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The contents of total phenolic, flavonoid, and antioxidant activity of ethyl acetate extract of Juniperus phoenicea L. (Cupressacees) leaves growing in East Libya

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ABSTRACT

Background: the harmful effect of antioxidative stress can be inhabited by the natural antioxidants present in many medicinal plants. These plants contain compounds including phenolics and flavonoids responsible for reducing the effects of free radicals, that can be used as a substitute to treat many diseases.

Aim: This study aims to quantitively determine the total phenolic contents (TPC), total flavonoids contents (TFC), and the reducing power assay of ethyl acetate extract leaves extract of *Juniperus phoenicea L*. plant. *Juniperus* is a member of the Cupressaceae family which has been used as a folk medicine in Libya. the plant was collected from the Al-Jabal Al Akhdar area, east Libya.

Methodology: The powdered dried leaves of Juniperus were extracted continuously by the Soxhlet extractor, the extract was obtained and evaporated to dryness by a rotatory evaporator. The extracts were investigated for reducing power and the presence of phenolic and flavonoid compounds. The TPC was determined using the colorimetric method, expressed as pyrogallol equivalents and the absorbance was measured at 765 nm. The TFC was determined calorimetrically and the results were expressed as quercetin equivalents and the absorbance of color was determined at 510 nm. While the reducing power was measured spectrophotometrically at 700 nm, ascorbic acid was used as standard and expressed as (µg/ml).

Results: the levels of TPC, TFC, and reducing power increased with increasing concentration of extract.

Conclusion: The extracted contents of phenolic compounds, flavonoid compounds, and reducing power were lower than the standard pyrogallol, quercetin, and ascorbic acid respectively.

Key words : Antioxidant activity, extraction, Juniperus phoenicea L., ethyl acetate, leaves extract.

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INTRODUCTION

Thousands of years ago, a variety of medicinal complaints and human diseases were treated by plants and until now numerous species have been stated to carry pharmacological and medicinal activities as they contain chemical constituents of healing values [1][2][3]. In 2008, the World Health Organization (WHO) mentioned that more than eighty percent of world populations based on traditional medicine using plants in their primary healthcare needs [3][4], that the use of this type of traditional plant-based systems has become more and more increasingly common practice in the developing countries [5], as a result of lack of access to conventional medicine and poverty. The extract of many plants offers a wide range of secondary metabolites such as polyphenols tannins, volatile oils, flavonoids, alkaloids, glycosides, and terpenoids [6][7], which are responsible for a wide variety of biological in vitro activities including antibacterial, antifungal and antiprotozoal activities] 8[. Libya has a huge wealth of medicinal herbs which are spread throughout a massive area, especially in the Al-Jabal Al-Akhdar (Green Mountain) region. These plants are used in Libyan folk medicine for their medicinal value. Al-Jabal Al-Akhdar contains a great diversity of plant species demonstrating the economic and medical importance]5[. Nowadays, researchers are searching for natural antioxidants that have no side effects on the health of humans. Research is proceeding to discover effective, newer, and harmless antioxidants to be used in foods and pharmaceuticals to displace the synthetic ones. The major sources of natural antioxidants are mainly medicinal plants. Recently, the investigations had confirmed the validity of many antioxidants uses and reported the phenolic contents of extracts from family Cupressaceae which include neolignans, flavonoids, and phenylpropanoids]9 [. There are many native plants in Mediterranean regions, that belong to the family Cupressaceae of the Juniperus genus [10]. In the region of Al-Jabal Al-Akhdar mountainous, the Phoenician juniper (J. phoenicea) is a bedrock species present in different types of habitats from wadis to slopes but it is not present in the peaks of the mountain. Locally, this plant is well known as "Araar"]11[. This plant is considered as one of the most valuable plants in traditional medicine with different uses such as rheumatism, treatment of diarrhea and diabetes. Also, the inhalation of this plant steam used to control arthritis and for bronchitis. This plant berries are used as a hypoglycemic agent orally. Meanwhile, the leaves are used as diuretic and against bronco-pulmonary disease]12[. Many studies have reported the reducing capabilities of J. phoenicea species grown in different geographical regions and also reported the antibacterial, hepatoprotective effects and antioxidant properties. Many compounds including phenolics, terpenoids, sterols, hydrocarbons and furanone glycosides have been isolated from J. phoenicea' berries and leaves grown in different countries. Also, deoxypodophyllotoxin and Podophyllotoxin were also found in Juniperus aerial parts of species several including J. phoenicea]13[. Therefore, this study aims to quantitively determine the total phenolic contents (TPC), total flavonoids contents (TFC) and reducing power of ethyl acetate extract leaves extract of Juniperus phoenicea L. plant collected from the Al-Jabal Al Akhdar area, east Libya.

MATERIALS AND METHODS:

Chemicals and Reagents

Chemicals such as ethyl acetate, copper sulfate, ascorbic acid, and monobasic dihydrogen phosphate were obtained from Merck company. Ferric chloride, sodium nitrite, aluminum chloride, sodium chloride, and sodium carbonate were obtained from Farmitalia Carloerba. Dibasic monohydrogen phosphate, trichloroacetic acid, sodium nitrate, and sodium hydroxide were obtained from Redeal De Haennagtca. Potassium ferricyanide was obtained from the NICE company.

Plant Materials Collection and Identification

The aerial parts of the plant were collected from the Al-Jabal Al Akhdar area, east Libya, during (Spring, 2018). The botanical identification of Juniperus phoenicea ssp. L. was identified by the aid of the botany department at the University of Benghazi. The leaves only were collected from the aerial parts, air-dried at ambient temperature, grounded, and kept in a tightly closed jar until extraction.

Extraction method of J. phoenicea leaves

The dried leaves of the J. phoenicea plant were powdered in the grinder and an amount equivalent to 40 g was extracted with 250 ml of ethyl acetate solvent continuously by Soxhlet extractor apparatus (size 29-24), the hot Soxhlet extraction process was done for 8 hours. The gained extract was evaporated to dryness at 20-30 ºC by a rotatory evaporator (RE2000). The extract was kept in a dry clean container until the analysis. The extract was investigated for reducing power and the presence of phenolic and flavonoid compounds]14[.

Total phenolic contents determination

The preparation of calibration curve of pyrogallol.

The total phenolic content was determined using the colorimetric method and expressed as pyrogallol equivalents according to the method anticipated by Singleton et al. in 1999, with some adjustments. Serials of pyrogallol standard solutions in ethyl acetate accounting (100, 200. 300, 400, 500 µg/mL) were prepared. Then the Folin-Cicalteau (FC) test was performed, to each 100 µl of different concentrations of pyrogallol standard solution, a 2ml of de-ionized water was added, then mixed with " 600 " µl of FC reagent. The tubes were allowed to stand for 5 min at room temperature, and 2 ml of sodium carbonate (20%) were added and kept for 1 min at a boiling water bath. While, the blank was prepared by adding 100 µl of ethyl acetate, 2ml de-ionized water, " 600 " µl of Folin-Cicalteau reagent, and 2 ml of sodium carbonate (20%). The standards and the blank were cooled and the absorbance of generated blue color was measured at 765 nm by Aquarins (CE700) spectrophotometer Cecil instruments. The mean of three replicate absorbance of different standard solutions was taken and the pyrogallol standard calibration curve was prepared]15[.

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The preparation of J. phoenicea leaves extract for total phenolic contents.
The different concentrations of Juniperus ethyl acetate extract (100, 200. 300, 400, 500 mg/L) were prepared, the same procedure designated for standard pyrogallol was followed. The average absorbance of three measurements was used to determine the mg/g of phenolic contents as pyrogallol equivalents per extract dry weight. The total phenolic content was compared to pyrogallol (100 to 500 mg/L), which is a highly phenolic natural compound and the results were expressed as pyrogallol equivalents in milligram(mg) per extract dry weight in gram(g).

Total Flavonoid Content
The preparation of calibration curve of quercetin.
Different concentrations of standard quercetin were prepared (100 to 500 mg/L), Then total flavonoids content was determined calorimetrically by adding 4 ml of de-ionized water to 1 ml of 100, 200, 300, 400, and 500 mg/L of the standard solutions. 0.3 ml of sodium nitrite solution (5%) was added, followed by 0.3 ml of aluminum chloride solution (10%). After that, the mixture was incubated for 5 min at ambient temperature, and then 2 ml of sodium hydroxide (1 M) were added to the mixture. Directly, the reaction mixture volume was made to 10 ml with de-ionized water. The blank was prepared in the same way as standard but without active constituents. The absorbance of both the blank and developed pink color of standard solutions was determined at 510 nm. A calibration curve was prepared with quercetin and the results were expressed as quercetin equivalents [16].

The preparation of J. phoenicea leaves extract for quercetin.
Juniperus ethyl acetate extract was prepared in different concentrations (100, 200. 300, 400, 500 mg/L), the same procedure described for standard quercetin was followed. The absorbance of three measurements was recorded and the average was taken to plot the calibration curve and the results were expressed as quercetin equivalents in milligram(mg) per extract dry weight in gram(g).

Reducing power assay
The preparation of calibration curve for ascorbic acid.
The reducing power assay was determined spectrophotometrically according to the method proposed by Oyaizu (1986). 2.5 ml of 100, 200, 300, 400, and 500 mg/L of ascorbic acid were mixed with 2.5 ml of sodium phosphate buffer solution (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [K3Fe (CN)6] (1%), then the mixture was incubated for 20 min at 50o C. After that, 2.5 ml of trichloroacetic acid (10% w/v) were added, the mixture was centrifuged for 8 min at 1000 rpm using (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (5ml) was mixed with 5 ml of de-ionized water and 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Ascorbic acid was used as standard [17].

The preparation of J. phoenicea leaves extract for ascorbic acid.
Serials of different Juniperus ethyl acetate extract concentrations were prepared (100, 200. 300, 400, 500 mg/L), the procedure designated for standard ascorbic acid was followed for extract solution of different concentrations. The absorbance of three measurements was recorded and the results were expressed as (µg/ml) against ascorbic acid.

RESULTS:

Total Phenolic content
The amount of phenolic content in the Juniperus ethyl acetate extract was compared to pyrogallol, which is a highly phenolic natural compound. The total phenolic contents of Juniperus ethyl acetate leaves extracts were ranged from (0.047±0.005 at 100 µg/ml of pyrogallol) to (1.022±0.005 at 500 µg/ml of pyrogallol). While, in comparison to pyrogallol equivalent (PE), the quantity of TPC were ranged from 21.36 to 84.55 mg PE per g of dry extract weight (Table 1).

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Table 1: Total phenolic content for Juniperus ethyl acetate leaves extract and pyrogallol phenolic compound.

Conc."μg/ml"	Absorbance (Mean ± Standard Deviation)		TPC in mg/g, in pyrogallol equivalent "mg/g"
	Pyrogallol	Ethyl acetate extract	
100	0.292±0.005	0.047± 0.005	21.36
200	0.494±0.003	0.095± 0.005	43.18
300	0.797±0.007	0.106± 0.003	48.18
400	0.857±0.002	0.120± 0.002	54.55
500	1.022±0.005	0.186± 0.005	84.55

Total flavonoid content

All data were compared to quercetin, which is a flavonoid natural standard and the result showed a lower flavonoid content for our extract. The total flavonoids contents of Juniperus ethyl acetate leaves extracts were ranged from (0.019±0.009 at 100 μg/ml of quercetin) to (1.022±0.005 at 500 μg/ml of quercetin). Whereas, in comparison to quercetin the quantity of TFC were ranged from 14.62 to 90 mg QE per g of dry weight (Table 2).

Table 2: Total flavonoid content of Juniperus ethyl acetate extract and quercetin as flavonoid compound.

Conc."μg/ml"	Absorbance (Mean ± Standard Deviation)		Quercetin equivalent weight "mg/g"
	Quercetin	Ethyl acetate extract	
100	0.236±0.003	0.019±0.009	14.62
200	0.337±0.026	0.042±0.006	32.31
300	0.442±0.087	0.054±0.006	41.54
400	0.542±0.004	0.079±0.006	60.77
500	0.588±0.006	0.117±0.007	90

Reducing power assay

All data were compared to ascorbic acid which has high reducing power ability and the result shows low reducing power assay for *Juniperus* ethyl acetate leaves extract. The reducing power ranged from (0.075±0.012 at 100 μg/ml of pyrogallol) to (0.349±0.015 at 500 μg/ml of pyrogallol) (Table 3)**.**

Table 3: Reducing power assay for Juniperus ethyl acetate extract and ascorbic acid

Conc."μg/ml"	Absorbance (Mean ± Standard Deviation)	
	Ascorbic acid	Ethyl acetate extract
100	0.293±0.012	0.075±0.012
200	0.382±0.032	0.152±0.012
300	0.445±0.008	0.184±0.036
400	0.693±0.10	0.298±0.020
500	0.992±0.005	0.349±0.015

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DISCUSSION:

The largest class of secondary metabolites are represented by phenolic components that are also recognized as exceptional antioxidants. The antioxidant activity of these compounds depends on their redox potential. The numerous bioactivities of phenolic compounds are in control for their biological properties such as anti-inflammatory, anticarcinogenic, antimicrobial, and therapeutic effects [6]. Folin – Ciocalteu (F.C) reagent was used to determine the total polyphenol in sample extracts. This reagent oxidases phenolate, resulting in the production of complex molybdenum-tungsten blue which can be detected spectrophotometrically at 765 nm. F.C. reagent is employed routinely in studying phenolic antioxidants[18].

The total phenolic contents of ethyl acetate Juniperus leaves extract was the lowest in comparison to the phenolic contents of methanol, ethanol, and acetone Juniperus leaves extracts as reported in Elmhdwi *et al*., (2015) study [19]. In which, the phenolic content of acetone extract was the highest and higher than the pyrogallol standard. Meanwhile, the phenolic content of ethanol extract was less than that of the acetone extract but still higher than the pyrogallol standard. While the phenolic content of methanol extract was the lowest and lower than pyrogallol standard. But, it has higher phenolic content than the ethyl acetate extract in this study. Also, in El Jemli *et al*., (2016) study, the aqueous extract of *J. phoenicea* leaves had the lowest (TPC) in comparison to aqueous leaves extracts of other three studied species of the family Cupressaceae and the TPC of ethyl acetate leaves extract in this study (84.55 mg PE/g of dry weight) was lower than that of aqueous extract (116.35 ± 9.71) μg GAE/mg edw[9]. In addition, Ennajar *et al*., (2009), studied the phenolic contents of three different leaves extracts. In which, the TPC was the highest in ethanolic extract (217±2 g GAE/kg of dry material), then methanolic extract (169±2 g GAE/kg of dry material) and followed by ethyl acetate extract (116±2 g/kg at 300 µg/ml of pyrogallol). That was higher than the TFC of ethyl acetate extract found in this study (48.18 g/kg at 300 µg/ml of pyrogallol) [20]. In Menaceur *et al*., (2013) work, the TP content of *J. phoenicea* ethanol extract was (308 mg GAE/ g), which was higher than the highest concentrations of ethyl acetate leaves extract (84.55 mg pyrogallol per g dry weight extract) in the current study[7]. In addition, Keskes *et al*., (2014) mentioned that the total phenolics contents of three *J. phoenicea* leaves extract showed that the lowest content was found in the nonpolar hexane extract (162.3±3.2 mg GAE/g) [21], then in ethyl acetate extract (180.8±3.6 mg GAE/g), and finally in the polar methanol solvent extract, which has the highest content (265.0±5.8mg GAE/g) of TP. Similarly, the highest phenolic contents of this study (84.55 mg pyrogallol per g dry weight extract) were lower than that measured for our ethyl acetate extract.

Flavonoids are extensively dispersed in various amounts in spices, vegetables, and fruits in the plant kingdom. They are used to avoid many diseases in human beings including cancers, diabetes, and coronary heart diseases[6]. The total flavonoids contents (TFC) can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex using aluminum chloride which can be monitored spectrophotometrically at 510 nm [18].

The results in this study showed a lower flavonoid content for Juniperus leaves extracts by ethyl acetate**.** In Elmhdwi *et al*., (2015) study, the acetone Juniperus leaves extracts had higher (TFC) in comparison to quercetin as a flavonoid compound, while, the ethanol and methanol leaves extract was nearly comparable to quercetin standard, these results different to this study results that the (TFC) of ethyl acetate *Juniperus* leaves extract was lower than quercetin standard [19]. Consequently, the total flavonoids contents of Juniperus ethyl acetate leaves extracts were lower than that of the acetone, ethanol, and methanol leaves extracts. Also, the results of this study were different from the results stated in El Jemli *et al*., (2016) study, in which the aqueous leaves extract of *J. phoenicea* had the lowest (TFC) (6.69±0.22 μg (QE)/mg edw) in comparison to aqueous leaves extract of other three studied species of family Cupressaceae. Therefore, the lowest concentration of TFC (14.62 mg QE per g of dry weight extract) of our ethyl acetate *Juniperus* leaves extract was higher than the value stated of aqueous leaves extract of the same species (*J. phoenicea)*[9].

On other hand, In Ennajar *et al*., (2009), the flavonoid contents studied in three different leaves extract of *Juniperus phoenicea L.* stated that the TFC was the highest in ethanolic extract (29.3±1.3 g GAE/kg of dry material), then ethyl acetate extract (24.6±0.7 g GAE/kg of dry material) and followed by methanolic extract (20.9±1.0 g GAE/kg of dry material) [20]. In the current study, the ethyl acetate extract had higher (TFC) 41.54 g/kg at 300 µg/ml of quercetin than the TFC of the three leaves extracts solvents of the same species. In Keskes *et al*., (2014) work. the total flavonoids contents of three *J. phoenicea* leaves extract depended on the polarity of solvents. The lowest contents were found in the nonpolar hexane extract (96.00±0.48 mg QE/g), then in ethyl acetate extract (104.00±0.80 mg QE/g) the TFC increased, and finally, in polar methanol solvent (176.00±0.52 mg QE/g), the TFC was the highest [21]. Accordingly, the highest concentration of TPC (90 mg/g at 500 µg/ml of quercetin) in our studied ethyl acetate leaves extract was lower than the lowest TFC found in the hexane leaves extract of the same species.

The reducing power of bioactive compounds was reflected by the electron donation capacity as indicated by different studies [22]. The presence of reducers (i.e. antioxidants) causes the conversion of the Fe^{+3} / ferricyanide complex used in this method to the Fe^{+2}/ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, a higher absorbance indicates a higher reducing power[18].

All data in this study shows low reducing power assay for *Juniperus* ethyl acetate leaves extract. In El Jemli *et al*., (2016) work,[9] the reducing power of four leaves aqueous extracts of plants from family Cupressaceae including *J. phoenicea* were

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studied, and they found that *J. phoenicea* had the second-highest RP (46.85 ± 0.42 μg/mL) which is higher than the highest reducing power of this study ethyl acetate leaves extracts.

CONCLUSION:

The *Juniperus* ethyl acetate extract contents of phenolics, flavonoids compounds, and reducing power were lower than the pyrogallol, quercetin, and ascorbic acid standard solution respectively. Consequently, they are considered low in *Juniperus* ethyl acetate extract. The variations in TPC, TFC concentration, and reducing power of the same extracting solvent and plant species are due to different factors including geographical region, extraction methods, plant harvesting time, and condition. These factors and others could cause different chemical constituents' concentration and availability.

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