

HPLC analysis for identification and quantification of phenolic acids and flavonoids in *Juniperus excelsa*

N. El-Achi¹, Y. Bakkour^{1,2}, H. El-Nakat^{1*}, F. El-Omar²

¹Department of Chemistry, Faculty of Science, University of Balamand, Deir El-Balamand, El-Koura, Lebanon

²Laboratory of Applied Chemistry, Faculty of Science III, Lebanese University, P.O. Box 826, Tripoli, Lebanon

*Corresponding Author

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ABSTRACT

The present study was designed to define the phenolic profile, flavonoid profile and the biological potentials of the ethanol extract of the berries of *Juniperus excelsa* growing in Lebanon. A total of 11 phenolic compounds, including gallic acid, vanillic acid, hydroxybenzoic acid, sinapic acid, ellagic acid, myrcetin, and hesperitin were identified and quantified in *Juniperus excelsa* using Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The ethanolic extract of the berries of *J. excelsa* was examined for the antioxidant activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. The extract showed a strong scavenging activity with an IC₅₀ 48.90µg/ml compared to the synthetic antioxidant butylatedhydroxytoluene (BHT) (IC₅₀ 91.40µg/ml). This strong antioxidant activity can be attributed to the total phenolic content (17.95mg/g of extract) and the total flavonoidal content (3.817mg/g of extract) of the extract that were determined using the Folin-Ciocalteau (FC) and aluminum chloride methods, respectively.

Keywords: *Juniperus excelsa*; Antioxidant activity; Phenolic acids; Flavonoids; RP-HPLC.

INTRODUCTION

Phenolic compounds form a large group of plant secondary metabolites which are released by plants for defense and adaptation (Furstenberg-Hagg, et al., 2013).

All phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids. Vanillic acid, gallic acid, sinapic acid, ferulic acid, p-coumaric acid and p-hydroxybenzoic acids are the most common acids which are present in plants including fruits and vegetables (Shahidi and Naczki, 2004). On the other hand, flavonoids are polyphenols bearing a C₆-C₃-C₆ carbon framework. The major subclasses which are common in diets are flavanol, flavanones, flavones, isoflavones, flavonols, and anthocyanidins (Inbaraj, et al., 2010). In recent years, there is an increased interest in phenolic acid and flavonoids because of their strong antimicrobial and antioxidant activities that have positive impacts on human health (Amzad Hossain, et al., 2011; Rached, et al., 2010) including their protective role

against cancer and heart diseases. It is important, therefore, to determine their quantity and variety in different medicinal plants.

Juniperus excelsa (Family-Cupressaceae) is used to treat respiratory tract diseases ranging from common cold to pneumonia, urinary tract inflammations, renal and gall bladder stones, and rheumatism (El Beyruthy, et al., 2008; Öztürk, et al., 2011; Sanchez de Medina, et al., 1994).

Reactive oxygen species (ROS) are present in the human body as a result of food metabolism, exposure to pollution and radiation. Since ROS directly contribute to aging, DNA damage and cancer, it is important to look for antioxidants that tend to donate a hydrogen atom to these ROS and thus neutralizing their effect (Evans, et al., 2006). The commonly used synthetic antioxidants to preserve food quality are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). However, these antioxidants were reported to be possible carcinogens to humans (WHO, 2002). So, it is vital to replace these antioxidants by natural alternatives like phenolic acids and flavonoids especially that several studies have elucidated the high antioxidant activity of phenolic compounds (Amzad Hossain, et al., 2011; Barros, et al., 2010; Proestos et al., 2006; Rached, et al., 2010). The antioxidant activity of *Juniperus excelsa* essential oil tested by Bakkour et al. (2013), showed that the essential oil was capable of reducing the DPPH, but with limited activity compared to that of BHT.

A number of analytical methods have been used for the separation and identification of phenolic compounds. Most of these protocols depend on High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC/MS) (Chu, et al., 2001; Deng, et al., 1999). The sample preparation for GC/MS requires derivatization which is very sensitive to moisture and temperature; thus quantification is not easily performed using this technique. Since HPLC doesn't require previous derivatization for the analysis, it is considered to be the most commonly used technique (Chen, et al., 2001).

In this study the antioxidant activity, total phenolic and total flavonoid content of the ethanolic extract was assessed using DPPH, FC, and aluminum chloride colorimetric assays respectively. RP-HPLC was utilized for the identification and quantification of each of the phenolic compounds in the berries using the external standard method.

Accordingly, our objectives were to develop a RPHPLC method for qualitative and quantitative determination of phenolic acids and flavonoids in berries of *J. excelsa* and to determine the antioxidant activity of the extract in a way to verify, scientifically, the usage of this plant in herbal medicine. To our best knowledge, this is the first report that focuses on the phenolic compounds of *J. excelsa*.

MATERIALS AND METHODS

Plant material: 2000g of *Juniperus excelsa* berries were collected from Makmel Mountain in June 2012 and authenticated by Dr. Ali Chakas Botanist, Lebanese University, Lebanon.

Chemicals: The solvent used for extraction of *Juniperus excelsa* was analytical grade ethanol; while those used for the HPLC analysis were HPLC grade acetonitrile and Milli-Q (Millipore Australia Pty. Ltd) distilled water.

Aluminum chloride, potassium acetate, FC reagent, Na₂CO₃, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) and the standards used (such as Gallic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, hydroxybenzoic acid, sinapic acid, ferulic acid, p-coumaric acid, cinammic acid,

myrcetin, hesperetin, quercetin, naringenin, chrysin and ellagic acid) were all purchased from Sigma Aldrich (Steinheim, Germany) .

Determination of Total Flavonoids: Total flavonoid content of the ethanolic extract was determined using the aluminum chloride colorimetric method (Amzad Hossain, et al., 2011) with slight modifications. 0.5ml of ethanolic extract, 0.1ml of aluminum chloride (10%), 0.1 mL of potassium acetate (1M) and 4.3 mL of distilled water were mixed together then incubated for 30 minutes. The Absorbance was measured at 415 nm using a Shimadzu UV-1800 Spectrophotometer. Quercetin was used to prepare the calibration curve. Results were expressed in mg of quercetin/g dry sample.

Determination of Total Phenolic: Estimation was done by colorimetric assay (Barros, et al., 2010) with slight modifications. 1ml of the ethanolic extract was mixed with 0.5ml of Folin-Ciocalteu reagent (1N) and diluted by 5ml of distilled water. After 5 minutes, 350 μ l of Na₂CO₃15% was added. The tubes were allowed to stand for 1.5h in the dark for color development. Absorbance was measured at 725nm by Shimadzu UV-1800 Spectrophotometer. Gallic acid was used to prepare the calibration curve. Results were expressed in mg of gallic acid/g dry sample.

HPLC Analysis: A simple and quick reversed phase method for determination of phenolic acids and flavonoids was developed. Chromatography analysis was performed with the use of liquid chromatographic system, which consisted of Prominence Liquid Chromatographic Shimadzu instrument with UV- Detector-SPD-20A. The separation was carried out on Ascentis RP-Amide (15 cm x 4.6 mm ID, 5 μ m particles) reversed phase column. Column temperature was maintained at 25°C. The mobile phase was a gradient elution of water containing 0.085 % orthophosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml/min. The gradient program of solvent A in B (v/v) was as follows: 0-30 min 85 % A; 30-35 min 65% A; 35-60 min 15% A. The injection volume for all samples was 20 μ l. For detection, chromatograms were monitored at 280 nm. Identification of phenolic acids and flavonoids was based on retention times in comparison with standards. The quantification was carried out using the external standard method. Stock solution of standard compounds at concentration 1mg/ml each was prepared in acetonitrile, and several dilutions with acetonitrile were made. The solution of standards at various concentrations (25-125mg/l) was injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of each compound was calculated from peak area according to calibration curves. The amount of each phenolic acid and flavonoid was expressed as mg/g dry sample.

Antioxidant Activity of the Ethanolic Extract: The radical scavenging activity of the BHT and a range of 0.04-0.4 mg/ml solutions of ethanolic extract were tested using the same protocol mentioned by (Prakash, et al., 2001), but with slight modifications. DPPH radical (1.75 mM) was added and the total volume was adjusted to 4ml by ethanol. The reaction mixtures were shaken and then incubated at room temperature in the dark for 60 minutes. The DPPH radical inhibition was measured at 517nm by using a Shimadzu UV -1800 Spectrophotometer.

RESULTS

Total phenolic and total flavonoid content: The calibration curve that represents the variation of the absorbance at 725 nm as a function of the range of concentrations of Gallic acid, used as a standard, showed a good linearity with a fit $R^2 = 0.999$. Similarly, the calibration curve representing the variation of the absorbance at 415nm as a function of the range of concentrations of Quercetin revealed a fit of $R^2 = 0.997$.

Accordingly, the total phenolic content of the *Juniperus excelsa* berries was 25.90mg gallic acid/g dry weight; whereas its total flavonoid content was 3.833mg quercetin/g extract. The results show that the berries contain significant amounts of phenolic compounds including flavonoids.

HPLC Analysis: After extraction, the content of phenolic substances was determined by HPLC quantitative analysis. The HPLC chromatogram of *Juniperus excelsa* is presented in Figure 1. and the amounts of identified phenolic compounds are listed in Table 1. The major constituents of the extract are phenolic acids whereas flavonoids are present in relatively lower levels. This is in accordance with the results obtained by the aluminum chloride and FC colorimetric methods. The most abundant phenolic acids were Gallic acid (11.3mg/g), Cinnamic acid (5.45mg/g) and ellagic acid (3.18mg/g). Quercetin (0.36mg/g) and hesperetin (0.38mg/g) were the main identified flavonoids.

Antioxidant capacity: Degree of inhibition was calculated by following equation:

$$\% \text{ DPPH Inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

The ethanolic extract of the *Juniperus excelsa* inhibited the activity of DPPH radical in a dose response relationship. In this case, the IC₅₀ of the extract was 48.90µg/ml which was significantly less than that of BHT (91.40µg/ml). Accordingly, the ethanolic extract is a better antioxidant than the commonly used synthetic BHT and can be considered a potential natural additive.

DISCUSSION

The phenolic compounds, including flavonoids, of the extract of the berries of Lebanese *Juniperus excelsa* were quantified using the FC and aluminum chloride spectrophotometric methods. The chemical composition of the extract was determined using quantitative HPLC using the external standard method. The extract was found to be rich in phenolic acids and flavonoids which have important pharmacological impact, thus supporting the potential medicinal applications of these berries. Gallic acid has been shown to be anti-inflammatory (Stich, et al., 1982), antimutagenic (Ohno, et al., 1999), analgesic (Krogh, et al., 2000). Sinapic acid has been reported to protect from myocardial infarction (Roy et al., 2012), inhibit KCN-induced hypoxia and inhibit scopolamine-induced memory impairment (Karakida, et al., 2007).

The extract exhibited a significantly strong antioxidant activity compared to the synthetic food additive BHT. This can be attributed to the relatively high levels of phenolic compounds, mainly gallic acid (Table 1), which have strong radical scavenging activity as they favor the donation of hydrogen to the radical in order to form a highly stable phenoxy radical (Srinivasan, et al., 2007).

CONCLUSION

This work suggests the usage of extract of the berries of *Juniperus excelsa* in food industry as natural food additives.

REFERENCES

- Amzad Hossain, M., Mizanur Rahman, S.M., (2011): Total phenolics, flavonoids and antioxidant activity of tropical fruit pineapple. *Food Res. INT.*, 44 (3): 672-676.
- Bakkour, Y., El-Achi, N., Tabcheh, M., El-Nakat, H., El Omar, F., (2013): Chemical composition and antioxidant activities of the essential oils from green and ripe berries of *Juniperus excelsa* growing in Lebanon. *J. Pharm. Life Sci.*, 4(2): 2362-2367.

- Barros L, Heleno SA, Carvalho AM, Ferreira ICFR (2010): Lamiaceae often used in Portuguese folk medicine as a source of powerful antioxidants: Vitamins and phenolics, *LWT-Food Science and Technology* 43 (3), 544-550
- Chen, H., Zuo, Y., Deng, Y., (2001): Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *J. Chromat. A*, 913: 387-395.
- Chu, T.Y., Chang, C. H., Liao, Y.C., Chen, Y.C., (2001): Microwave accelerated derivatization processes for the determination of phenolic acids by gas chromatography-mass spectrometry. *Talanta*, 54: 1163-1171.
- Evans, M.D., Cooke, M.S., (2006): Oxidative damage to DNA in non-malignant disease: biomarker or biohazard? *Genome dynamics*, 02: 53-66.
- Deng, Y., Fan, X., Delago, A., Nolan, C., Furton, K., Zuo, Y., (1998): Separation and determination of aromatic acids in natural water with preconcentration by capillary zone electrophoresis. *J. Chromat. A*, 817: 145-152.
- Fürstenberg-Hägg, J., Zagrobelny, M., Bak, S., (2013): Plant Defense against Insect Herbivores. *Int J Mol Sci. May 2013; 14(5): 10242–10297.*
- El Beyruthy, M., Arnold, N., (2008): Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon. *J.Ethnopharmacol.*, 120: 315-334.
- Inbaraj, B.S., Lu, H., Kao, T.H., Chen B.H., (2010): Simultaneous determination of phenolic acids and flavonoids in *Lycium barbarum* Linnaeus by HPLC-DAD-ESI-MS. *J Pharm.Biomed. Anal.*, 51: 549–556 .
- Karakida, F., Ikeya, Y., Tsunakawa, M., Yamaguchi, T., Ikarashi, Y., Takeda, S., Aburada, M., (2007): Cerebral protective and cognition-improving effects of sinapic acid in rodents. *Biol. Pharm. Bul.*,30(3): 514-519.
- Krogh, R., Yunes, R., (2000): Structure–activity relationships for the analgesic activity of gallic acid derivatives. *Farmaco*, 55: 730-735.
- Ohno, Y., Fukuda, K., Takemura, G., Toyota, M., Watanabe, M., Yasuda, N., (1999): Induction of apoptosis by gallic acid in lung cancer cells. *Anticancer Drugs*, 10: 845-851.
- Öztürk, M., Tümen, I., Uğur, A., Aydoğmuş-Öztürk, F., Topçu, G., (2011): Evaluation of fruit extracts of six Turkish juniperus species for their antioxidant, anticholinesterase and antimicrobial activities. *J. Sci. Food Agric.*, 91(5): 867–876.
- Prakash, A., Rigelhof, F., Miller, E., (2001): Antioxidant Activity. *Medallion Laboratories Analytical Progress*. Minneapolis Minnesota, USA. www.medallionlabs.com
- Proestos, C., Boziaris, I.S., Nychas, Komaitis, M., (2006): Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry*, 95: 664-671.
- Rached, W., Benamar, H., Bennaceur, M., Marouf, A., (2010): Screening of the antioxidant potential of some Algerian indigenous plants. *Journal of Biological Sciences*, 10(4): 316-324.
- Roy, S.J., Mainzen Prince, S.P., (2012): Protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats. *Food Chem. Toxicol.*, 50(11): 3984-3989.
- Sanchez de Medina, F., Gamez, M.J., Jimenez, I., Jimenez, J., Osuna, J.I., Zarzuelo, A., (1994): Hypoglycemic activity of juniper berries. *Planta Med.*, 60: 197-200.
- Shahidi, F., Naczki, M., (2004): Phenolics in Food and Nutraceuticals. CRC Press LLC, New York, pp. 84.
- Srinivasan, M., Sudheer, A.R. Menon, V.P., (2007): Ferulic acid: Therapeutic potential through its antioxidant property. *J. Clin. Biochem. Nutr.*, 40(2): 92-100.
- Stich, H.F., Rosin, M.P., Brison, L., (1982): Inhibition of mutagenicity of a model nitrosation reaction by naturally occurring phenolics, coffee and tea. *Mutat. Res.*, 95: 119-128.
- World Health Organization. (2002): Food safety and food borne illness. Retrieved June 12, 2012, from <http://www.who.int>.

Table- 1: Phenolic compounds of *Juniperus excelsa*.

Compound	mg/g dry weight	Retention time
Gallic acid	11.3	2.185
Vanilic acid	0.58	10.339
Hydroxybenzoic acid	0.46	12.109
Sinapic acid	0.78	13.814
Ferulic acid	0.575	15.213
p-coumaric acid	0.08	16.56
Cinnamic acid	5.45	26.25
Myrcetin	0.22	33.76
Hesperetin	0.38	36.599
Quercetin	0.36	37.117
Ellagic acid	3.18	39.438

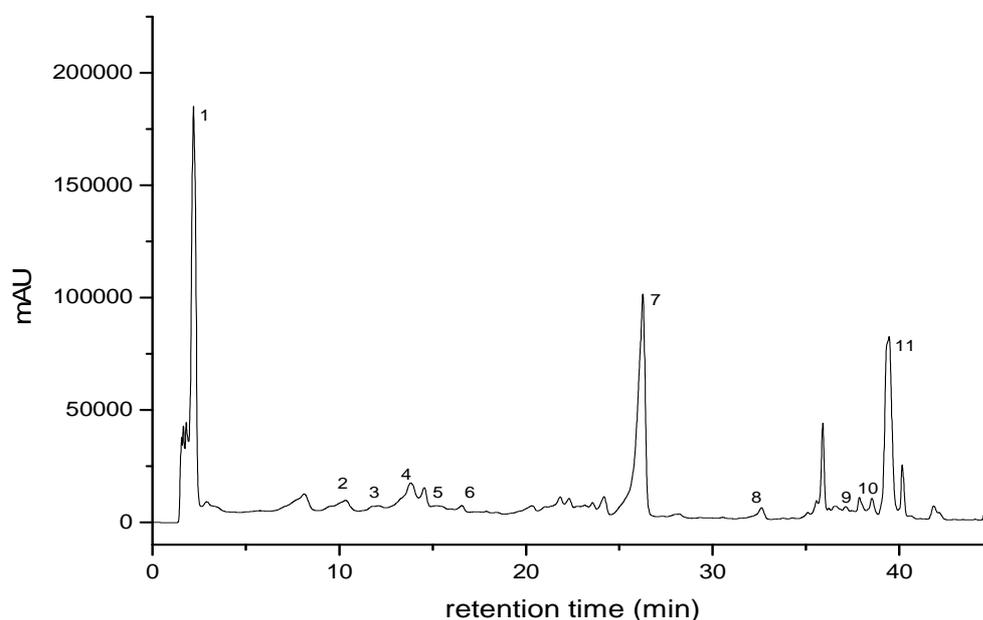


Figure- 1: HPLC chromatogram of the ethanolic extract of *Juniperus excelsa*.

- (1): Gallic acid, (2): Vanilic acid, (3): Hydroxybenzoic acid, (4): Sinapic acid, (5): Ferulic acid, (6): p-coumaric acid, (7) cinnamic acid, (8): Myrcetin, (9): Hesperetin, (10): Quercetin, (11): Ellagic acid, (12)