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Phenolic composition, enzyme inhibitory, and antioxidant activity of *Bituminaria bituminosa*

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Abstract This study aimed to evaluate the *in vitro* antioxidant and enzyme inhibitory activities of ethyl acetate, methanol, and water extracts of *Bituminaria bituminosa*. In phosphomolybdenum assay, the methanol extract showed the highest activity (166.78 μmol TEs/g dry plant). The water extract exhibited the highest scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2-azino-bis (3-ethylbenzothiazloine-6-sulphonic acid) (ABTS$^{\bullet+}$). In addition, it exhibited the highest activity in cupric ion reducing (CUPRAC) and ferric reducing antioxidant power (FRAP) assays (41.26 and 46.82 μmol TEs/g dry plant). The extracts did not show cholinesterase and tyrosinase inhibitory activity. However, α-glucosidase inhibition assay resulted in the superiority of water extract (1233.86 μmol ACEs/g dry plant). In the case of α-amylase inhibitory assay, the ethyl acetate extract showed the highest activity (53.65 μmol ACEs/g dry plant). The water extract exhibited the highest phenolic content (31.70 μmol GAEs/g dry plant). In contrast, the methanol extract was found rich in flavonoid compounds (5.29 μmol REs/g dry plant). The water extract contained considerable amounts of rosmarinic acid, luteolin, quercetin, and rutin. Therefore, it can be used as a source of new and alternative antioxidant and enzyme inhibitory agents.

Keywords: *Bituminaria bituminosa*, antioxidant activity, enzyme inhibitory activity, phytochemistry

Introduction

Bituminaria bituminosa (*L.*) C. H. Stirton is a perennial wild legume plant and widely distributed throughout the Mediterranean basin. It is commonly named as "Arabian pea" or "pitch trefoil" by the local people (1,2). It is mainly used as forage shrub (1,3,4). It is also used as a hair restoration agent in Madeira Island. Infusions prepared from the fresh leaves are used for the treatment of fever and urinary infections (1,5,6). *B. bituminosa* has been reported to have considerable quantities of phytochemicals having pharmaceutical activities such as furanocoumarins (psoralen, angelicin), pterocarpans (erybraedin C, bitucarpin A), and flavonoids (daidzin, isoorientin) (1,7-9).

Phenolic acids and flavonoids play a preventive role in the development of some cancers and heart diseases. Phenolic compound mixture extracted from red wine can effectively enhances the antioxidant status of plasma in humans (10-12). These compounds are commonly found in many species and herbs. So far, many phenolic compounds having strong antioxidant activity have been identified in the plant species (10).

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) regulate the level of acetylcholine in many tissues. AChE is the most important cholinesterase and mainly distributed throughout the synapses of the brain and neuromuscular junctions (13,14). Accumulation of malignant β-amyloid plaques in the brain tissues of patients with Alzheimer's disease (AD) has been proven to be in association with the increased amounts of AChE and BChE (14,15). Therefore, cholinesterase inhibitors are expected to be useful agents in preventing the development of neurodegenerative diseases such as AD (14,16).

Diabetes mellitus is an important metabolic disorder caused by the deficiency of insulin (17,18). Inhibiting or delaying intestinal carbohydrate digestion can effectively reduce the symptoms of type 2 diabetes mellitus. α-Amylase and α-glucosidase inhibitors can reduce the rate of digestion and delay the absorption of digested products from the intestinal tract (17,19).

Melanin plays an important role in skin pigmentation. When exposed to the ultraviolet radiation, skin cells produce melanin by tyrosinase-mediated melanogenesis (17,20). In recent years, the cosmetic industry has focused on tyrosinase inhibitors such as kojic acid and hydroquinone because of their potent bleaching actions.

However, these compounds have been found to cause severe skin inflammations. Plant-based secondary metabolites could serve as natural anti-pigmentation agents in the cosmetic industry (17,21).

This study aimed to evaluate the *in vitro* antioxidant and enzyme inhibitory activities of ethyl acetate, methanol, and water extracts of *B. bituminosa*. Antioxidant activities of the extracts were analyzed using phosphomolybdenum, radical scavenging, reducing power, and metal chelating assays. Inhibitory activities of the extracts were tested on AChE, BChE, α-amylase, α-glucosidase, and tyrosinase. In parallel to the experiments, phytochemical compositions of the extracts were also evaluated using qualitative and quantitative chromatographic techniques.

Materials and Methods

Chemicals Ferric chloride, Folin–Ciocalteu's reagent and methanol were purchased from Merck (Darmstadt, Germany). DPPH, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), AChE (Type-VI-S, EC 3.1.1.7), BChE (EC 3.1.1.8), 3,4-dihydroxy-L-phenylalanine (L-DOPA), tyrosinase, acetylthiocholine iodide (ATCI), butyrylthiocholine chloride (BTCl) and phenolic standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Plant material Aerial parts of *B. bituminosa* were collected from Sarimsak Mountain, Gokceli village, Andirin-Kahramanmaras-Turkey on 24 June 2015. The senior taxonomist Dr. Olcay Ceylan from the Department of Biology, Mugla University, Mugla-Turkey identified the plant material taxonomically. The voucher specimen was deposited at the Herbarium of the Department of Biology, Mugla University, Mugla-Turkey (GPS coordinates of the collection area: 906 m, 37°33'21.00"N 36°21'53.00"E, Voucher number: MUH 2114).

Preparation of extracts The extracts were prepared according to the method of Sarikurkcu *et al.* (22). Yields of the ethyl acetate, methanol, and water extracts were determined as 4.94, 10.67, and 19.12% (w/w), respectively.

Quantification of phenolic compounds using reversed-phase high-performance liquid chromatography (RP-HPLC) Phenolic compositions of the extracts were determined according to the method of Sarikurkcu *et al.* (22).

Determination of total phenolic and flavonoid compounds Total phenolic and flavonoid contents were determined according to the method of Zengin *et al.* (23).

Total antioxidant activity by phosphomolybdenum method Total antioxidant activities of the samples were evaluated using phosphomolybdenum method described by Zengin *et al.* (24).

Radical scavenging activity Effects of the samples on DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ were measured according to the methods available in literature (24,25).

Reducing power Reducing powers of the samples were investigated using CUPRAC (26) and FRAP assays (24).

Metal chelating activity on ferrous ions Metal chelating activities of the samples on ferrous ions were analyzed using the method given in the literature (24).

Enzyme inhibitory activity Inhibitory activities of the samples on α-amylase, α-glucosidase, and tyrosinase were analyzed using the methods given in the literature (24).

Statistical analysis All the assays were done in triplicate. Results obtained from all the assays were presented as mean and standard deviation values (mean±SD). One-way analysis of variance and Tukey's honest significant difference post hoc test with α=0.05 [Statistical Package for Social Sciences (SPSS) v. 22.0, SPSS Inc., Chicago, IL, USA] were used to determine the statistical differences among the assays. Correlation analyses were performed using a two-tailed Pearson's correlation test.

Results and Discussion

Phytochemical composition The ethyl acetate, methanol, and water extracts of *B. bituminosa* were analyzed for the quantification of phenolics and flavonoids (Table 1). In the first case, the water extract was found to be rich in phenolics (31.70 μmol gallic acid equivalent (GAEs)/g dry plant). It was followed by the methanol and ethyl acetate extracts, respectively. In the latter case, the methanol extract was found to be rich in flavonoids (5.29 μmol rutin equivalent (REs)/g dry plant). In this assay, the water extract also contained considerable amounts of flavonoids (3.17 μmol REs/g dry plant). In contrast, the ethyl acetate extract was found to be quite poor in flavonoids (0.31 μmol REs/g dry plant).

In addition to the qualitative analyses, quantities of twenty-three phytochemicals were determined in the ethyl acetate, methanol, and water extracts of *B. bituminosa* (Table 2). As can be seen from Table 2, the extracts did not contain caffeic acid, chlorogenic acid, eriodictyol, ferulic acid, gallic acid, hesperidin, kaempferol, *o*-coumaric acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, sinapinic acid, and vanillin. In general, the ethyl acetate extract was found to be poor in all of the compounds. The water extract contained the highest amounts of benzoic acid, luteolin, quercetin, rutin, syringic acid, and *trans*-cinnamic acid (152.68, 95.25, 98.05, 155.48, 50.43, and 16.81 μg/g dry plant, respectively). In contrast, the methanol extract had considerable amounts of (-)-epicatechin, apigenin, and rosmarinic acid (151.90, 90.09, 242.02 μg/g dry plant,

Table 1. Amounts of total phenolics and flavonoids of *B. bituminosa* extracts[1)]

Solvent	Total phenolics (μmol GAEs/g dry plant)[2)]	Total flavonoids (μmol REs/g dry plant)[3)]
Ethyl acetate	17.35±0.43[c]	0.31±0.01[c]
Methanol	24.79±0.80[b]	5.29±0.06[a]
Water	31.70±0.25[a]	3.17±0.10[b]

[1)]Data marked with different subscript letters within the same column indicate significant difference statistically (p<0.05)
[2)]GAEs, gallic acid equivalents
[3)]REs, rutin equivalents

respectively). Among the standards screened, rosmarinic acid was determined to be the most abundant compound in the extracts. It was followed by rutin, benzoic acid, and (-)-epicatechin, respectively. As far as our literature survey could ascertain, phytochemistry of *B. bituminosa* has previously been studied by several research groups. According to these studies, furanocoumarins (angelicin and psoralen) and pterocarpans (erybraedin C, bitucarpin A) were the most prominent compounds (3,8,9,27-29). In addition to these compounds, flavonoids, phenolic acids, lignans, saponins, and other volatile compounds (mainly terpenoids) have been reported (1,7,30).

Antioxidant activity *B. bituminosa* extracts were analyzed for their chelating effects (Table 3). As can be seen from the table, metal chelating assay resulted in the superiority of the water extract (9.63 μmol EDTAEs/g dry plant). In this assay, the ethyl acetate extract exhibited the weakest activity (0.96 μmol EDTAEs/g dry plant). The chelating effects of the extracts were observed to statistically differ among each other (p<0.05).

Antioxidant activities of the extracts were analyzed using a phosphomolybdenum assay (Table 3). In this assay, the methanol extract showed the highest activity (166.78 μmol trolox equivalent (TEs)/g dry plant). The water and ethyl acetate extracts also exhibited considerable antioxidant activities (153.01 and 112.42 μmol TEs/g dry plant).

The extracts were also screened for their DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging abilities (Table 4). The water extract exhibited the highest scavenging activity on both radicals. In the case of DPPH$^{\bullet}$ scavenging assay, activity of the water extract was measured as 29.41 μmol TEs/g dry plant. It was followed by the methanol and ethyl acetate extracts, respectively. DPPH$^{\bullet}$ scavenging activities of the extracts were found to be statistically different from each other (p<0.01). In the latter case, the water extract exhibited quite strong scavenging activity on ABTS$^{\bullet+}$ (60.23 μmol TEs/g dry plant). It was closely followed by the methanol extract (59.68 μmol TEs/g dry plant). No statistical difference was observed between the ABTS$^{\bullet+}$ scavenging potentials of the methanol and water extracts (p>0.01). As happened in the first case, the ethyl acetate extract exhibited the weakest activity (33.57 μmol TEs/g dry plant).

In this study, we also investigated the reducing power potentials of

Table 2. Amounts of selected phytochemicals in *B. bituminosa* extracts[1)]

No	Phenolic components	Concentration (μg/g dry plant)		
		Ethyl acetate	Methanol	Water
1	(-)-Epicatechin	ND[2)]	151.90±14.14	ND
2	(+)-Catechin	ND	ND	ND
3	Apigenin	38.53±0.42[c]	90.09±1.54[a]	77.04±1.41[b]
4	Benzoic acid	16.30±0.14[c]	120.13±0.57[b]	152.68±0.42[a]
5	Caffeic acid	ND	ND	ND
6	Chlorogenic acid	ND	ND	ND
7	Eriodictyol	ND	ND	ND
8	Ferulic acid	ND	ND	ND
9	Gallic acid	ND	ND	ND
10	Hesperidin	ND	ND	ND
11	Kaempferol	ND	ND	ND
12	Luteolin	8.90±0.07[c]	37.10±0.28[b]	95.25±4.24[a]
13	*o*-Coumaric acid	ND	ND	ND
14	*p*-Coumaric acid	ND	ND	ND
15	*p*-Hydroxybenzoic acid	ND	ND	ND
16	Protocatechuic acid	ND	ND	ND
17	Quercetin	17.29±0.98[c]	68.90±3.54[b]	98.05±2.83[a]
18	Rosmarinic acid	22.23±0.28[c]	242.02±7.07[a]	198.90±5.66[b]
19	Rutin	13.34±0.27[c]	104.23±1.41[b]	155.48±0.99[a]
20	Sinapinic acid	ND	ND	ND
21	Syringic acid	1.98±0.05[c]	40.63±0.18[b]	50.43±2.83[a]
22	*trans*-Cinnamic acid	2.96±0.28[c]	7.07±0.85[b]	16.81±0.71[a]
23	Vanillin	ND	ND	ND

[1)]Data marked with different subscript letters within the same row indicate significant difference statistically (p<0.05).
[2)]ND, Not detected.

B. bituminosa extracts using CUPRAC and FRAP assays (Table 4). The extracts showed a similar activity profile in both assays. The water extract exhibited the highest activity (41.26 and 46.82 μmol TEs/g dry plant). It was followed by the methanol and ethyl acetate extracts, respectively. Data obtained from both assays were found to be statistically different from each other (p<0.01). In addition, the reducing power of the water extract in FRAP assay was found to be slightly greater than that of its activity in CUPRAC assay.

As can be seen from the phytochemistry section of this paper, the ethanol and water extracts contained considerable amounts of benzoic acid. According to our literature survey, benzoic acid and its derivatives have remarkable antioxidant activity (31). According to a study conducted by Tuyen *et al.* (32), benzoic acid derivatives contribute the antioxidant activity of *Quercus salicina*. In addition, according to another study reported by our research team, pure *p*-hydroxybenzoic acid showed a promising total antioxidant activity (95.26%), which was found to be higher thant those of butylated hydroxytoluene (BHT) and butylatedhydroxyanisole (BHA) (33). According to the same report, antioxidant activities of cinnamic and syringic acids were determined as 93.14 and 91.94%, respectively (33). Therefore, antioxidant activity of *B. bituminosa* could be attributed to the presence of these compounds. However, to clearly identify the compounds responsible for the antioxidant activity, biological activity guided chromatographic fractionation techniques should be applied as the next step of this study.

According to what our literature survey could ascertain, $DPPH^{\bullet}$ scavenging and cupric ion reducing power of the *n*-butanol extract of *B. bituminosa* have previously been reported by Azzouzi *et al.* (7). In the first case, the free radical scavenging potential of the extract was measured as 0.26 μg/mL (IC_{50}). In the case of CUPRAC assay, the activity value was determined as 0.10 L/mg. $ABTS^{\bullet+}$ scavenging, reducing power (via FRAP assay), and metal chelating activities of *B. bituminosa* have not previously been reported elsewhere. Moreover, antioxidant activity of this species has not previously been analyzed by phosphomolybdenum assay. Therefore, data presented here could be assumed as the first report on this species.

Enzyme inhibitory activity Inhibitory activities of *B. bituminosa* extracts were analyzed on AChE, BChE, α-amylase, α-glucosidase, and tyrosinase (Table 5). As can be seen from the table, the extracts exhibited no activity on cholinesterases and tyrosinase. In contrast, the extracts showed various degrees of inhibitory activities on α-amylase and α-glucosidase. Inhibitory activities of the extracts on α-glucosidase were found to be greater than those of their inhibitory activities on α-amylase. α-Glucosidase inhibitory assay was resulted in the superiority of the water extract (1233.86 μmol ACEs/g dry plant). In α-amylase inhibition assay, the ethyl acetate extract showed the highest activity (53.65 μmol ACEs/g dry plant). It was followed by the methanol and water extracts, respectively.

Our research group has mainly focused on the antioxidant and enzyme inhibitory activities of plant species. According to our experiences in this field, the extracts consisting of low-polarity compounds (for example ethyl acetate and/or acetone extracts)

Table 3. Metal chelating and total antioxidant (by phosphomolybdenum method) activities of *B. bituminosa* extracts[1)]

Solvent	Chelating effect (μmol EDTAEs/g dry plant)[2)]	Phosphomolybdenum (μmol TEs/g dry plant)[3)]
Ethyl acetate	0.96±0.01^{c}	112.42±2.97^{c}
Methanol	5.55±0.11^{b}	166.78±0.78^{a}
Water	9.63±0.10^{a}	153.01±5.24^{b}

[1)]Data marked with different subscript letters within the same column indicate significant difference statistically (p<0.05)
[2)]EDTAEs, ethylenediaminetetraacetic acid (disodium salt) equivalents
[3)]TEs, trolox equivalents

Table 4. Radical scavenging activity and reducing power potential of *B. bituminosa* extracts[1)]

Assays	Ethyl acetate	Methanol	Water
$DPPH^{\bullet}$ (μmol TEs/g dry plant)[2)]	7.71±0.02^{c}	14.08±0.08^{b}	29.41±0.04^{a}
$ABTS^{\bullet+}$ (μmol TEs/g dry plant)	33.57±0.03^{b}	59.68±0.58^{a}	60.23±1.46^{a}
CUPRAC (μmol TEs/g dry plant)	10.96±0.32^{c}	22.27±0.91^{b}	41.26±1.91^{a}
FRAP (μmol TEs/g dry plant)	7.02±0.36^{c}	19.69±0.54^{b}	46.82±4.29^{a}

[1)]Data marked with different subscripts within the same row indicate significant difference statistically (p<0.05).
[2)]TEs, trolox equivalents

Table 5. Enzyme inhibitory activity of *B. bituminosa* extracts[1)]

Assays	Ethyl acetate	Methanol	Water
AChE inhibition (μmol GALAEs/g dry plant)[2)]	NA[5)]	NA	NA
BChE inhibition (μmol GALAEs/g dry plant)	NA	NA	NA
α-Amylase inhibition (μmol ACEs/g dry plant)[3)]	53.65±0.21^{a}	42.64±2.74^{b}	17.72±0.44^{c}
α-Glucosidase inhibition (μmol ACEs/g dry plant)	566.96±72.71^{c}	638.78±3.97^{b}	1233.86±24.42^{a}
Tyrosinase inhibition (μmol KAEs/g dry plant)[4)]	NA	NA	NA

[1)]Data marked with different subscripts within the same row indicate significant difference statistically (p<0.05)
[2)]GALAEs, galanthamine equivalents
[3)]ACEs, acarbose equivalents
[4)]KAEs, kojic acid equivalents
[5)]NA, Not active

Table 6. Correlation coefcients between the assays of *B. bituminosa*[1)]

Assays	AAIA	AGIA	TAP	MCA	DPPH	ABTS	CUPRAC	FRAP	TFC
AGIA	-0.979								
TAP	-0.549	0.367							
MCA	-0.968	0.896	0.741						
DPPH	-0.999**	0.982	0.537	0.964					
ABTS	-0.748	0.597	0.965	0.891	0.739				
CUPRAC	-0.997*	0.961	0.610	0.984	0.996	0.796			
FRAP	-0.999**	0.976	0.560	0.971	0.999*	0.757	0.998*		
TFC	-0.380	0.183	0.982	0.600	0.366	0.898	0.447	0.392	
TPC	-0.971	0.902	0.733	0.999**	0.968	0.885	0.986	0.974	0.590

[1)]Data represents Pearson Correlation Coefcient R. (AAIA, α-amylase inhibitory activity; AGIA, α-glucosidase inhibitory activity; TAP, total antioxidant activity by phosphomolybdenum method; MCA, metal chelating activity; DPPH, $DPPH^{\cdot}$ scavenging activity; ABTS, $ABTS^{\cdot+}$ scavenging activity; CUPRAC, CUPRAC reducing power potential; FRAP, FRAP reducing power potential; TPC, total phenolic content; TFC, total flavonoid content)

* indicates $p<0.05$.

** indicates $p<0.01$.

exhibited greater activity than those of the other extracts containing polar phytochemicals. Superiority of α-amylase inhibitory activity of the ethyl acetate extract of *B. bituminosa* was found to be consistent with our previous reports (34,35).

In contrast, α-glucosidase inhibitory activity of the water extract, which consists of the polar phytochemicals, was found to be quite interesting. According to a study reported previously by our research group (36), the water extract of *Clinopodium vulgare* subsp. *vulgare* has exhibited a similar activity profile. Therefore, we think that the polar phytochemicals have greater inhibitory potential on α-glucosidase than those of the non-polar phytochemicals. In order to find the compound(s) responsible for the inhibitory activities of the extracts, bioactivity guided chromatographic analyses should be conducted as the next step of this study.

As far as our literature survey could ascertain, Tesauro *et al.* (27) have studied the human topoisomerase I inhibitory activity of erybraedin C, a natural compound from *B. bituminosa*. However, AChE, BChE, α-amylase, α-glucosidase, and tyrosinase inhibitory activities of *B. bituminosa* have not previously been reported. Therefore, data presented here could also be assumed as the first report for the literature.

Correlation coefficients between the assays To reveal the coherence between the data obtained from all of the parameters, correlation coefficients were individually calculated for each test couple (Table 6). As can be seen from the table, $DPPH^{\cdot}$ scavenging activities of the extracts were found to be quite consistent with their reducing power potentials (correlation coefficients between CUPRAC and DPPH assays: 0.996, $p<0.05$ and between FRAP and $DPPH^{\bullet}$ assays: 0.999, $p<0.01$). In general, a positive correlation was determined between the biological activity assays and total phenolic contents of the extracts. We also determined a strong correlation between the total phenolic content and α-glucosidase inhibitory activity (0.902, $p<0.05$), metal chelating activity (0.999, $p<0.01$), $DPPH^{\bullet}$ scavenging activity (0.968, $p<0.05$), CUPRAC (0.986, $p<0.05$), and FRAP assays (0.974, $p<0.05$). A negative correlation was determined between the α-amylase inhibitory activity and other parameters.

In conclusion, as can be seen from the results presented above, the water extract of *B. bituminosa* exhibited considerable antioxidant activity in radical scavenging and reducing power assays. It also showed the highest activity in α-glucosidase inhibitory assay. According to the correlation coefficients presented above, biological activity potentials of the extracts were found to be highly in correlation with their phytochemical profiles. The water extract was found to be rich in the phenolic compounds while the methanol extract contained a considerable amounts of flavonoids. In addition, the water extract was found to have considerable amounts of rosmarinic acid, luteolin, quercetin, and rutin. To reveal the compound(s) responsible for the biological activities studied here, bioactivity guided chromatographic analyses should be employed as the next step.

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Disclosure The authors declare no conflict of interest.

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