Chemical Composition and Antioxidant Activity of Extracts from the Inner Bark of *Berberis vulgaris* Stem

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Extracts from the inner stem bark of *Berberis vulgaris* were analyzed for their antioxidant activity using the 1,1-dipheny-2-picrylhydrazyl (DPPH) method and compared with ascorbic acid (AA) and butylated hydroxytoluene (BHT). The most active extracts were analyzed for their chemical composition using gas chromatography-mass spectrometry. Acetone extract was found to be the most active as an antioxidant agent at 98.61%, which was higher than the value of vitamin C (93.03%) at the concentration of 0.16 mg/mL. The major components identified in the acetone extract were tetracosanoic acid, methyl ester (26.36%), followed by phthalic acid, diisooctyl ester (20.93%), 1,2-bis(trimethylsiloxy) ethane (10.26%), and 1,2-benzendicarboxylic acid, diisononyl ester (8.70%). The dissolved water:methanol (1:1 *v/v*) partitioned from acetone extract afforded 12 fractions; among them, fraction F11 was found to have good antioxidant activity (95.41%) at the concentration of 0.16 mg/mL. The major compounds identified in F11 were N-methyl-4-(hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoloine (28.82%), 9-α-hydroxy-17β-(trimethylsilyl-oxy)-4-anderostene-3-methyloxime (13.97%), ribitol, pentaacetate (9.76%), 1-methyl-4-[4,5-dihydroxyphenyl]-hexahydropyridine (6.83%), and 2-ethylacridine (4.77%).

Keywords: Berberis vulgaris inner stem bark; Acetone extract; Fraction; GC/MS

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INTRODUCTION

The barberry (*Berberis vulgaris* L., family Berberidaceae) plant grows in Asia and Europe. Various parts of this plant, including the roots, bark, leaves, and fruit, have been used extensively for medicinal purposes and are known for possessing antiarrhythmic and sedative effects in Iranian traditional medicine (Fatehi *et al.* 2005; Javadzadeh and Fallah 2012). *B. vulgaris* was macerated along with *Foeniculum vulgare* in Ancient Egypt to ward off pestilent fevers (Chevallier 2001), and was also used in Catawba for peptic ulcer disease.

Barberry is extensively used as a food additive, and the juice is recommended to cure cholecytitis (Zargari 1983). The *B. vulgaris* plant is a shrub, growing approximately 1 to 3 m tall, with spiny, yellow wood, and obviate leaves bearing pendulous yellow flowers succeeded by oblong red berries (Zargari 1983; Amin 1991; Ciulei *et al.* 1993; Dewick 2002; Damaschin and Analiza 2006).

The pharmacological, biochemical, and anti-cancer effects of berberine (isoquinoline alkaloid present in roots, rhizome, and outer bark) were reported in several studies concerning the importance of medicinal plant species in the *Berberis* genus (*B. aquifolium, B. vulgaris, B. aristata, etc*.) (Marinova *et al.* 2000; Kim *et al.* 2003; Mahata *et al.* 2011; Wu *et al.* 2011). Based on previous reports, the majority of the medical and toxic properties of barberries are related to the different alkaloids existing in the different parts of the plant (Končić *et al.* 2010; Javadzadeh and Fallah 2012). Barberry crude extract and the active alkaloid, berberine, have been promising for the treatment of hepatic oxidative stress, Alzheimer's disease, and idiopathic male factor infertility (Abd El-Wahab *et al.* 2013). The ethanolic extract of dried powdery roots can be regarded as non-toxic, as it does not inhibit the growth of normal peripheral blood mononuclear cells that can induce cancer cells (Abd El-Wahab *et al.* 2013). Anthocyanins and barberry fruit extract have been found to have inhibitory effects on capillary permeability (Cohen-Boulakia *et al.* 2000) and epidermal growth factor (Meiers *et al.* 2001), as well as anticholinergic and antihistaminergic effects (Tomosaka *et al.* 2008).

Barberry fruits phenolic compounds, including anthocyanins and carotenoid pigments, have antioxidant and cytoprotective activities (Sabir 1971; Shamsa *et al.* 1999; Zhou and Mineshita 2000; Freile *et al.* 2003; Mahady *et al.* 2003; Kuo *et al.* 2004; Han and Lee 2005; Tomosaka *et al.* 2008). Extracts from different parts (bark, fruits) of *B. vulgaris* have been studied for their antibacterial and antifungal activities (Jain and Kar 1971; McCartney 1989; Parekh and Chanda 2005; Ghareeb *et al.* 2013; Mahmoudvand *et al.* 2014). A larger number of these plants and their isolated constituents have beneficial therapeutic effects, such as a reduction in hepatitis C virus (HCV) symptoms, anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial, and immunomodulatory activities (Huffman 2003; Ghareeb *et al.* 2013).

Barberry fruits have been reported to have a strong antioxidant capacity (Özgen *et al.* 2012). There were several studies related to the antioxidant activities of extracts from *B. vulgaris*. The ethanolic extracts from the roots, twigs, and leaves provide some antioxidant activities, which was determined to be caused by the scavenging effect on the DPPH free radical; the antioxidant activity was well correlated with the content of main plant antioxidants, phenols, and flavonols (Končić *et al.* 2010). The leaves and fruit have good antioxidant activity, which was revealed when using a DPPH free radical scavenging assay, and probably involves the high flavonoid content (Hadaruga *et al.* 2010).

To the best of our knowledge, there have been no reports on the chemical composition of the inner bark of the *B. vulgaris* stem extract. However, the chemical composition of this part has been reported. Previous phytochemical analyses of the root or stem bark extract of *B. vulgaris* has yielded the presence of protoberberines, bisbenzyl-isoquinoline alkaloids (berbamine, tetrandrine, chondocurine berberine, (-)-tejedine, jatrorrhizine, columbamine, berberubine, oxicanthine, palmatine, vitamin C, resin, and tannins), and flavonoids (quercetin and kaempferol) (Akhtar *et al.* 1978; Akhter *et al.* 1979; Dewick 1993; Ivanovska and Philipov 1996; Suau *et al.* 1998; Fatehi *et al.* 2005; Damaschin and Analiza 2006; Aghbashlo *et al.* 2008). Therefore, this novel approach investigated the antioxidant activity of some extracts, as well as the fractions of the stem inner bark extracts of *B. vulgaris* and compared them with ascorbic acid (AA) and butylated hydroxytoluene (BHT). The most active extract was analyzed for its chemical composition using gas chromatography-mass spectrometry (GC/MS).

EXPERIMENTAL

Plant Materials

Fresh stems of *B. vulgaris* were collected from Siahbishe, Chalous, and Mazandaran, Iran in May of 2013. The plant material was identified by Khosrow Ashrafi, Assistant Professor, Department of Wood Science and Paper Technology, Karaj Branch, Islamic Azad University, Karaj, Iran, and a voucher specimen was deposited in the Herbarium College of Agricultural and Natural Resources, Karaj Branch, Islamic Azad University, Karaj, Iran. The inner bark was separated from the stems and air-dried to achieve an 8.0% moisture content.

Extraction and fractionation

The inner bark of stems were cut into small pieces and chopped using a laboratory electrical rotary mill to obtain bark flour. The flour size was between 40 and 60 mesh. Approximately 50 g of this flour was placed into the 5 extraction thimbles, and then five independent extracted using pure acetone (300 mL in a 500-mL balloon) and a Soxhlet-type apparatus for 8 h. The combined extract was concentrated using a Heidolph Laborota 4001 rotary-evaporator apparatus (at 40 °C to reach total solvent evaporation) for approximately 15 min. Then, the extracts were collected, dried over anhydrous sodium sulphate, and stored at 4 °C until further analysis. The solid extractive weight was 2.72 g. Subsequently, 2.0 g of the solid extractives were dissolved in water:methanol (1:1 *v/v*) where the marc or the residue was discarded then the supernatant poured into a separatory funnel, followed by the addition 50 mL of *n*-hexane. The mixture was shaken by hand for 10 min. Half a gram of the water:methanol aqueous supernatant extractive was used for column chromatography with silica gel, Merck KGaA 64271 Darmstadt, Germany. The 12 fractions were labeled F1 to F12 (Fig. 1). The 3 × 10 mL eluent volume was used in the chromatographic separation for each solvent.

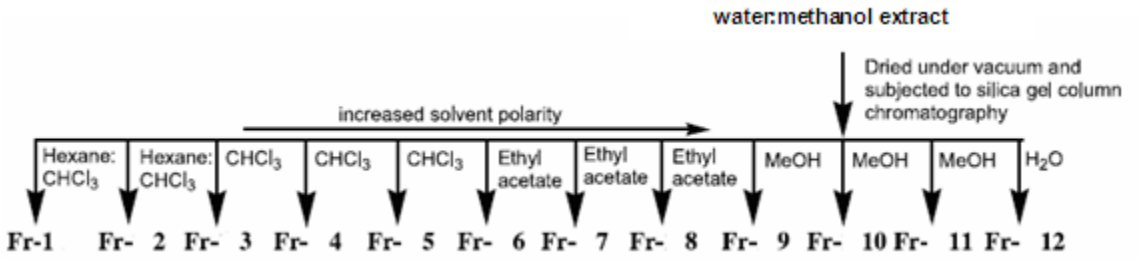

Fig. 1. Isolation scheme of active constituents of *B. vulgaris* from the water:methanol extract of inner stem bark.

Free radical scavenging activity by DPPH assay

The free radical scavenging activities of the acetone and water:methanol extracts, as well as the fractions from F1 to F12 of the stem inner bark powders, were determined using the 1,1-dipheny-2-picrylhydrazyl (DPPH) method (Karau *et al.* 2013). For this method, a stock solution was prepared by dissolving 2.4 mg of DPPH free radical in 100 mL of methanol. The stock solution was stored at 20 °C. The working solution was prepared by diluting the DPPH stock solution with methanol. Then, 1250 µL of the working solution was combined with 250 µL of the methanol extract from the medicinal plant (1 mg/mL). Serial dilutions were carried out with the stock solutions (1 mg/mL) of

tested extract to obtain concentrations of 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 mg/mL. The experiment was performed in triplicates, and the average absorbance was recorded for each concentration. The reaction mixture was mixed for 10 s and left to stand at room temperature in a dark place for 30 min. The absorbance was measured at 517 nm, using a UV scanning spectrophotometer. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as the reference standards and were dissolved in methanol to make the stock solutions with the same concentration (1 mg/mL). The control samples were prepared with the same volume of solution, without test compounds and the referenced standards. Pure methanol (Sigma-Aldrich, Germany) was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation,

$$\text{Inhibition} = 100(Ac - As)/Ac \quad (1)$$

where the percentage inhibition value was calculated from the absorbance of the control, *Ac*, and of the sample, *As*.

The controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

Analysis of extracts

Gas chromatography-mass spectrometry (GC/MS) analysis of the acetone and F11 extracts were performed using split mode (30:1 and 10:1) injection. One microlitre of the silylated extract, 30 µL N,O-bis-(trimethylsilyl)triflouroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) reagent, and approximately 30 µL of pyridine were run on a HP 6890 (Hewlett Packard, USA) gas chromatograph fitted with a cross-linked 5.0% PH ME siloxane HP-5 capillary column (dimensions: 30 m x 0.25 mm, 0.50 µm coating thickness) and coupled with a model 5975B mass detector. The GC/MS operation conditions were as follows: injector temperature 250 °C; transfer line 290 °C; oven temperature program 50 to 250 °C (5 °C/min); carrier gas: He at 1.4 mL/min; mass spectra: electron impact (EI+) mode 70 eV with a mass range of 40 to 450 m/z; and ion source temperature at 250 °C. Individual components were identified using Wiley 275 L and NIST05 mass database matching and by comparing the retention times and mass spectra of constituents with published data (Julian and Konig 1988; Adams 1995, 2001). Retention indices (R_I) were determined with reference to a homologous series of normal alkanes, using the following formula (Kovats 1958),

$$R_I = 100\,[(n + (N\text{-}n) \times \log t_{1R}\,(x) - \log t_{1R}\,(C_n)/\log t_{1R}\,(C_N) - \log t_{1R}\,(C_n)] \quad (2)$$

where R_I is the retention index of the compound of interest, t_{1R} is the net retention time (t_R-t_0), t_0 is the retention time of solvent (dead time), t_R is the retention time of the compound of interest, C_n and C_N are the number of carbons in the n-alkanes eluting immediately before and after the compound of interest, and *N* and *n* are the number of carbon atoms in the n-alkane eluting immediately before and after the compound of interest.

Statistical analysis

Data of antioxidant activity were statistically analyzed using the SPSS program.

RESULTS AND DISCUSSION

Antioxidant Activity

Statistically, there were significant differences among the treatments (F1→F12, water:methanol, acetone, BHT, vitamin C, and their concentrations (Tables 1 and 2).

The acetone extracts exhibited high antioxidant activity overall (Fig. 2). The lowest antioxidant activity, 91.66%, was observed at the concentration of 0.005 mg/mL, which was higher than for vitamin C (88.93%) at the same concentration. The highest activity came from acetone extract (98.61%) at 0.16 mg/mL, which was also higher than the value for vitamin C (93.03%) at the same concentration. The same trend was observed with the reference, BHT.

Additionally, among the assayed fractions, F11 and F12 showed high antioxidant activity at 95.41% and 93.65%, respectively, when the concentration was 0.16 mg/mL. Looking at Fig. 2, the more polar compounds seem to have the higher antioxidant activity, because following the extractions with non-polar solvents the fraction F11 retains an antioxidant activity comparable to acetone fraction.

Previously, the hexane fraction of the crude methanol extract from the shade dried plant material was the most active fraction, with an IC_{50} of 72 μg/mL (Kolář *et al.* 2010), in the case of inhibition with acetylcholinesterase. Berberine and methanolic barberry crude extract showed a significant reduction in their antioxidant abilities and radicals scavenging effects, especially on hydroxyl and DPPH radicals (El-Sayed *et al.* 2011). Furthermore, *B. vulgaris* extract, as well as berberine chloride, inhibited DPPH oxidation in the range of 13 to 46% more than control level (Abd El-Wahab *et al.* 2013).

Leaves, fruit, and stem extracts of *B. vulgaris* were reported as being active in the DPPH radical-scavenging assay, but the active constituents involved were not determined (Heinrich *et al.* 2005). However, Cannabisin G and (±)–lyoniresinol were responsible for the antioxidant activity of *B. vulgaris* root bark extract (Tomosaka *et al.* 2008).

Table 1. Statistical Analysis of the Effect of Treatments and Concentrations on the Antioxidant Activity of Inner Bark Extractives of *B. vulgaris*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	213074.833	95	2242.893	107.137	0.000
Intercept	881822.434	1	881822.434	42122.475	0.000
Treatment	175054.301	15	11670.287	557.461	0.000
Concentration	18020.254	5	3604.051	172.157	0.000
Treatment * Concentration	20000.278	75	266.670	12.738	0.000
Error	4019.467	192	20.935		
Total	1098916.733	288			
Corrected Total	217094.300	287			

Table 2. Mean±SD of the Antioxidant Activity (%) as Affected by Different Treatments of Inner Bark Extractives of *B. vulgaris* and Comparing them with BHT and Vitamin C

Treatment	Concentration (mg/mL)					
	0.005	0.01	0.02	0.04	0.08	0.16
F1	19.97±0.53	25.43±3.83	25.55±6.44	26.13±3.83	26.59±0.87	27.29±7.49
F2	26.64±1.25	31.66±0.54	35.84±0.72	37.99±3.58	38.11±1.25	44.20±1.61
F3	23.84±1.42	25.26±1.77	25.62±0.71	26.81±2.67	27.40±5.33	44.60±15.83
F4	25.92±1.25	25.44±2.51	27.24±1.43	27.24±0.71	29.51±0.54	32.25±0.00
F5	8.69±0.97	10.89±2.33	13.61±1.16	13.61±6.61	15.56±7.00	29.70±19.64
F6	29.13±6.83	39.68±3.77	41.12±1.98	44.60±1.07	50.00±5.39	53.59±7.19
F7	35.44±1.39	56.74±0.39	60.84±1.39	62.69±2.77	69.44±0.79	81.48±8.13
F8	42.33±1.90	50.63±13.66	52.24±1.73	53.40±10.89	54.67±9.68	56.86±7.44
F9	74.14±2.12	75.14±2.86	75.81±2.49	75.89±1.87	77.55±0.49	79.63±3.36
F10	53.55±2.18	57.58±5.91	62.81±3.72	67.60±0.77	68.46±1.15	68.46±1.15
F11	69.72±10.55	86.39±3.90	87.30±2.06	92.81±1.61	93.11±1.83	95.41±0.45
F12	63.88±3.57	72.61±3.57	84.92±7.93	87.43±0.99	90.60±2.58	93.65±1.19
water:methanol	25.75±0.37	27.65±0.75	28.15±0.57	50.37±0.75	74.24±2.65	85.98±2.27
Acetone	91.66±0.00	92.92±0.21	96.33±0.21	97.47±0.21	97.85±0.21	98.61±0.95
BHT	13.38±3.22	21.59±3.03	43.30±0.57	55.80±1.33	87.12±2.65	92.42±2.27
Vitamin C	88.93±0.81	89.75±2.45	89.89±0.23	90.16±.41	91.39±0.40	93.03±2.04

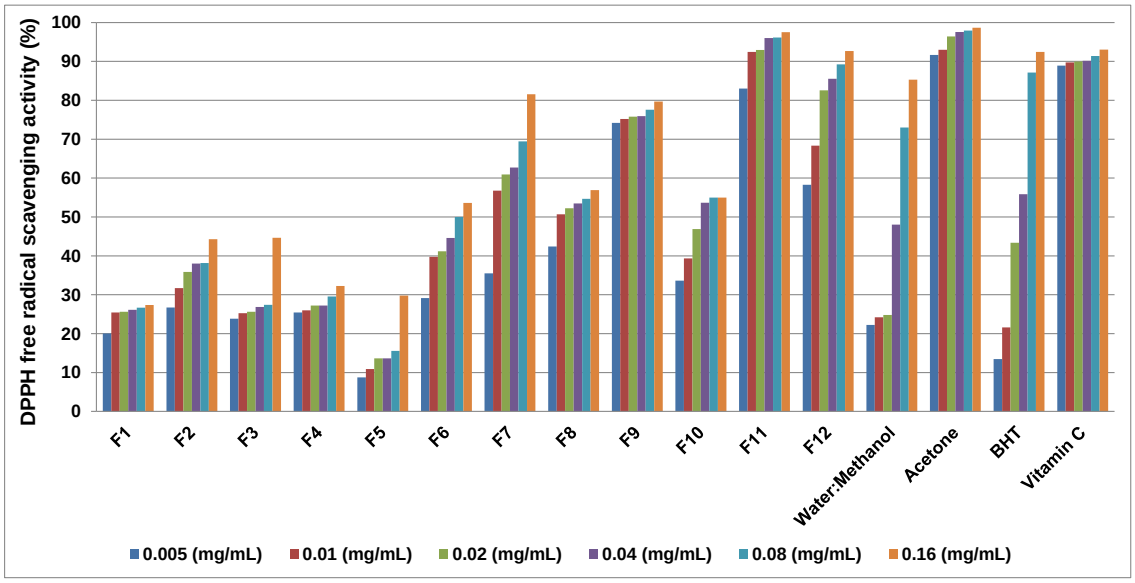

Fig. 2. The antioxidant activity of extracts from the inner bark of *B. vulgaris*

Inner Bark Extract

The suggestable chemical constituents identified in the acetone extract are presented in Table 3. The acetone extract of the fresh stem inner bark of *B. vulgaris* exhibited a yellowish extract with a pleasant aroma, and a yield of 8.0% (v/w). Ten components, comprising 100% of the inner bark extract, were identified. The major components of the inner bark extract were tetracosanoic acid, methyl ester (26.36%), phthalic acid, diisooctyl ester (20.93%), 1,2-bis(trimethylsiloxy)ethane (10.26%), and 1,2-benzenedicarboxylic acid, diisononyl ester (8.70%). Among the identified components, three of the components comprising 55.99% of the extract were fatty acids

(aliphatic compounds), others included: sesquiterpene (5.22%) two aromatic amines (8.36%), benzoxepine derivatives (7.04%), oxetane compounds (6.80%), ammoxidized hydroquinone derivatives (10.26%), and ketone compound (6.34%).

Table 3. The Suggestable Chemical Composition of Acetone Inner Stem Bark Extract of *B. vulgaris*

Component	Retention Time (min)	Retention Indices	Area (%)
1,2-Bis(trimethylsiloxy)ethane	10.230	982	10.26
1-Amino-2-(hydroxymethyl)anthraquinone	31.460	1783	5.57
Benzaldehyde, 2-{(1-(2-{[1-(2aminophenyl)methylidene] amino}phenyl)methylidene]amino}	33.575	1886	2.79
1,2-Benzenedicarboxylic acid, diisooctyl ester	45.024	2547	20.93
Tetracosanoic acid, methyl ester	48.278	2726	26.36
2-(4-Methylphenyl)-3-(1-hydroxyhexyl)oxetane	48.388	2731	6.80
2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-	48.964	2756	7.04
Phenyl(I-p-diphenylmethylphenyl-3,3-dimethyl)butyl ketone	49.436	2777	6.34
1,2-Benzenedicarboxylic acid, diisononyl ester	49.585	2784	8.70
Epi-ligulyl oxide	50.568	2821	5.22
Total	-	-	100

Analyses of the extract showed that predominantly volatile organic compounds in the form of fatty acid esters were found. Two-(4-methylphenyl)-3-(1-hydroxyhexyl) oxetane is an oxetane derivative that is used in medicinal chemistry. Early studies in rats involving 3,3-diethyloxetane and other simple oxetanes revealed their anesthetic, sedative, and anticonvulsant properties (Wuitschik 2008). For the coptidis, the use of an alternative biosynthesis route with proline, phenylalanine, catechollactate, and 2-mono-isobutyrin was proposed to synthesize its major bioactive alkaloids. It was observed that different fatty acids might be needed to modulate the biosynthesis of the bioactive secondary metabolites of medicinal herbs (Teo *et al*. 2011).

In addition, sesquiterpene, epi-ligulyl oxide, and derivatives from ammoxidized hydroquinone, *i.e.*, 1,2-bis(trimethylsiloxy)ethane, were detected in barberry bark extracts for the first time. The epi-ligulyl oxide compound has been characterized in agarwood and eaglewood oil as a novel compound (Naf *et al*. 1992; Mei *et al*. 2008).

The suggestable chemical constituents identified in F11 are presented in Table 4. The major compounds were N-methyl-4-(o-hydroxybenzyl)-1,2,3,4-tetrahydroiso-quinoline (28.82%), 9-α-hydroxy-17β-(trimethylsilyloxy)-4-androstene-3-methyloxime (13.97%), ribitol, pentaacetate (9.76%), 1-methyl-4-[4,5-dihydroxyphenyl]-hexahydro-pyridine (6.83%), and 2-ethylacridine (4.77%). Most of the identified compounds were derived from isoquinoline alkaloids and one compound (ribitol, pentaacetate) was derived from carbohydrates.

Table 4. The Suggestable Chemical Composition of F11 Inner Stem Bark Extract of *B. vulgaris*

Component	Retention Time (min)	Retention Indices	Area (%)
1-Methyl-4-[4,5-dihydroxyphenyl]-Hexahydropyridine	28.904	1664	6.83
N-Methyl-4-(o-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline	31.427	1781	28.82
2-Ethylacridine	32.333	1825	4.77
9-α-Hydroxy-17β-(trimethylsilyloxy)-4-androstene-3-methyloxime	33.562	1886	13.97
2-(4-Methylphenyl)-Indolizine	35.360	1978	4.22
Silicic acid, diethyl bis(trimethylsilyl) ester	40.677	2276	4.05
1,2-Bis(trimethylsilyl)benzene	43.012	2418	3.86
Ribitol, pentaacetate	45.018	2547	9.76
N-Cyano-N',N',N'',N''-tetramethyl-1,3,5-triazinetriamine	45.218	2560	4.20
Total	-	-	80.48

The alkaloid fraction has been found in some species of *Berberis* in both the stem and bark ethanolic extracts (Rocha *et al*. 2005). In the review article by Imanshahidi and Hosseinzadeh (2008), the most important constituents found in the stem bark of the plant were the isoquinoline alkaloids, acanthine, berbamine, berberine, columbamine, jatrorrhizine, and magnoflorine. Berberine and berbamine are the most biologically active compounds and are widely distributed in almost all *Berberis* species (Gorval and Grishkovets 1999; Dev 2006; Rashmi *et al*. 2008).

CONCLUSIONS

1. The extracts from the inner stem bark of *Berberis vulgaris* exhibited high antioxidant activity and the values were higher than the standard antioxidant compounds (vitamin C and BHT).
2. The suggestable major components identified in the acetone extract by GC/MS were tetracosanoic acid, methyl ester, phthalic acid, diisooctyl ester, 1,2-bis(trimethyl-siloxy)ethane, and 1,2-benzenedicarboxylic acid, diisononyl ester.
3. Water:methanol (1:1 v/v) was divided into 12 fractions: Among these, fraction F11 exhibited high antioxidant activity and contained the following suggestable main chemical compounds by GC/MS: N-methyl-4-(o-hydroxybenzyl)-1,2,3,4-tetrahydro-isoquinoline, 9-α-hydroxy-17β-(trimethylsilyloxy)-4-androstene-3-methyloxime, ribitol, pentaacetate, 1-methyl-4-[4,5-dihydroxyphenyl]-hexahydropyridine, and 2-ethylacridine.
4. It can be concluded that the extracts from the inner bark of *Berberis vulgaris* possess high antioxidant activity and most of the identified compounds are isoquinoline alkaloids.

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