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CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITY OF LEAVES EXTRACTS OF *CISTUS LADANIFERUS* (L)

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ABSTRACT

The present study aims to assess the in vitro antioxidant activity in the methanol ,water, ethanol extracts and the decoction of leaves of *Cistus ladaniferus* (L). The antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP) and chelation power on ferrous ions. The quantitative analysis of total flavonoids and phenols by the colorimetric method shows that polar extracts (methanol and water) and the decoction is the most extracts rich in polyphenols compared with the ethanol extract. The methanolic extract of *Cistus ladaniferus* has better antiradical capacity (IC50 = (0.047 ± 0.01 mg / ml) in comparison with ascorbic acid (IC50= 0.041 ± 0.07mg / ml). As for the ethanolic extract, it has an ability to reduce iron much lower than that of ascorbic acid. In general, the evaluation of antioxidant indicated that the leaves extracts of *Cistus ladaniferus* (methanol, water, decoction) showed strong activity to scavenge DPPH radicals, a high reducing power of iron, but very little chelating effect.

KEYWORDS: Cistus ladaniferus; Antioxidant activity; DPPH; FRAP; Chelation power on ferrous ions.

INTRODUCTION

Cistus ladaniferus is a very fragrant shrub, up to 2 to 3 meters in height. It belongs to the family of Cisitaceae, represented by seven genera (Cistus, Fumane. Halmium, Tuberaria. Helianthemum, Hudsonia and Lechea), the genus includes 16 species, particularly prevalent in the Mediterranean (Talavera et al., 1993). Cistus ladaniferus spread to Portugal, Spain, Italy, Algeria, and Morocco (Mariotti et al., 1997).

Research has shown that the *Cistus ladaniferus* has antifungal effects (Barros et al., 2013), antiviral, antinflammatoire, gastroprotective, antitumor (Skoric, 2012). Flavonoids and tannins are the main compounds of cistus ladaniferus, they are largely appreciated by their potential beneficial health effects, such as antioxidant (Amensour et al., 2010), antimicrobial (Guvenc et al., 2005). The cistus ladaniferus aqueous extract has an antidiarrheal activity and antipropulsive (Aziz et al., 2011). It was also considered curative and preventative against hypertension (Belmoukhtar et al., 2009).

MATERIAL AND METHODS

Plant materials

The plant was harvested in the period from April to June 2013. The plant material was dried in the shade and dry place at room temperature for some days before being crushed and preserved thereafter until with its use.

Preparation of extracts

Total aqueous extract was prepared using the traditional method (decoction): 10 g of dry powder of the tested plants are boiled with 100 ml of distilled water for 15 minutes. After cooling, the extract is filtered, and then evaporated to dryness under reduced pressure at 60° C using a rotary evaporator. The other extract (ethanolic, methanol, water) are obtained by first out with solvents of increasing polarity. The various fractions are filtered and evaporated to dryness. The obtained residues are then dissolved in distilled water.

Determination of Total Phenolic Content

The quantitative determination of total polyphenols is performed according to the method described by Singleton et coll. (1965) using the Folin-Ciocalteu reagent. This yellow color reagent consists of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) yellow.

In the oxidation of polyphenols, the Folin-Cioclateu reagent is reduced to a new molybdenum-tungsten blue color complex (Ribéreau-Gayo, 1968). The color intensity is proportional to the oxidized phenolic compound rate (Boizot, 2006).

A volume of 200 .mu.l of each sample at various concentrations is mixed with 1 ml of Folin-Ciolcalteu (10%). After 4 min, 800µl of sodium carbonate (Na_2CO_3) (75mg / ml) are added. After 2 h incubation at room temperature the absorbance is measured at 765 nm.

A calibration curve is prepared using gallic acid as standard, the results are expressed in microgram equivalents of gallic acid per milligram extract (EG mcg / mg). Each sample is repeated three times.

Determination of Total Flavonoid Content

Determining flavonoids in the different extracts was performed using the colorimetric method of aluminum trichloride (Bahorun et al., 1996). 1 ml of each sample and standard (prepared in methanol to the organic extracts and distilled water for the aqueous extract) is added to 1 ml of $AlCl_3$ (2% in methanol). After 10 min of incubation, the absorbance is read at 430 nm. The results are expressed as micrograms per milligram equivalent quercetin extract (EQ mcg/mg).

Determination of Condensed Tannin Content

The dosage of the condensed tannins was performed according to the method of the acidic medium by vanillin described (Price et al., 1978).

A volume of 400µl of each extract is supplemented with 3 ml of a methanol solution of 4% vanillin, then 1.5 ml of concentrated hydrochloric acid is added. After 15 min of reaction, the absorbance is read at 550nm. The concentration of condensed tannins is derived from a calibration range established with catechin. The results are expressed as micrograms per milligram equivalent catechin extract (mcg EC / mg).

Determination of DPPH free-radical scavenging activity

The antioxidant activity of the tested extracts was determined by the method of the DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the protocol described by Sanchez-Moreno et al., (1998). The procedure of evaluation of the antioxidant capacity is performed as follows: A volume of 50 .mu.l of each solution extracts at different concentrations of the extracts at different concentrations (0.05-2 mg / ml) and ascorbic acid is added to 1 95 ml of the methanol solution of DPPH (0,025g / l). The negative control is prepared in parallel by mixing 50 .mu.l of methanol with 1.95 ml of the methanolic solution of DPPH. After incubation in the dark for 30 min and at room temperature reading absorbence at 515 nm using a spectrophotometer. The positive control is represented by a solution of a standard antioxidant (ascorbic acid), the absorbance was measured in the same conditions as the samples. For each concentration, the test is repeated three times. Percent inhibition is calculated using the following equation: % IP = (c Abs - Abs t / c Abs)* 100.

Abs c: Absorbance of control. Abs t: Absorbance of the test.

Ferric-reducing antioxidant power assay (FRAP)

The reducing power of various extracts of the plants tested was evaluated according to the Oyaizu method (1986). This method is based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}).

1 ml of different concentrations (0.05-2,5 mg / ml) of each diluted extract in distilled water was mixed with 2.5 ml of a phosphate buffer solution 0.2 M (pH 6.6) and 2.5 ml a potassium ferricyanide solution $K_3Fe(CN)_6$ 1%.

The mixtures were incubated at 50 ° C for 20 min. After, 2.5 ml of trichloroacetic acid (10%) is added. The whole is centrifuged at 3000 rpm for 10 min. At the end, 2.5 ml of the supernatant of each concentration is mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$, 6:20 (0.1%). Absorbance is measured at 700 nm against a blank. The ascorbic acid is used as a positive control acid whose absorbance was measured in the same conditions as the test samples.

Chelation power on ferrous ions

The in vitro evaluation of the power of chelating different extracts was performed according to the method described by Decker et al. (1990) who used ferrozine as a stable compound. This compound forms with the Fe^{2+} ions ferrozine a free-Fe^{2+} complex of intense purple color. Over the coloring of the solution containing the extract tested is intense, the chelating power of the extract was important. This is why an increase in the absorbance of the complex Fe^{2+}-ferrosine indicates the presence of antioxidants chelators (Le et al., 2007).

A volume of 500.mu.l of solutions of extracts or standard chelator (EDTA) at different concentrations are added to 100.mu.l of $FeCl_2$ (0.6 mM) and 900.mu.l of methanol. After 5 min of incubation, 100 .mu.l of ferrosine (5 mM) were added, the mixture stirred and allowed to stand for 10 minutes. The absorbance of the complex Fe^{2+} -ferrosine is measured at 562 nm. The iron chelating power is expressed as a percentage using the following equation:

Chelating power (%) = (OD control - OD sample / OD control) * 100.

Statistical analysis

All results are expressed as mean ± standard deviation (SD). Comparing the groups with the control is performed using the software Primer of biostatistic. Values are considered significantly different at the threshold level of 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Determination of Total Phenolic Content, total flavonoids and condensed tannin content of *Cistus ladaniferus* extracts.

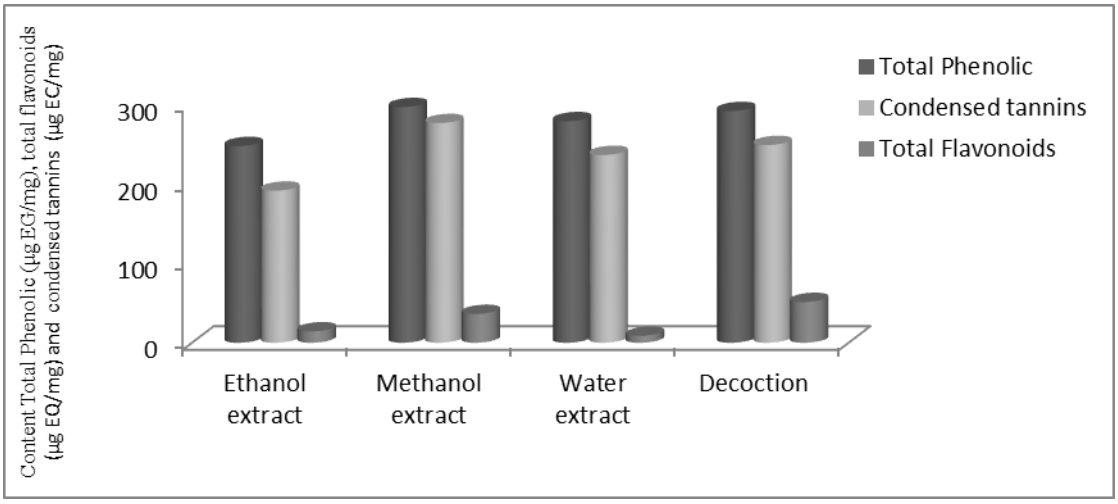

Figure 1: Determination of Total Phenolic Content, total flavonoids and condensed tannins content of *Cistus ladaniferus* extracts.

The levels of total phenols species studied were first reported in equivalent milligrams of gallic acid and determined by the type of equation: y=0,010x+0.031 knowing that $R^2 = 0.995$.

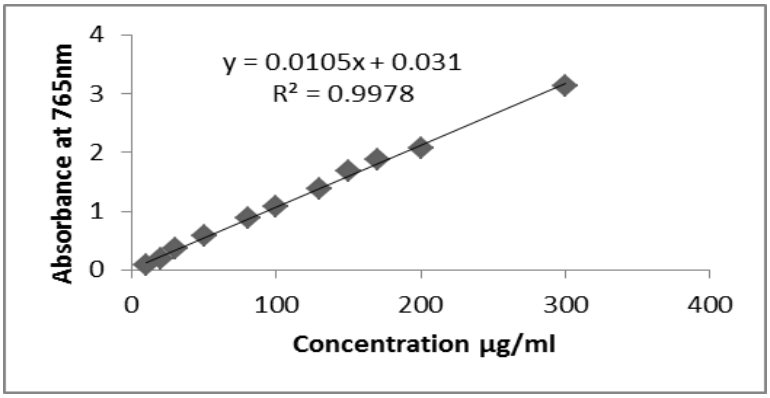

Figure 2: Calibration curve of gallic acid.

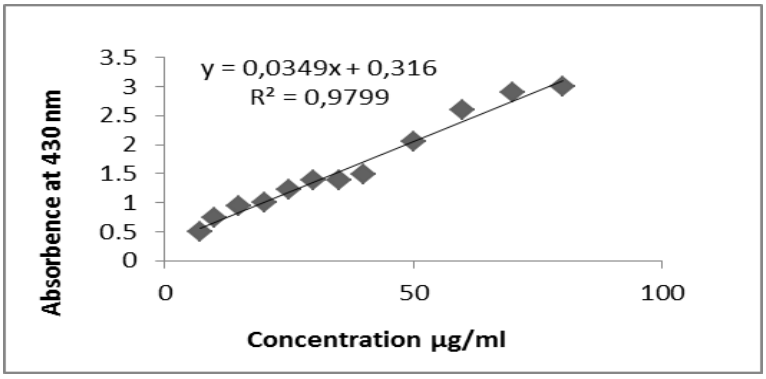

Figure 3 : Calibration curve of Quercetin.

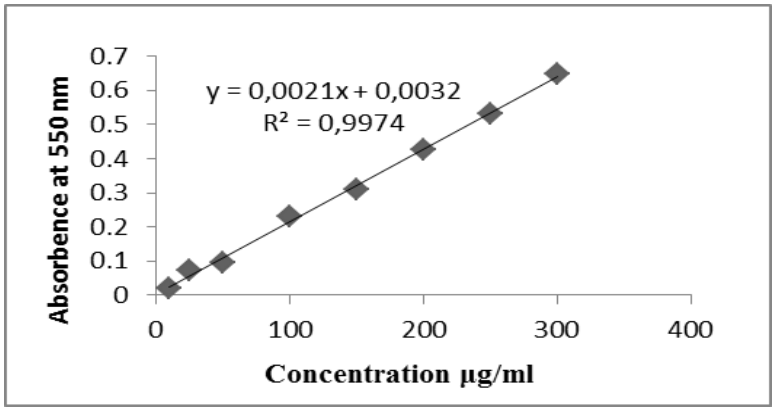

Figure 4: Calibration curve of Catechin.

The results show that the polar extracts (methanol and water) and the decoction is the most extracts rich in polyphenols compared with the ethanol extract. *Cistus ladaniferus*, its total methanol and water extracts contain the same high content of phenolic compounds (296.5 ± 0.005, 291.5 ± 0.001µg EAG / mg) respectively.

Flavonoids The rates of the four extracts of the studied plant were obtained from the calibration curve which follows an equation: y = 0.0349 x + 0, 316 knowing that R^2=0.993 (Figure 3).

The results of determination of total flavonoids by the aluminum trichloride method reveals that the water extract, methanol extract and decoction is as the richest in flavonoids extracted. Thereafter comes the ethanolic extract. The results of determination of total flavonoids by the aluminum trichloride method reveals that the water extract, methanol extract and decoction is as the richest in flavonoids extracted. Thereafter comes the ethanolic extract.

The amounts corresponding tannins of each extract were first reported in micrograms equivalent of catechin and determined by the type of equation: y = 0.0021 x + 0.0032 where R^2 = 0.997 (Figure 4). The results of tannin assay revealed that the methanol extract is the richest in tannins and the water extract and decoction. For cons, the ethanolic extract has a low content of condensed tannins compared to other extracts.

Antioxidant Activity

DPPH Radical Scavenging Activity

The anti-radical activity is performed by the method of radical 2,2-diphenyl-1, picrylhydrazyl (DPPH). According Bozin et al. (2008) the radical DPPH is one of the most commonly used substrates for rapid and direct assessment of antioxidant activity due to its radical form of

stability and simplicity of the analysis. This reduction in capacity is determined by a decrease in absorbance induced by anti-radical substances. Ascorbic acid, taken as a reference antioxidant.

The anti-radical activity profiles obtained (Figure 5) show that extracts of *Cistus ladaniferus* have a dose-dependent anti-radical activity, the IC50 for each of the different extracts were determined (Figure 6).

At a concentration of 2 mg / ml inhibitions percentages are of the order of 98.74% for the methanol extract followed by the water extract and the decoction with a percentage of 97.80% and 97.53% respectively. The ethanolic extract has a percent inhibition of the order of 96.64% (Figure 5).

The results show that the methanol extract exhibits antioxidant activities similar to that of ascorbic acid, which exerts an inhibitory effect of 100% at the same concentration 2 mg / ml (Figure 5).

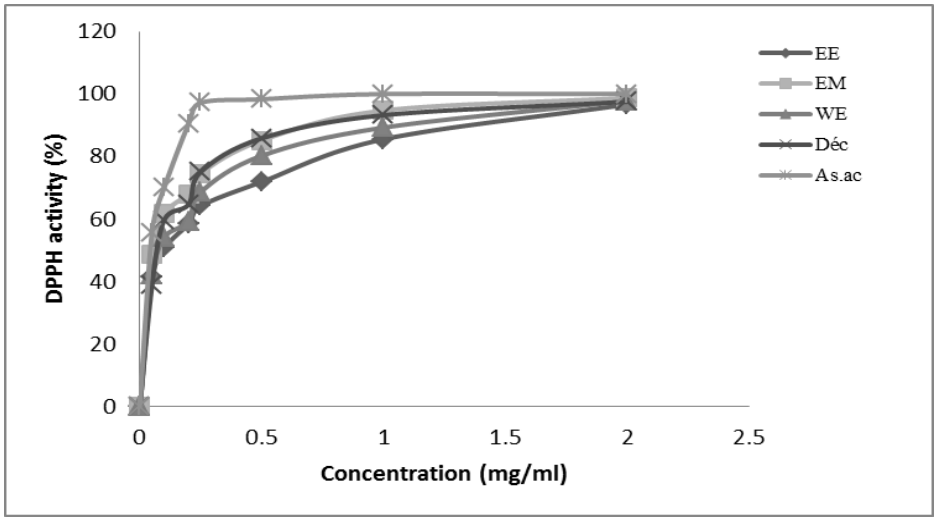

Figure 5: Evolution de l'activité antiradicalaire des extraits *Cistus ladaniferus* extracts and ascorbic acid fonction de la concentration. WE: water extract, ME: Methanol extract, EE: ethanol extract, DEC: decoction, Asac: Ascorbic acid (reference for DPPH radical scavenging activity))

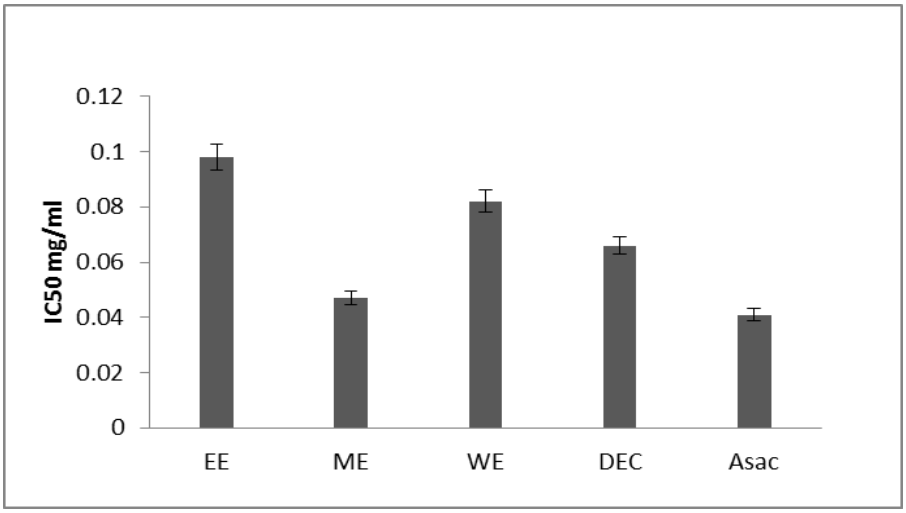

Figure 6: Comparison between IC50 (mg/ml) of plant extracts tested of *Cistus ladaniferus* and IC50 (mg/ml) ascorbic acid (WE: water extract, ME: Methanol extract, EE : ethanol extract, DEC : decoction, Asac: Ascorbic acid (reference for DPPH radical scavenging activity)).

All extracts exhibit anti-radical power to the DPPH (Figure 6). Signalon more than the value of the IC50 becomes shorter, the extract is considered as a powerful antioxidant. The results obtained with this test show that the methanol extract of *Cistus ladaniferus* presents a significant IC50 (0.047 ± 0.01 mg / ml) which results in excellent radical scavenging effects. Comparing this value with that of the standard antioxidant, show that the methanol extract of *Cistus ladaniferus* is as active as ascorbic acid. The decoction (0.066 ± 0.05 mg / ml) and the water extract (0.082 ± 0.03 mg / ml) showed a scavenging power of the DPPH radical greater than that obtained by the ethanol extract (0.098 ± 0.03 mg / ml). These results suggest that the extracts contain scavengers of free radicals.

The antioxidant ascorbic acid showed a very good activity in the range of 0.041mg / ml, which is statistically similar to the activities of all extracts *Cistus ladaniferus*. Our results agree with those found by Benayad (2013) which showed that the water extracts of *C. ladaniferus* and methanol exhibit strong antioxidant activity, this activity reaches 100% at a concentration of 10 ug / ml for both extracts.

Similarly Amensour et al. (2010); Zidane et al. (2012) also confirm that the methanol extract of *C. ladaniferus* has the highest activity against other tested extracts. Our results confirm those published by Babili et al. (2013) which show that the total water extract proves the most powerful in trapping DPPH radical.

Iron Reduction

This method is based on the ability of polyphenols to reduce ferric iron Fe $^{3+}$ ferrous iron Fe $^{2+}$. The results shown in Figure 7 have shown that the capacity reduction is proportional to the increase of the concentration of our samples.

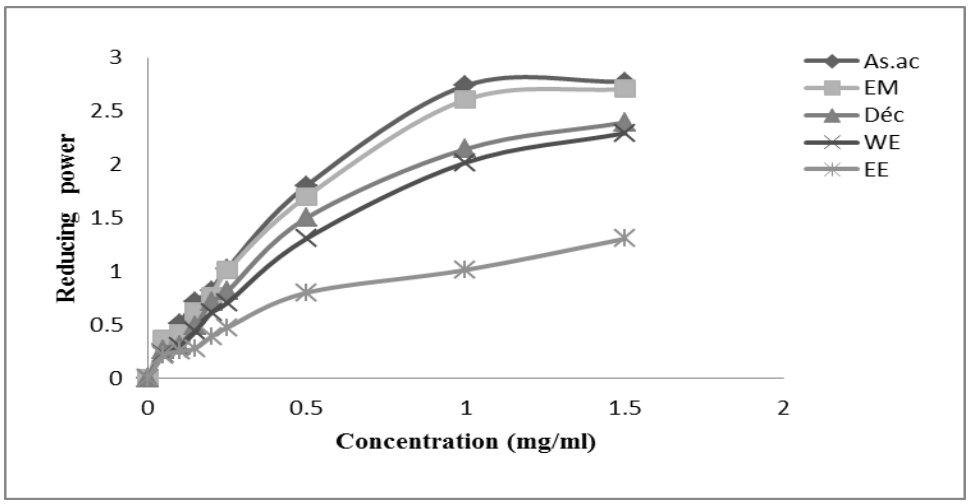

Figure 7: Reducing power of the extracts of *Cistus ladaniferus* and ascorbic acid (WE: water extract, ME: Methanol extract, EE: ethanol extract, DEC: decoction, Asac: Ascorbic acid (reference for reducing power)).

The results in Figure 7 show that the methanol extract of leaves of *C. ladaniferus* represents the highest activity to reduce the iron with a maximum optical density of 2.706 at the concentration 1.5 mg / ml. It is similar to that of ascorbic acid which exhibits an optical density of 2.774 for the same concentration.

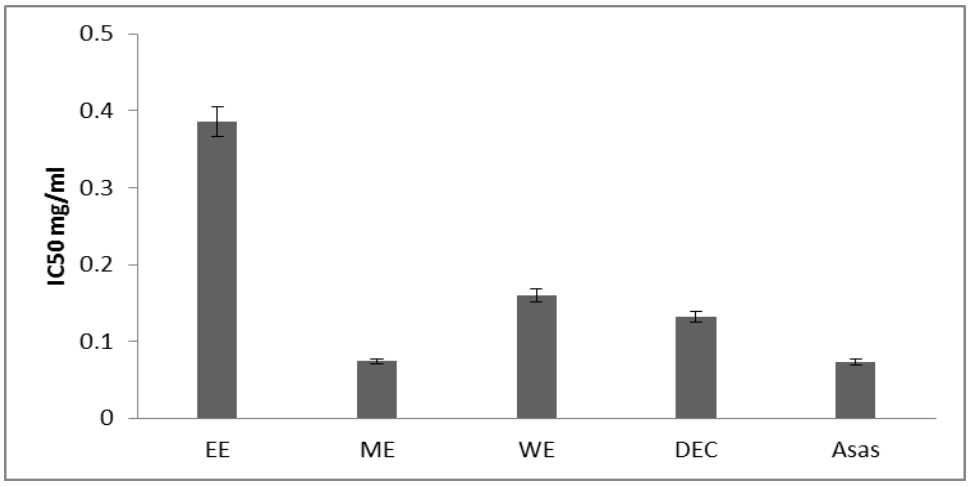

Figure 8: Comparison between the IC50 (mg/ml) extracts of *Cistus ladaniferus* and IC50 (mg/ml) ascorbic acid (WE: water extract, ME: Methanol extract, EE: ethanol extract, DEC: decoction, Asac: Ascorbic acid (reference for reducing capacity)).

We determined the IC50 concentration for comparing the reducing activity of different extracts. The IC50 is the lowest encountered in the methanol extract of the leaves of *Cistus ladaniferus* (0.074 ± 0.04 mg/ml) compared to that of Ascorbic acid (0.073 ± 0.02 mg/ml). The iron removal efficiency is inversely proportional to the IC50 value.

The results show that the methanol extract of *Cistus ladaniferus* and ascorbic acid have almost the same reducing activity. However, these values are not statistically significant (p> 0.05). The sequence for chelating power was Ascorbic acid> methanol >decoction > water > ethanol (Figure8).

Chelating Activity

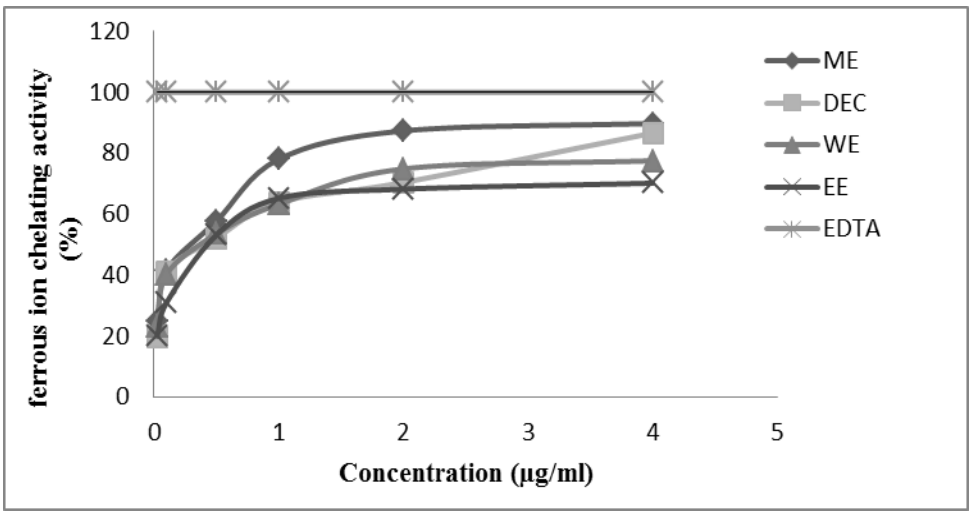

Figure 9: Activity chelating extracts from leaves of *Cistus ladaniferus* and EDTA (Each value represents the mean ± SD of three trials).

The absorbance obtained show that all tested extracts of plants has very low activity chelating vis-à-vis the ferrous ion (Figure 9).

At a concentration of 4000 mg / ml, the methanol and water extracts exert a weak chelating power of the order of 64% and 63% respectively. These values are statistically smaller than the EDTA giving a maximum chelation of 100% at a concentration of 15µg / ml (Figure 9).

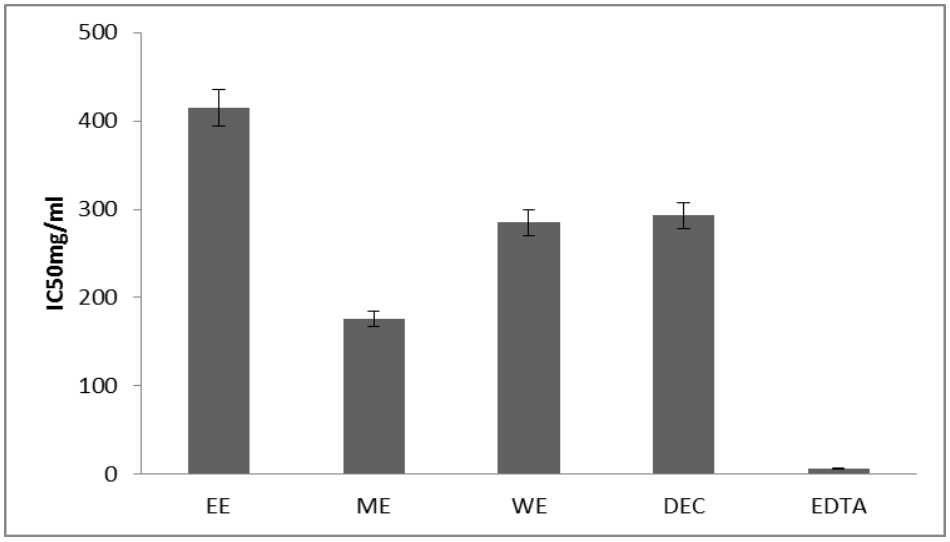

Figure 10: Chelating activity (EC 50 mg/ml) and EDTA extracts standard (WE: water extract, ME: Methanol extract, EE: ethanol extract, DEC: decoction, EDTA: reference for ferric ion-chelating effect)).

The chelating effect of the extracts of the tested plants is measured in this study by the direct reduction of $Fe^{3+}(CN)_6$ in a ferrous $Fe^{2+}(CN)_6$ which is determined by spectrophotometric detection of the complex $(Fe^{3+})_4 [Fe^{2+}(CN)_6]_3$ having a strong absorption at 700 nm (Le et al., 2007). The yellow color of the reaction mixture changes to green depending on the intensity of the reducing power of the sample studied (Zou et al., 2004).

It appears that all the extracts tested plants have very low chelating activity. The differences between the water extract, methanol extract, decoction and EDTA are statistically significant ($p < 0.05$). (Figure 10).

CONCLUSION

The antioxidant activity was evaluated using (2,2-diphenyl-1-picrylhydrazyl) (DPPH), the reduction of iron FRAP (ferric reducing antioxidant power) and power iron chelator. The results obtained in this study indicate that all tested extracts of plants have antioxidant activity and a major iron chelator power but very little chelating activity. In fact, extracts from the leaves of *Cistus ladaniferus* (methanol, water, decoction) has a high activity to scavenge DPPH radicals and a high reducing power of iron compared to other extracted from plants tested.

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