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Polyphenols content and antioxidant power of fruits and leaves of *Juniperus phoenicea* L. From Tounfite (Morocco)

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Abstract:

The natural antioxidants have attracted considerable attention of researchers because of their beneficial roles in human health. In the framework of the development of new antioxidants from natural sources, we are interested in this work in studying the phenolic compounds and evaluating the antioxidant properties of *Juniperus phoenicea* L. leaves and fruits collected in the mountains of Eastern High-Atlas (Tounfite). The phytochemical screening has allowed highlighting the existence of polyphenols, tannins, flavonoids, sterols and triterpenes, and oses and holosides in both studied samples. Mucilages are also present in these samples but in small quantities, while alkaloids and saponins are entirely absent. Extracts from leaves and fruits have been obtained by different extraction solvents: water, methanol, chloroform, ethyl acetate and n-butanol. The contents of total phenols and flavonoids were evaluated using gallic acid and quercetin as standard respectively. The ethyl acetate extracts of the leaves and fruits contain high levels of total phenolic estimated 2,91mg/g and 5,35mg/g equivalent of gallic acid (GAE) respectively. As for the flavonoid content, the ethyl acetate extracts have also reached the increased levels 11,82mg/g and 2,66mg/g equivalent of quercetin (QE). The antioxidant activities by DPPH test were quantified by spectrophotometry; the results showed that the ethyl acetate extracts from the leaves and fruits exhibit an antioxidant activity with inhibitory concentrations 50% (IC_{50}) of 94,4µg/ml and 185,83µg/ml respectively compared to that of vitamin C (47,76µg/ml).

The antioxidant test allowed concluding that the leaves and fruits have a strong anti-radical activity that was assumed to be related closely to the content of polyphenols, which are generally very good antioxidants and that the leaves show a higher activity than fruits.

Keywords: *Juniperus phoenicea L., ethyl acetate extract, phytochemical screening, Antioxidant Activity.*

1. Introduction

For a long time, natural products, especially those from vegetal origin, have always been an important source of therapeutic agents. The man has found out the beneficial properties of medicinal and aromatic plants (MAP) by a progressive approach and after the use of plants with failures and successes. About 25%

of drugs, available for the treatment of diseases, are derived from natural products (plants, animals, bacteria and fungi) [1]. The discovery of radical chemical species, present normally in the body, has revolutionized our understanding of biological mechanisms. These free radicals are produced by various physiological mechanisms, as they are useful for the organism at reasonable dose; but the production can become excessive or result from exogenous toxic phenomena and the bodies will have to protect themselves from these excesses by various antioxidant systems. The antioxidant compounds like vitamin E (tocopherol), vitamin C (ascorbate) and polyphenols, which are powerful antioxidants vis-à-vis the free radicals, act by scavenging radicals and capturing the unpaired electron, turning them into molecules or stable ions [2-3]. This effectiveness in stopping radical reactions, by neutralizing free radicals, is mainly due to their phenolic structures with the presence of hydroxyl groups. The phenolic compounds are a complex group of natural substances that have attracted considerable attention by researchers due to their beneficial roles on human health [4]. Indeed, they are involved in the prevention and the treatment of diseases related to oxidative stress such as cancer, diabetes, hypertension, and Alzheimer's disease by fighting oxidant stress [4-5-6]. These phenolic compounds are particularly an interesting way to limit this oxidative damage. This research aims at valorizing the local flora to develop new compounds or active principles with therapeutic interest. In this respect, we are interested to perform a phytochemical screening followed by determination of total polyphenols and flavonoids from the leaves and the fruits of *Juniperus phoenicea* L. in order to evaluate the antioxidant activity of their extracts, using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test.

2. Materials and Methods

2.1. Phytochemical tests

2.1.1. Plant material

The plant used, selected to perform the phytochemical study and evaluate the antioxidant activity, is represented by leaves and fruits of *J. phoenicea* L. which were harvested in October 2014 in Tounfite (Province of Midelt) in the mountains of Eastern High Atlas (Morocco). This plant has been identified by Mr M. Ibn Tatou Professor in Botany and Plant Ecology Laboratory at the Scientific Institute of Rabat (Morocco). The leaves and dried fruits were pulverized and then used for the preparation of various extracts.

2.1.2. Reactions of characterization

The phytochemical screening is the main way to highlight the great families of the secondary metabolites in the extract from the studied plant. This screening focused on either the formation of insoluble complexes using precipitation reactions or on the formation of colored complex by using the color reactions. The tests of characterization of different chemical groups were conducted according to the experimental protocols by Bruneton J., 2009 [7] and Sofowora et al. [8] by performing the most used pharmaceutical operations in general practice: the decoction; infusion and maceration. The research of alkaloids was performed by precipitation reactions with the general reagents (Mayer and Dragendorff), while the reaction by ferric perchloride (2%) was used for the detection of polyphenols.Flavonoids were detected by the cyanidin reaction while the reaction of Stiasny was used to detect thegallicand catechintannins. Confirming the inexistence of saponins was conducted by measuring the foam index whereas the reaction of Liebermann Buchard allowed detecting the sterols and triterpenes. Concentrated sulfuric acid and saturated alcohol with thymol have revealed the monosaccharides and holosides; absolute ethanol is used to characterize the mucilages.

2.1.3. Preparation of extracts from leaves and fruits of J. phoenicea L.

For solid liquid extraction of total phenols and flavonoids in the solvents, 60 grams of ground material from a dry pulverized sample was macerated in 300ml of aqueous methanol solution (80%) at room temperature every 48 hours (3 replicates). After filtration and vacuum concentration, the aqueous phase was subjected to successive extractions (splitting) of liquid-liquid using organic solvents with increasing polarity (chloroform, ethyl acetate and n-butanol).

2.1.4. Determination of phenolic total

The amount of total phenols in the extracts of *J. phoenicea* L. leaves and fruits was determined by the method described by Abbas Ali Dehpour et al. 2009 [9] slightly modified. They used the Folin-Ciocalteau method to determine the polyphenols content of a plant extract. Different concentrations: 0,08; 0,04; 0,16; 0,32; 0,48; 0,6; 0,96 and 1,28g/ml, were prepared, in volumetric flasks, from 50mg/l of gallic acid by adding to each solution a volume of 1,5ml of Folin-Ciocalteu (10%). The mixture was stirred and allowed to stand for 6 minutes before the addition of 1,5ml of Na_2CO_3 solution (7,5%). The solutions were adjusted with distilled water to a final volume of 100ml, shaken immediately and kept in the dark for 2h at room temperature. The absorbance of each solution was determined at 760nm with a spectrophotometer Shimadzu UV-MINI 1240. The quantitative analysis of total phenols in our phenolic extracts samples was carried out by adapting the same procedure used for the preparation of the curve calibration, replacing gallic acid with a volume of extract to an appropriate concentration. The total polyphenols concentrations of each extract was calculated from the regression equation of the calibration range established with gallic acid (y=0,095x+0,003) (Figure 1). The results are expressed in milligrams of gallic acid equivalent/gram of dry matter (GAE mg/g plant). These results were used to estimate the total polyphenols contents contained in the leaves and fruits of *J. phoenicea* L. The content of total phenols is calculated using the following formula:

$$T = \frac{\mathrm{C} \times \mathrm{V}}{\mathrm{m}_{\text{dried matter}}} \times \mathrm{D}$$

C: Concentration measured by calibration curve (figure 1); V: Volume of the overall sample and D: Dilution factor.

2.1.5. Determination of flavonoids

The quantification of flavonoids was carried out by a colorimetric method adapted by Djeridane et al. 2006 [10]. From the methanolic solution (0,1g/l) of quercetin, different concentrations: 5, 10, 15, 20, 25 and 30µg/ml were prepared in volumetric flasks (50ml) by adding to each solution 20ml of distilled water. After 5 min, 100µl of aluminum trichloride ($AlCl_3$) at 10% (w/v) is added. The solutions were adjusted to 50ml with pure methanol, shaken immediately and then kept in the dark for 30 minutes at room temperature. The absorbance of each concentration was determined by a spectrophotometer at 333nm as mentioned previously for the determination of total phenolic content. Quantitative analysis of flavonoids in our extracts was carried out by adapting the same procedure used for the preparation of the calibration curve, replacing the quercetin by a volume of the extract until an appropriate concentration. The flavonoids concentrations of each extract were calculated from the regression equation of the calibration range established with quercetin (y= 0,073x– 0,081) (Figure 4):

$$T = \frac{\mathrm{C} \times \mathrm{V}}{\mathrm{m}_{\text{dried matter}}} \times \mathrm{D}$$

C: Concentration measured by the calibration curve (figure 4); V: Volume of the overall sample and D: Dilution factor.

2.2. Antioxidant activity

The experiment was performeds mentioned above by the spectrophotometer at 515nm. The solution of DPPH (1,1-diphenyl-di-picrylhydrazyl) at 6.10^{-5}M is obtained by dissolving 2,4mg of the powder in 100ml of ethanol while the samples were prepared by dissolving in ethanol at 1,60mg/ml [11]. The test is carried out by mixing 2,8ml of the prepared solution DPPH with 200µl of the ethyl acetate extract or standard antioxidant (ascorbic acid) at different concentrations (0 to 200µg/ml). After 30 minutes of incubation in the dark at room temperature, the absorbance is read at 515nm against a control containing only ethanol. The control consists of DPPH free extract, and the values obtained are then converted into percentages of inhibition using the following formula:

$$AA\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

AA%: Percentage of antioxidant activity; $A_{control}$: Absorbance of the solution containing only radical DPPH solution

A_{sample} : Absorbance of the sample solution to be tested in the presence of DPPH

The graph of the absorbance variation according to the concentration of extract allowed determining the IC_{50} (concentration corresponding to the loss of 50% of free radicals activity).

3. Results and discussion

3.1. Phytochemical tests

3.1.1. Reactions of characterization

The phytochemical tests were conducted on various extracts prepared from the leaves and the fruits of *J. phoenicea* L. using the decoction, infusion and maceration with specific reagents. The results have shown the presence of some secondary metabolites in the leaves and the fruits of *J. phoenicea*, these results are summarized in Table 1.

Table 1. Results of characterization reactions of different chemical groups in the extracts of various parts of the plant

Chemical group	Leaves	Fruits
Polyphenols	+++	+++
CatechinTannins	+	+
Gallic Taninns	+	+
Flavonoids	+++	++
Sterols and triterpenes	+	+
Oses and holosides	+	++
Mucilages	+	+/-
Alkaloids	-	-
Saponins	-	-

(+): Weak presence (++): Moderate presence

(+++): Abundance (-): Absence

The achieved results reveal that the studied *J. phoenicea* leaves and fruits have the same chemical families with a strong abundance of flavonoids in leaves compared to the fruits that are richer in monosaccharides and holosides than the leaves while alkaloids and saponins are entirely absent.

3.1.2. Yield of extraction

The solid liquid extraction by maceration of phenolic compounds with methanolic solution (80%), followed by fractionation by different pure solvents of increasing polarity (chloroform, ethyl acetate and n-butanol), allowed determining the yields of the extracts (Figure 1).

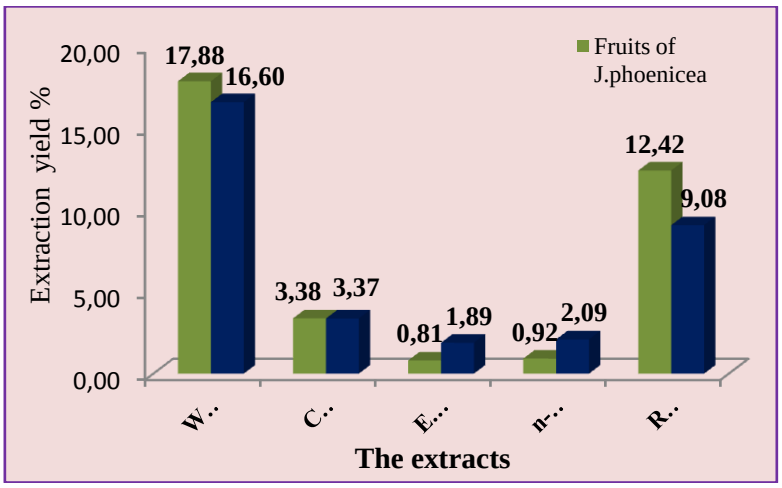

Figure 1. Yields of leaves and fruits extracts of *Juniperus phoenicea* L.

According to the obtained results, we found that the yields of the crude extracts from fruits and leaves, conducted by methanol (80%), reached 17,88% to 16,60% respectively, followed by those of residual aqueous phase obtained by liquid-liquid extractions; whereas, the chloroformic extracts from fruits and leaves recorded similar yields in the range of 3,38% and 3,37% respectively; these rates are higher than those of the ethyl acetate and n-butanol extracts in which the yields of the leaves and fruits are very close.

3.1.3. Determination of total phenols

The total of phenols is determined from the linear regression equation of the calibration curve expressed as mg of gallic acid equivalent /g dry matter (Figure 2). Figure 3 summarizes the results, obtained by a UV-visible spectrophotometer, relating to the total phenols contents of hydromethanolic crude extracts (Water/Met. E), the chloroform extracts (CHCl3. E), ethyl acetate extracts (EthAc. E), butanol extracts (But. E) and residual aqueous phase extracts (RA. E) of *J. phoenicea* L. leaves and fruits. The different fractions obtained from the extracts of leaves and fruits contain the total phenolics but with different amounts (Figure 3).In effect, the first solid-liquid extraction using a methanol-water mixture (80%) showed that the total phenols occurring in the leaves with amounts about 2,99mg GAE/g of dry matter greater than that of fruits (0,61mg GAE/g). Then, the liquid-liquid extraction, using pure solvents of increasing polarity (chloroform, ethyl acetate and n-butanol), revealed that the higher rate of the phenolic compounds was detected for both leaves and fruit in the ethyl acetate extracts: 5,35mg GAE/g dry matter of fruit respectively and 2,91mg GAE/g dry matter of the leaves, followed by butanol extracts where the total phenol content of fruit is almost twice that of the leaves, while the residual aqueous fraction was the poorest in total phenols.

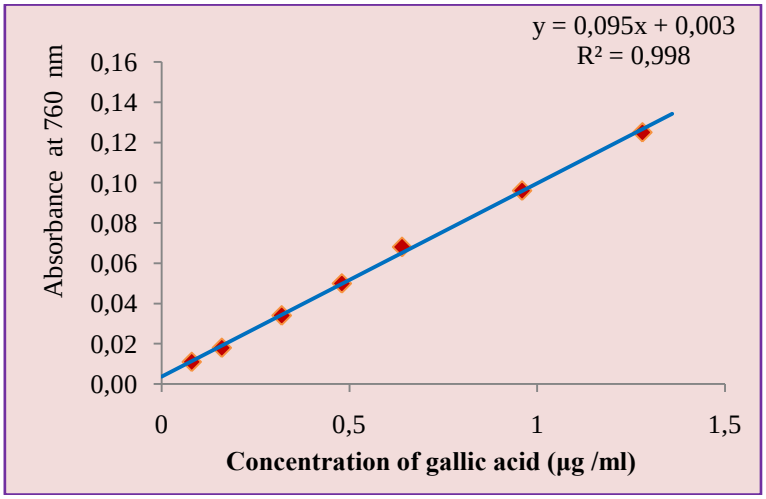

Figure 2. Curve of calibration of gallic acid

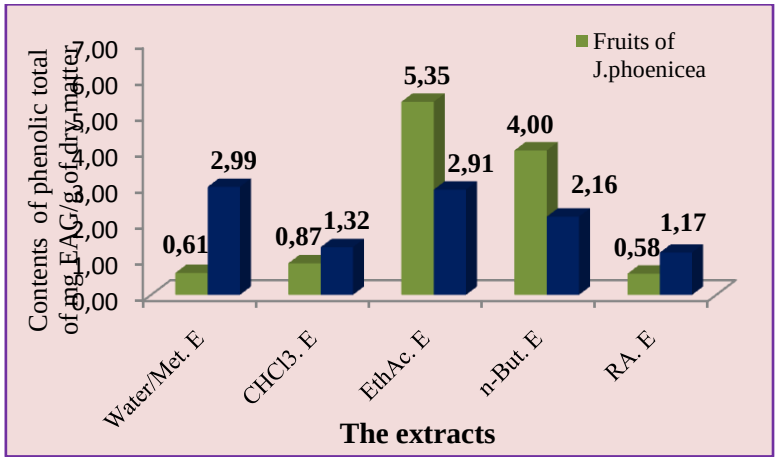

Figure 3. Contents of total phenols of *J. phoenicea* L. leaves and fruits

3.1.4. Determination of flavonoids

The quantitative analyses of flavonoids are determined from the linear regression equation of the calibration curve expressed in mg equivalent of quercetin/g dry matter (Figure 4).

Figure 5 illustrates a comparison of the total flavonoids content from the leaves and fruits of *J. phoenicea* in different studied extracts.

The quantitative determination of flavonoids in different phases, obtained by several solvents, revealed that the leaves seem to get a more significant content of flavonoids than fruits (Figure 5). In addition, our results are in agreement with those obtained by Ennajar (2009) [12].

Several factors can affect the content of phenolic compounds such as geographic and climatic factors, genetic factors, the degree of maturation of the plant and the storage [13-14]. However, the different phases do not have the same richness in these compounds, which suggests that some solvents are more suitable than others for the extraction of flavonoids. For both studied samples, it appears that the ethyl acetate allowed the extraction of the majority of flavonoids (2,36mg QE/g dry matter for leaves and 0,53mg QE/g for fruits) compared to other solvents, followed by n-butanol with a rate of 1,86mg QE/g for leaves and 0,28mg QE/g for fruits. The residual aqueous phase of each sample presented the lowest content of total flavonoids.

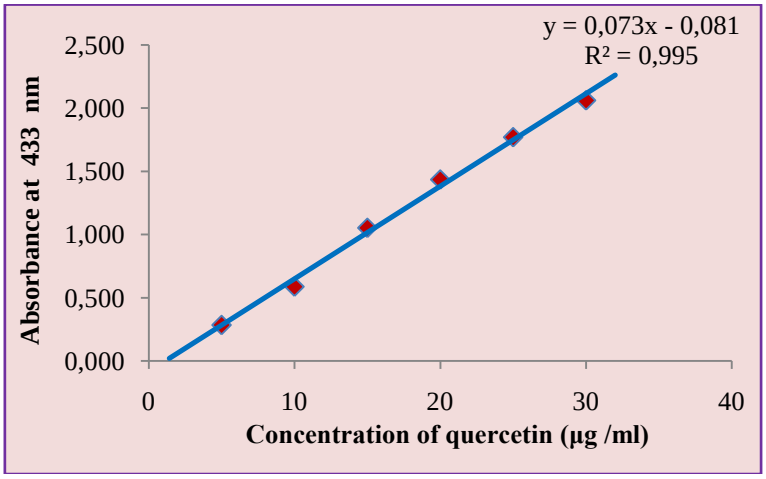

Figure 4. Calibration curve of quercetin.

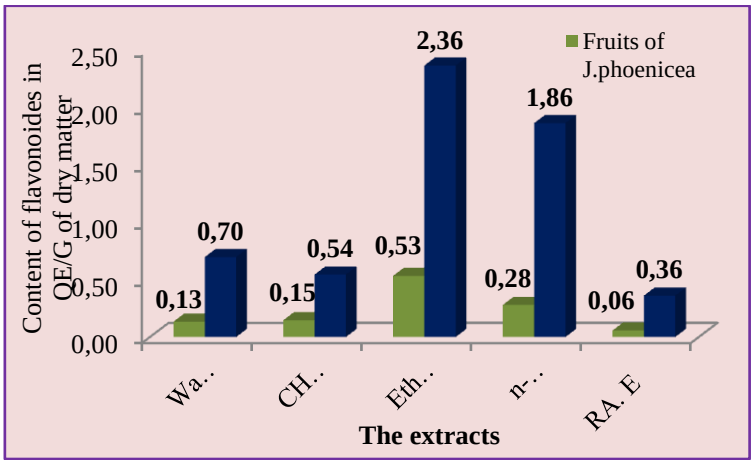

Figure 5. Levels of flavonoids from leaves and fruits of *J. phoenicea* L.

3.2. Antioxidant Activity

3.2.1. Free radical DPPH˙ scavenging

The antioxidant activity of ethyl acetate extracts, from the fruits and the leaves of *J. phoenicea*, is determined by the free radical DPPH reduction method. This is usually the most widely used substrate for quick and direct evaluation of the antioxidant activity of various plant extracts due to its stability at free radical form and the simplicity of the analysis. The chemical compound 2, 2-diphenyl-1-picrylhydrazyl is one of the first free radicals used to study the structure-antioxidant activity relationship of phenolic compounds [15]. It has an unpaired electron on a nitrogen atom. Reducing this radical is accompanied by its passage from the purple color, characteristic of the DPPH˙ solution, to yellow one (DPPH-H) measured by spectrophotometry at 515nm. This reduction in capacity is determined by a decrease in absorbance induced by anti-radical substances [16-17]. According to the obtained values, we calculated the inhibition percentage susing the formula previously given. Then, the results allowed obtaining the curves as shown in Figure 6.

From these results, the percentage of inhibition of the free radical increases with increasing concentration either for vitamin C, which is used as positive control due to its high antioxidant activity, or for the ethyl acetate extracts of the fruit and leaves. For all the applied concentrations, the percentage inhibition of vitamin C is higher than that of ethyl acetate extracts of the fruit and leaves, whereas the percentage inhibition of the leaves is higher than that of fruit. From the concentration 128µg/ml in which ascorbic acid

exerted a maximum anti-radical effect about 93,581% towards the free radical DPPH˙, the ethyl acetate extracts of the fruits and leaves showed an anti-radical activity with inhibition percentages 39,087% and 63,623% respectively.

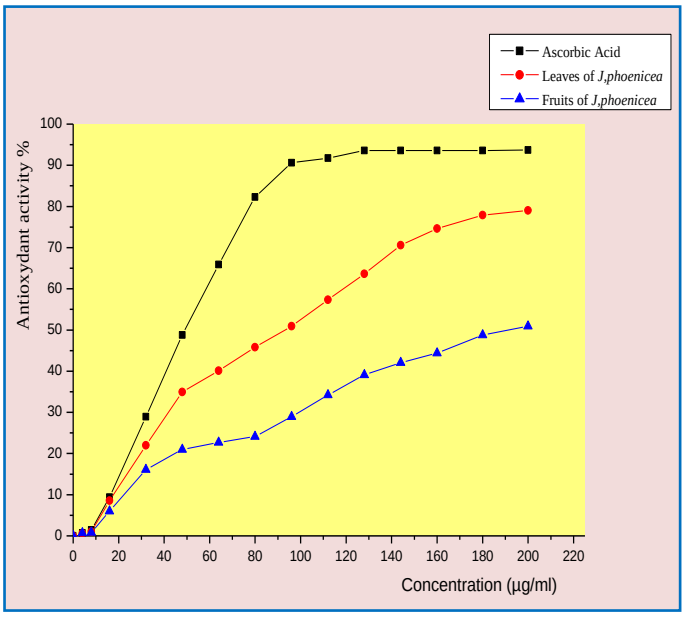

Figure 6. Inhibition Percentage of ethyl acetate extracts from the leaves, the fruits and ascorbic acid.

3.2.2. Determination of inhibitory concentration: IC_{50}

The IC_{50} values determined graphically in µg/ml express the effective concentration of the antioxidant extract required for scavenging and reducing 50% of DPPH˙ radical. The more the value of the IC_{50} is smaller, the more the extract is considered as a potent antioxidant [18] (Figure 7).

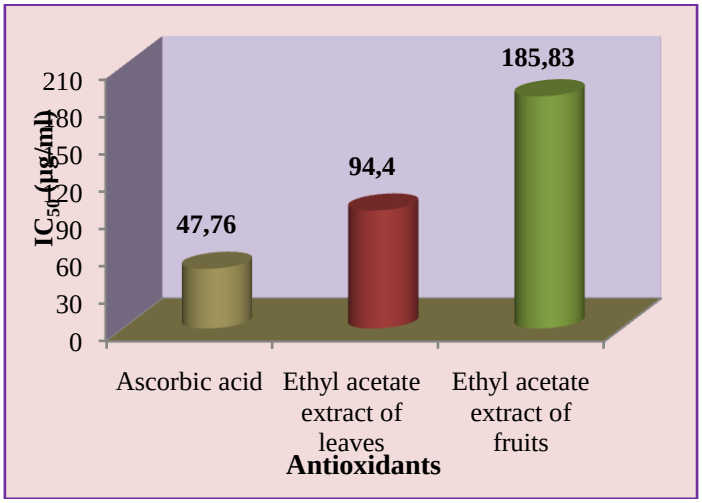

Figure 7. Result of antioxidant test expressing the effective concentration 50% in µg/ml

According to the achieved results, the ethyl acetate extracts of the leaves and fruits are endowed with important antioxidant power. This high activity of these extracts is attributed to their richness in phenolic compounds, which have the highest content of dosed molecules (polyphenols, flavonoids). A study done by Kang et al. [19] suggested that the polar molecules present in plant extracts contribute to the increase of the

anti-radical activity. The reducing power of the ethyl acetate extract of the *J. phoenicea* leaves (IC_{50} = 94,4µg/ml) is more important than that of fruits (IC_{50} = 185,83µg/ml) but relatively lower than that of ascorbic acid which remains a most effective antioxidant with an IC_{50}= 47,76µg/ml. These values can be compared with those of the same species obtained by Ennajar et al. 2009 [12], 273±4mg/ml for ethyl acetate extract from the leaves and 642±7µg/ml for fruits. Furthermore, they also showed that the methanolic extract of the leaves exhibited an anti-radical activity IC_{50}=8,5 ± 0,3mg/l compared to that of vitamin C (4,4 ± 0,2mg/l).

4. Conclusion

Medicinal plants always remain the reliable source of active principles known for their therapeutic properties. The phytochemical screening using reactions of characterization allowed identifying different secondary metabolites present in plant extracts. The dosage in the different phases obtained from the leaves and fruits of *J. phoenicea* showed the abundance of polyphenols and flavonoids, very interesting antioxidant natural substances in pharmacology. The results of the $DPPH^{\cdot}$ assay indicated that the ethyl acetate extracts of the various used parts of *J. phoenicea* exhibit very significant anti-radical activities, and that the antiradical activity of leaves is higher than that of fruits. This difference in activity may be related to quantitative and/or qualitative diversity of phenolic compounds present in the extract of each plant part. To evaluate more this plant, the isolation and the identification of bioactive compounds responsible for antioxidant activity are necessary.

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