Available free online at www.medjchem.com

Mediterranean Journal of Chemistry **2014**, 2(5), 639-647

Secondary metabolites and antioxidant activity of seed extracts from *Solanum elaeagnifolium* Cav.

Houda Feki, Imed Koubaa and Mohamed Damak*

Laboratory of Chemistry of Natural Products, Faculty of Science of Sfax, University of Sfax, BP 1171. 3000, Sfax, Tunisia.

Abstract: The aim of this study was to screen various solvent extracts of seeds of *Solanum elaeagnifolium* to display the phytochemical composition, the total phenolic content, the total flavonoid content and the antioxidant capacity in order to find possible sources for future novel antioxidants in food and pharmaceutical formulations. Various extracts of seeds of *Solanum elaeagnifolium* were obtained by maceration. The total phenolic content of the different extracts was determined by Folin-Ciocalteu method, and the total flavonoid content was quantified using a method based on the formation of a flavonoid–aluminium complex. The antioxidant activity was essayed through some in vitro models such as the antioxidant capacity by phosphomolybdenum method, radical scavenging activity using 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay and reducing power assay. The acetone extract showed the highest total phenolics (580.99 ± 20.56 mg gallic acid equivalents/g of extract), and the highest total flavonoids (207.61 ± 2.62 mg quercetin equivalents/g of extract). This extract showed an antioxidant activity higher than that of α-tocopherol.

These results suggest the potential of *Solanum elaeagnifolium* against free-radical-associated oxidative damage.

Keywords: Antioxidant activity, *Solanum elaeagnifolium*, total phenolics, total flavonoids, DPPH, reducing power, secondary metabolites.

Introduction

Natural antioxidants have received great attention and have been studied extensively, since they are effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT), which are suspected of being carcinogenic and causing liver damage [1]. It is believed that an increased intake of food, which is rich in natural antioxidants, is associated with a lower risk of degenerative diseases, particularly cardiovascular diseases and cancer [2]. Phenolic compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step [3]. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups [4].

Flavonoid constituents possess a wide spectrum of chemical and biological activities, including radical scavenging properties. Indeed, Shimoi et al. (1996) reported that plant flavonoids that show antioxidant activity *in vitro* also function as antioxidants *in vivo* [5].

In this paper, and as a part of our investigations into some medicinal plants known in Tunisia [6-12], we report the phytochemical composition of seeds of *S. elaeagnifolium*, the total

**Corresponding author:*
E-mail address: Mohamed.Damak@fss.rnu.tn
DOI: http://dx.doi.org/10.13171/mjc.2.5.2014.05.01.22

phenolic content of the different extracts using Folin-Ciocalteu method, the total flavonoid content using the method based on the formation of a flavonoid–aluminium complex and the antioxidant activity using some in vitro models such as the antioxidant capacity by phosphomolybdenum method, radical scavenging activity using 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay and reducing power assay in order to find possible sources for future novel antioxidants in food and pharmaceutical formulations.

To our knowledge, the research of the phytochemicals and antioxidant activity of *S. elaeagnifolium* was not investigated before.

The genus *Solanum* belonging to Solanaceae family [13-14], consists of approximately 2000 species [15]. The specimen *Solanum elaeagnifolium*, commonly named *silverleaf nightshade* or *trompillo* [16] is a perennial shrub, widely distributed in Asia, Africa, Australia, and tropical and subtropical America [17-18]. It is native to countries bordering the Mediterranean Sea. No report indicates its uses in traditional, but it has been implicated in reduced weight gains, lowered animal production, teratogenic effects [19-20] and neurological disorders in ruminants [21]. This specimen is used as a source of steroidal alkaloids in the pharmacochemical industry [22].

Experimental Section

Plant material

Solanum elaeagnifolium was collected in December 2011 (final stage of maturity) at Monastir, Tunisia. It was identified by Pr. M. Om Zin **(**Institut Supérieur Agronomique de Chott Meriem, Sousse, Tunisie). Voucher specimen (N°LCSN112) was deposited at the Laboratory of Chemistry of Natural Products, Faculty of Sciences of Sfax, University of sfax, Tunisia.

The fruit is an irregularly dehiscent berry, initially spherical, green (with white patches) becoming yellow to orange (approximately 0.3 g weight and 10-15 mm of diameter) at maturity. A single plant generally produces 40-60 fruits, each containing 60-120 seeds, smooth, flat, greenish-brown, 2-3 mm in diameter, closely resembling those of tomatoes [23].

Phytochemical composition of seeds of *S. elaeagnifolium*

Quantification of chemical compounds from the seed of *S. elaeagnifolium*

Saponins

They were extracted according to the method worked out by Bouchelta et al [24]. 5 g of seeds were delipided during 2 h by 150 mL of n-hexane. After elimination of the organic phases, the precipitates obtained were macerated in 50 mL of absolute ethanol under magnetic agitation at the ambient temperature during 24 h. The ethanolic phases were evaporated at 40 °C by the rotavapor. The dried residues were extracted three times by 50 mL from distilled water/petroleum ether mixture (V/V) heated at 50 °C in water bath during 30 min. The aqueous phases were mixed then treated by 10 mL of butanol during 30 min. The organic phases were evaporated at 40 °C and were weighted.

Tannins

The extraction of tannins from *S.elaeagnifolium* was obtained according to the method of Zhang et al [25]. Five grams of seeds was milled into powder. The powder was extracted with 100 mL acetone–water (70/30, V/V), and the mixture was stirred continuously for 72 h at room temperature. Then, the mixture was filtrated and evaporated under vacuum at 40 °C to

remove acetone. The remaining solution was first washed with 30 mL dichlomethane to remove liposoluble substances, and then extracted twice with 30 mL of ethyl acetate). The resulting water layer was evaporated to dryness, and weighted.

Proanthocyanidin

Proanthocyanidin-rich extract was obtained according to Hayder et al[26]; by macerating 100 g of powdered S. *elaeagnifolium* seeds in 500 mL acetone/water mixture (1:4 v/v) during 24 h with continuous stirring at room temperature. The extract was filtrated then concentrated under low pressure. The remaining aqueous phase was treated with an excess of NaCl, and separated from precipitated material. The supernatant was extracted with ethyl acetate. Organic layer was concentrated, dried over anhydrous sodium sulphate, and finally treated with an excess of chloroform. The precipitate so formed was separated by centrifugation at 10,000 *g* for 15 min and weighted.

Total alkaloids

They were obtained by triple liquid–liquid extraction according to the method of Harbone[27], the seed of *S. elaeagnifolium* were extracted by maceration in 150 mL of absolute ethanol during 5 h. The ethanolic extract was then evaporated under vacuum at 40 °C by Buchi Rotavapor R-200. The dry residue was taken again by 20 mL of chloroform and acidifiet by HCl at 5% to pH 3; they were let rest during 30 min at the ambient temperature. The acid aqueous phase was then basified by NH_4OH at 5% to pH 9 and extracted twice with 20 mL of chloroform. Chloroformic phases were evaporated and the dry residues, made up of total alkaloids, were weighted.

Phytochemical screening, total flavonoids and total phenolics of extracts of seeds of *S. elaeagnifolium*

Preparation of organic extracts

Hexane, dichloromethane, ethyl acetate, acetone, methanol and aqueous extracts were obtained successive by maceration of seeds of *S. elaeagnifolium* at room temperature for 48h. The various types of organic extracts, with different polarities, were concentrated to dryness and the residues were stored at 4 °C. Their yields (%) are shown in Table 1.

Phytochemical screening

Chemical tests were carried out on various extracts of *S. elaeagnifolium* for the qualitative determination of phytochemical constituents using standard phytochemical screening methods[28-30].

Total flavonoids

Total flavonoid content was determined according to Djeridane et al [31], using a method based on the formation of a flavonoid–aluminium complex, having the maximum absorbance at 430 nm. 1 mL of diluted sample (1mg/mL) was mixed with 1 mL of 2% aluminium trichloride ($AlCl_3$) methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with a Jenway 6320D UV–Vis spectrophotometer.

The concentrations of flavonoid compounds were calculated according to the equation obtained from the standard quercetin graph.

The total flavonoid content was expressed as milligram of quercetin equivalent (QE) per gram of extract.

Total phenolics

Total phenolic content (TPC) of the extracts was determined by a colorimetric assay, according to the method described by Singleton et al [32]. Briefly, 1mL of sample (1mg/mL) was mixed with 1 mL of Folin–Ciocalteu reagent. After 3 min, a 1 mL of saturated sodium carbonate Na_2CO_3 solution was added to the mixture followed by the addition of 7 mL of distilled water. The mixture was kept in the dark for 90 min at room temperature. The absorbance of samples was measured at 725 nm. The concentrations of phenolic compounds were determined using a standard curve prepared with gallic acid. Total phenolic content (TPC) was expressed as milligram of gallic acid equivalent/g of extract.

Antioxidant properties

Determination of total antioxidant capacity (TAOC)

The assay is based on the reduction of Mo(VI) to Mo(V) by the extract. When positive a green phosphate/Mo (V) complex was obtained at acidic pH [33]. 0.1 mL of each extract was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min. The solution was allowed to cool at room temperature and the absorbance was measured at 695 nm against a blank. The total antioxidant capacity (TAOC) was expressed as milligram of α- tocopherol equivalent/g of extract.

DPPH radical scavenging assay

The antioxidant activity of extracts was measured in terms of hydrogen-donating or radical scavenging ability, using the DPPH method [34-36] with a minor modification. Briefly, 1.5 mL of DPPH solution (10^{-4} M, in 95% Ethanol) was incubated with 1.5 mL of extracts at various concentrations (0.01-0.1mg). The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. The control was prepared as above without any extract. The absorbance of the solution was measured at 517 nm against a blank. The radical scavenging activity (RSA) was measured as a decrease in the absorbance of DPPH and was calculated using the flowing equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{Sample (517 nm)}} / A_{\text{Control (517 nm)}}) \times 100$$

The extract concentration providing 50% inhibition ($IC_{50\%}$) was calculated from the graph of scavenging effect percentage against the extract concentration. BHT was used as standard.

Reducing power assay

The reducing power was determined according to the method of Oyaizu M. [37]. 1 mL of a methanol solution of each extract was added to 1mL of 200 mmoL/L sodium phosphate buffer (pH 6.6) and 1 mL of 10% trichloroacetic acid (w/v). The mixture was centrifuged at 1500 rpm for 10 min (Hettich Zentrifugen 2002, Germany) .The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank: higher absorbance indicates higher reducing power. α-tocopherol was used as positive control.

Results and Discussion

Extract yields

Extractions were carried out first with increasing polarity solvents in the aim to get fractions with different compositions. Then, bioactive compounds such as tannins, saponins alkaloids and proanthocyanidins were extracted. The yields are shown in Table 1.

The Methanol extract of seeds of *S.elaeagnifolium* presents the higher yields 7.7%, followed by the acetone extract 5.31% showing that seeds are rich in polar compounds. This was confirmed by the yields of tannins, saponins and alkaloids obtained by specific extractions.

Table 1. Yields of some bioactive compounds and extracts from *S. elaeagnifolium* seeds.

Bioactive compounds	Yields (%)
Tannins	2.83
Alkaloids	3.02
Saponins	2.86
Proanthocyanidins	0.025
Hexane extract	2.85
Dichloromethane extract	2.34
Ethyl acetate extract	2.50
Acetone extract	5.31
Methanol extract	7.70
Aqueous extract	2.13

Phytochemical Study

Phytochemical screening

The results of our assay on the crude extracts of seed of *S. elaeagnifolium* are shown in Table 2. It reveals that about all crude extracts showed the presence of alkaloids, saponins and tannins and were negative for carotenoids, tropolones and quinones. Less polar extracts are rich in sterol and triterpenes while polar extracts are rich in flavonoids. These results are in agreement with those of the specific extractions (Table 2). These molecules were known to show medicinal activities as well as exhibiting physiological activities [38].

Table 2. Phytochemical screening of various extract of seeds of *Solanum elaeagnifolium*

Extracts	A	B	C	D	E	F	G	H
Hexane	+	-	-	-	-	-	+	-
Dichloromethane	++	-	-	-	+	-	++	+
Ethyl acetate	+	-	-	-	+	-	++	+
Acetone	++	-	-	-	+	+	++	+
Methanol	-	-	-	-	+	+	++	++
Aqueous	-	-	-	+	+	-	++	++

A: Sterols/Triterpenes, B:carotenoids,C:tropolones,D:quinones,E:alkaloids,F:Flavonoids,G:Saponins, H:Tannins

Total flavonoid and phenolic contents

In this work, the method of Djeridane et al [31] to estimate the amount of flavonoid type compounds in the extracts. Table 3 shows the total flavonoid contents in the seed extracts of *solanum elaeagnifolium.*

The most flavonoid rich extract was found to be acetone extract (207.61 ± 2.62 mg/g), while hexane extract (9.05 ± 0.26 mg/g) was the poorest.

The amounts of total phenolics in the extracts of seed of *solanum elaeagnifolium* were determined spectrometrically according to the Folin–Ciocalteu procedure and calculated as gallic acid equivalents. The amounts of total phenols found in the plant various extracts are shown in Table 3. The acetone extract (580.99 ± 20.56 mg/g) has the highest phenolic content. While the least phenolics containing one was ethyl acetate extract (91.5 ± 4.11mg/g).

Flavonoids and phenolic compounds are well known as antioxidants. In various studies, antioxidant activity of plant extracts was found to be fairly high when they are rich in phenolic compounds[39]. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups[40].

Table 3. Total phenolic content (TPC) and total flavonoid contents in seed extracts of *solanum elaeagnifolium*

extract	Total flavonoids[a]	Total phenols[b]
Hexane	9.05 ± 0.26	177.63 ±10.60
Dichloromethane	10.10 ± 0.30	383.70 ± 6.85
Ethyl acetate	11.81 ± 0.23	91.50 ± 4.11
Acetone	207.61± 2.62	580.99 ± 20.56
Methanol	120.34 ± 2.12	399.70 ± 9.27
Aqueous extract	80.31 ± 0.55	352.29 ± 18.65

Averages± Standard Deviation was obtained from three different experiments.

[a] Total flavonoids expressed as quercetin equivalents: milligrams quercetin per gram (dry weight) extract.

[b] Total phenols expressed as gallic acid equivalents: milligrams gallic acid per gram (dry weight) extract.

Antioxidant properties

Owing to the complex reactive facets of phytochemicals, the antioxidant activities of plant extracts cannot be evaluated by only a single method, but at least two test systems have been recommended for the determination of antioxidant activity to establish authenticity[41]. For this reason the antioxidant activity of various extracts of seed of *S. elaeagnifolium* were determined by three spectrophotometric methods, total antioxidant capacity, reducing power assay and scavenging activity of DPPH radical.

The total antioxidant cacpacity of the *Solanum elaeagnifolium* was measured spectrophotometrically through phosphomolybdenum method, which was based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate /Mo (V) compounds with a maximum absorption at 695nm. The antioxidant capacity was estimated from the regression equation prepared from concentration versus optical density of samples referred to α- tocopherol. So the antioxidant capacities of extracts of *Solanum elaeagnifolium* were expressed as the number of equivalents of α- tocopherol table 4.

In the second method, the antioxidants react with the stable free radical DPPH (1, 1-diphenyl- 2-picrylhydrazyl, deep violet color) to convert it in to 1,1-diphenyl-2-picrylhydrazine with discoloration. The degree of discoloration indicates the free radical

scavenging potentials of the sample. DPPH scavenging activity is usually presented by IC50 value, defined as the concentration of the sample necessary to cause 50% inhibition, which was obtained by interpolation from linear regression analysis [42]. A lower IC_{50} value is associated with a higher radical scavenging activity.

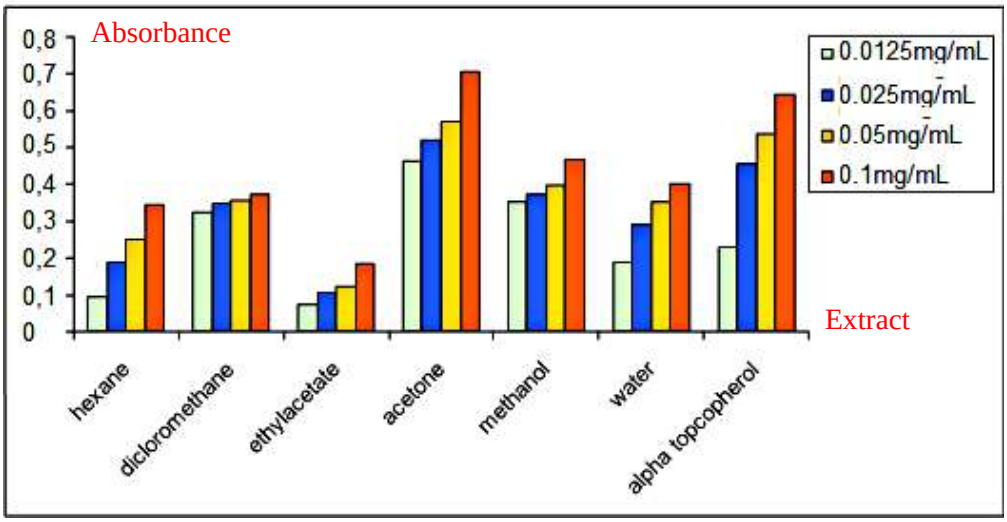
The two methods show high total antioxidant capacity and radical scavenging activity for the acetone, aqueous and methanol extracts (Table 4). These results may be explained by high levels of total phenolic and flavonoid contents.

The radical scavenging activity of these extracts was superior to that of BHT. The acetone extract shows the highest antioxidant power.

Table 4. Total antioxidant capacity (TAOC) and Antioxidant activity by DPPH, in seed extracts of *solanum elaeagnifolium*

extracts	TAOC(mg d'α-tocopherol/g of extract	DPPH (IC_{50}: mg/mL)
Hexane	11.14 ± 0.03	0.36
Dichloromethane	13.05 ± 0.08	0.185
Ethyl acetate	14.80 ± 0.2	0.21
Acetone	107.93 ± 0.65	0.025
Methanol	76.85 ± 0.23	0.042
Aqueous extract	62.16 ± 0.34	0.029
BHT	-	0.069

In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Then the amount of Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue ($Fe_4[Fe(CN^-)_6]_3$) at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Thus, Figure 1 shows that the reducing power followed the order: Acetone extract > methanol extract > aqueous extract > dichloromethane extract> hexane extract >Ethyl acetate extract. The reducing power of acetone extract was superior to that of α-tocopherol. This result fits with those of the two precedent methods.



Results were the averages obtained from two different experiments.

Figure1: Reduction powers of various extracts of seed of *S. elaeagnifolium* compared to α -tocopherol.

Conclusion

The acetone extract of *S. elaeagnifolium* seed contained high level of total phenolic and flavonoid compounds and were capable of inhibiting radicals and acting as reducing agents. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity.

Research is in progress to isolate and identify the antioxidant components in the acetone fraction.

Acknowledgements

This research was funded by the Ministry of higher education and scientific research in Tunisia.

References

1- D.V. Ratnam, D.D. Ankola, V. Bhardwaj, D.K. Sahana, R.M.N.V. Kumar, J. Controlled Release, **2006**, 113, 189-207.
2- J. Perez-Jimenez, F. Saura-Calixto, J. Agric. Food Chem, **2008**, 53, 5036-5040.
3- P.K. Agraval, Studies in Organic Chemistry, **1989**, 39, 564.
4- B.H. Havsteen, Pharmacol. Ther, **2002**, 96, 67-202.
5- K. Shimoi, S. Masuda, B. Shen, B. Furugori, N. Kinae, Mutat. Res, **1996**, 350, 153-161.
6- I. Koubaa, M. Damak, Fitoterapia, **2003**, 74, 18-22.
7- M. Kammoun, I. Koubaa, Y. Ben Ali, R. Jarraya, Y. Gargouri, M. Damak, S. Bezzine, Appl. Biochem Biotechnol, **2010**, 162 (3), 662-700.
8- N. Damak, N. Allouche, B. Hamdi, M. Litaudon, M. Damak, Nat. Prod. Res, **2011**, 26(2), 125-317.
9- A. Ben Hsouna, M. Trigui, R. Ben Mansour, R.M. Jarraya, M. Damak, S. Jaoua, Int. J. Food. Microbiology, **2011**, 148(1), 66-72.
10 - S. Miladi, R. Jarraya, M. Damak, J. Applied. Sci, **2008**, 8, 4689-4693.
11- S. Miladi, N. Abid, C. Debarnôt, M. Damak, B. Canard, M. Aouni, B. Selmi Nat. Prod. Res. **2012**, 26(11), 1027-32
12- I. Khlif, K. Hamden, M. Damak, N. Allouche, Chem. Nat. Comp, **2012**, 40, 799-802.
13- N. Gaston, G.P. Guibourt, Histoire naturelle des drogues simples ; ed. by J.-B. Baillière : Pais, **1849**.
14- D. Cauvet, Des Solanées ; ed. by G. Silbermann: California, **1864**, p.75.
15- X.M. vander Burgt, J.M. van Medenbach de Rooy, Springer, **1996**, 861.
16- M. Mellado, J.E. Garcıa, J.R. Arévalo, W. Pittroff, Arid. Environment, 2008, 72, 2034-2039.
17- J.W. Boyd, D.S. Murray, R.J. Tyrl, Economic *Botany,* **1984**, *38, 210*-217.
18- C.A. Chiale, J.L. Cabrera, H.R. Juliani, Phytochem, **1991**, 30, 1042-1043.
19- D.C. Baker, R.F. Keeler, W. Gaffield, Toxicon, **1989**, 27, 1331-1337.
20- R.F. Keeler, D.C. Baker, W. Gaffield, Toxicon, **1990**, 28, 873-884.
21- M.B. Porter, R.J. MacKay, E. Uhl, S.R. Platt, A. deLahunta, J. Am. Vet. Med. Assoc, **2003**, 223, 501-504.
22- S.0. Trione, M.A. Cony, Interciencia, **1988**, 13, 303.

23- OEPP/EPPO. *Solanum elaeagnifolium*, http ://www.eppo.org, (08.04.2010). EPPO Bull, **2007**, 37, 236-245.
24- A. Bouchelta, A. Boughdad, A. Blenzar, Biotechnol. Agron. Soc. Environ, **2005**, 9, 259-269.
25- S.Y. Zhang, C.G. Zheng, X.Y. Yan, W.X. Tian, Biochem. Bioph. Res. Co, **2008**, 371, 654-658.
26- N. Hayder, A. Abdelwahed, S. Kilani, R. Ben Ammar, A. Mahmoud, K. Ghedira, Mutat. Res, **2004**, 564(1), 89-95.
27- J.B. Harbone, Phytochemical methods: a guide to modern techniques of plant analysis; ed. by Chapman and Hall: London, **1998**, p. 302.
28- A. Sofowora, Medicinal plants and Traditional Medicine in Africa. Spectrum Books, Ibadan, **1993**, pp. 150.
29- J.B. Harborne, Phytochemical Methods: A Guide to Modern Technique of Plant Analysis. Chapman and Hall: London, **1973**, pp. 49-188.
30- G.E. Trease, W.C Evans, Pharmacognosy; ed. by Bailliere Tindall: London, **1989**, pp. 176-180.
31- A. Djeridane, M. Yousfi, B. Nadjemi, D. Boutassouna, P. Stocker, N. Vidal, Food Chem, **2006**, 97, 654-660.
32- V.L. Singleton, J.A. Rossi, Am. J. Enol. Vitic, **1965**, 16, 144-158.
33- P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem, **1999**, 269, 337-341.
34- W. Brand-Williams, M.E. Cuvelier, C. Berset, Technol, **1995**, 28, 25-30.
35- Y. Chen, M.F. Wang, R.T. Rosen, C.T. Ho, J. Agr. Food Chem, **1999**, 47, 2226.
36- G.H. Naik, K.I. Priyadarsini, J.G. Satav, M.M. Banavalicar, P.P. Sohoni, M.K. Biyani, H. Mohan, Phytochemistry, **2003**, 63, 97.
37- M. Oyaizu, Jpn. J. Nutr, **1986**, 44, 307.
38- N. Benhammou, F.A. Bekkara, T.K. Panovska, C. R. Chimie 12, **2009**, 1259-1266.
39- A. Cakir, A. Mavi, A. Yıldırım, M. E. Duru, M. Harmandar, C. Kazaz, J.Ethnopharmacol, **2003**, 87,73-83.
40- F. Oke, B. Aslim, S. Ozturk, S. Altundag, Food Chem, **2009**, 112, 874-879.
41- K. Schlesier, M. Harwat, V. Bohm, R. Bitsch, Free Rad. Res, **2002**, 36, 177-187.
42- D. Yang, Q. Wang, J. Jiang, T. Ying, Asia Pac. J. Clin. Nutr, **2007**, 16(1), 158-163.