard deviation

GC AND GC-MS ANALYSIS

GC and GC-MS analysis of the chloroform soluble fraction of ethanol extract from O. spinosa roots (Table 3) resulted in the identification of 31 constituents, which comprised mostly pterocarpanes (25.84%), saturated and unsaturated fatty acids (free and as esters, 21.6%), steroids (21.03%) and triterpenoids (19.16%).

Figure 3

Table 3. Compounds identified by GC and GC-MS analysis of chloroform fraction from .

Peak	*R_t*	*Compounds*	*M^+*	*Some important*	*% of*
1	10.020	Maltol	126	126, 97, 71, 43	4.00
2	25.283	Pentadecanoic acid	242	73, 129, 60, 43	0.64
3	26.228	Methyl palmitate	270	74, 87, 43	0.71
4	27.269	Palmitic acid	256	43, 73, 60, 57	9.5
5	28.967	Methyl linoleate	294	67, 263, 95, 81, 55	1.28
6	29.047,	Methyl linolenate	292	79, 108, 95, 67, 55	0.5
7	29.415	Methyl stearate	298	74, 87, 43	0.26
8	29.911	Linoleic acid	280	67, 95, 81, 55, 41	7.98
9	30.224	Stearic acid	284	43, 73, 57	0.78
10	34.980	Homopterocarpin	284	284, 269, 148	0.43
11	35.917	Medicarpin	270	270, 255, 105	9.4
12	36.214	Medicarpin-isomer	270	270, 255, 135	5.13
13	36.862	7-Hydroxy-2',4'-	286	164, 151, 121	0.67
14	36.950	Anhydrohomopisatin	282	282, 267, 141	1.67
15	37.351	Maackiain	284	284, 267 , 241, 162	8.01
16	38.680	Squalene	410	69, 81, 41	0.36
17	39.008	7, 2',4'-Trimethoxyisoflavan	300	164, 149,121	1.23
18	40.858	Ergostatriene	380	380, 143,135	1.53
19	41.363	Unidentified	-	-	0.43
20	42.331	Stigmasta-3, 5, 22-triene	394	394, 379, 255	2.33
21	42.676	Stigmasta-3, 5-diene	396	396, 381, 147, 81	1.6
22	45.318	24-ß-Methylcholesterol	400	43, 382, 315, 289,	1.17
23	46.111	Stigmasterol	412	412, 300, 285, 255	2.68
24	47.761	Sitosterol	414	414, 396, 329, 255	9.61
25	48.089	Trimethoxypterocarpan isomer	314	314, 299, 281, 229	1.2
26	48.481	ß-Amyrin	426	218, 203, 189	3.44
27	49.050	Lupenon	424	205, 314, 189	0.83
28	49.715	Taraxasterol	426	207, 189,	1.36
29	50.219	Stigmasta-3, 5-dien-7-one	410	174, 395, 269	1.21
30	51.444	Sitostenone	412	124, 289, 229	0.9
31	58.507	9,19-cyclo-27-Norlanostan-25-	484	95, 424, 409,	13.17

The triterpene 9, 19-cyclo-27-lanostan-25-on was the major constituent (13.17%), followed by ß-sitosterol (9.61%), medicarpin (9.4%), maackiain (8.01%) and linolic acid (7.98%). It should be noted that in the case of O. spinosa the

highest scavenging potential was found for chloroform fraction using two different in vitro assays DPPH (0.235 mmol g^{-1}) and ABTS (0.264mmol g^{-1}). An important point when assessing the antioxidant activity of plant extracts is to consider their interaction with other antioxidants, especially combinations of hydrophilic and lipophilic antioxidants may exert synergistic effects. Phytochemicals such as triterpenes, carotenoids, -tocopherol and vitamin C also contributed to the total antioxidant activities in the plant extracts. Triterpenoids are natural, biologically active compounds extracted from many plants. They possess anti-inflammatory, anticancer and antioxidant properties (21). The presence of triterpene 9, 19-cyclo-27-lanostan-25-on probably contributed to the highest activity of the chloroform fraction.

CONCLUSIONS

The present study elucidated for the first time antioxidant activity of the methanol extract, ethanol extract and fractions from O. spinosa. Particularly important are the results on the antioxidant activity and total phenolic compounds of the chloroform and ethyl acetate fractions. A correlation between radical scavenging capacities and total phenolic content was not observed.

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