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Antioxidant and free radical-scavenging properties of seeds flavonoids extract of *Cedrus atlantica Manetti* , *Linum usitatissimum* L. and *Ocimum basilicum* L. species

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ABSTRACT

This work aims to evaluate the antioxidant activity of seeds flavonoids extract of *Cedrusatlantica Manetti* (Pinaceae)**,** *Linum usitatissimum L.* (Linaceae) and *Ocimum basilicum L.* (Lamiaceae) species by using the 2,2-diphenyl-1-picrylhydrazyl(DPPH) method. In DPPH scavenging assay the *IC50*value of the extract was found to be respectively 0.40, 1.21 and 0.41 $mg.ml^{-1}$while to the IC_{50} value of the reference standard Butylated hydroxytoluene (BHT) was 0.003 $mg.ml^{-1}$. The seeds flavonoids extract of *C. atlantica Manetti* had a strong scavenger power of free radicals. This study suggests that the mean species may act as a providing antioxidant properties and offering effective protection from free radicals. Then, it's necessary to identify and isolate the compounds that are responsible to the antioxidant activity.

INTRODUCTION

Flavonoids are considered as mainly the most important phenolics, due to its wide range of chemical and biological activities, including antioxidant and free radical scavenging properties (Kahkonen *et al.,* 1999). In fact, many reports reflect, the antioxidant, scavengers of a broad spectrum of reactive oxygen species and inhibitors of lipid peroxidation effect of flavonoids (Williams *et al.,* 2004). Many of These compounds, which are widely distributed across the plant kingdom and currently used in large amounts in our daily diet, represent the most abundant antioxidants in the diet and they have gained tremendous interest as potential therapeutic agents against a wide variety of diseases, most of which involve oxidant damage (Ross and Kasum, 2002).

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Reactive oxygen species (ROS) leads to considerable oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA.The oxidative damage has been implicated in several chronic human diseases, namely cardiovascular diseases, rheumatism, diabetes mellitus and cancer (Pong 2003). Founded on development in free radical biology and thedeficiency of a specific function in fact, of effectual therapies for most chronic diseases, the utility of antioxidants in protection against these diseases is warranted. Antioxidants are chemical substances that reduce or prevent oxidation. They have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease, and several other diseases (Bandyopadhyay, 2007). Many studies have shown that phenolic compounds display antioxidant activity as a result of their capacity to scavenge free radicals (Seyoum *et al.,* 2006). Phenolic compounds can also act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system (Al-Azzawie and Alhamdani, 2006).

Cedrus atlantica Manetti, Members of the pine family (Pinaceae), especially species of Cedrus dominates many of Morocco forests; represent a potential antioxidant effect reported by many studies. *Ocimum basilicum* L. is a member of Lamiaceae, Basil extract has antimicrobial and antioxidant activities due to its phenolic and aromatic compounds (Jayasinghe *et al.,* 2003). The phenolics reported in basil are phenolic acids and flavonol glycosides (Kashyap *et al.,* 2011). The seeds of Linum usitatissimum L. are known to be a rich source of the polar glycosidic-dibenzyl-butanediol-type lignin secoisolariciresinol-diglucoside (SDG) which is considered avaluable food constituent due to its function as precursor for enterolignans (Westcott and Muir, 2003). These métabolites formed by the human enteral microflora have been demonstrated to possess chemopreventive activity against various tumors and cardiovascular disorders (Rickards-Bon and Thompson, 2003).

This work aims to evaluate the antioxidant activity of seeds flavonoids extract of three species (*Cedrus atlantica Manetti*, *Linum usitatissimum L.* and *Ocimum basilicum L.*) by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.

MATERIEL AND METHODS

Plant material

Seeds of Atlas cedar are collected from Forestry center « Azrou » as native coniferal species of Morocco (in November 2013), Lin seeds from cork oak Maâmora forest region and Basilic seeds from Taounate region. Then they are air-dried at 40°C with forced ventilation for two days. The Botanical identification and the Authenticated voucher specimens deposited in the Herbarium of The National Institute of Medicinal and Aromatic Plants.

Phytochemical screening

The identification of flavonoids in our extracts (using the samples of seeds ethanolic extract of each species) was done using the reaction Cyanidin test would detect compounds having the γ-benzopyrone nucleus (Farnsworth, 1966). In the presence of alcohol and hydrochloric magnesium appears coloration ranging from orange to red characteristic flavonoids. The principle is to react at equal volume to be extracted with a hydrochloric solution of alcohol.

A mixture to which is added some magnesium swarf then isoamyl alcohol, $1/5^{th}$ of the volume is compared to the hydrochloric alcohol.

Flavonoids extraction

For each accession, *Cedrus*, *Linum* and *Basilicum* dried seeds. One hundred grams of dried seeds were ground in fine powder and soaked in 50% aqueous ethanol for 4 h is heated to 90°C under reflux. The extract is filtered and the aqueous phase is then extracted with n-butanol and then acidified with 10% HCl to pH 3.The butanol layer was concentrated in vacuum by means of rotavaporand then evaporated to dryness at room temperature. The concentrated extract was then dissolved as extract three times with 200 ml of distilled water/ethyl acetate mixture (1v:1v) for one hour. The organic layer was basified with NaHCO3 to pH 9. After 15 min the residual organic phase (flavonoids) was concentrated in vacuum by means of rotavapor at 40°C (Lee et al, 1995)with slight modifications.

DPPH free radical-scavenging activity

The DPPH free radical-scavenging assay was carried out, as previously reported by Cheel *et al.* (2007) with some modifications. The flavonoids extract separated from *Cedrus*, *Linum* & *Basilicum* seeds at various concentrations (1, 0.5, 0.025 mg/ml), (2, 1, 0.5, 0.25 mg/ml) & (1, 0.0625, 0.03125mg/ml) respectively were added to a 4% DPPH* mixture 1v:1v solution in ethanol and the reaction mixture was shaken vigorously. After incubation for 30 min at room temperature in obscure, the absorbance at 517 nm was recorded spectrophotometrically. Extracts which displayed promising activity (≥50% decolorisation at 1mg/ml) were retested at lower concentrations using serial dilutions. BHT was used as a reference compound.

A control solution, without the tested compound, was prepared in the same manner as the assay mixture. All the analyses were done in triplicate. The degree of discolorisation indicates the free-radical scavenging (FRS), efficiency of the substances. The antioxidant activity of *Cedrus*, *Linum* and *Basilicum* extracts was calculated as an inhibitory effect (IE %) of the DPPH radical formation as follows:

$$\text{IE } \% = \frac{[\text{A517 (control)} - \text{A517(sample)}]}{\text{A517 (control)}} \times 100$$

A517 (control): Absorbance of the control (DPPH) at 517nm
A517 (sample): Absorbance of the Sample at 517nm
and expressed as *IC50*.
The *IC50* value was defined as the concentration (in mg/ml) of the flavonoid extract required to scavenge the DPPH radical by 50%.

Thin layer chromatography

The three extracts, were examined with thin layer chromatography about 5 µl of each extract (100 mg/ml) were deposited on fluorescence silica gel plates (0.2 cm thickness 60 F254) and migration was conducted with mixture of ethyl acetate/methanol/H2O (100.0/13.5/10.0). After drying plate, revelation was conducted using a UV lamp at 254 and 366 nm.

Statistical analysis

Data were collected and expressed as the mean ± standard deviation of three independent experiments and analysed for statistical significance from control, using the Dunnett test (SPSS 11.5 Statistics Software; SPSS, Chicago, IL). The criterion for significance was set at $p < 0.05$. IC50 values, from the data, were calculated by regression analysis.

Antioxidant tests

Although there is no standardized method to evaluate the antioxidant potential of foods and biological systems, it is recommended to evaluate the antioxidant activity by different methods FRAP, ABTS etc. (Frankel and Meyer, 2000), in this study we use a DPPH method for the aim to evaluate in general; the antioxidant activity.

RESULTS AND DISCUSSION

Determination of free radical scavenging activity by DPPH method

The radical scavenging assay was carried out according to (Mighri *et al.,* 2010), with slight modifications. Briefly, 100 µl of various concentrations of the extract was added to 10 ml of an ethanol/DPPH solution. The mixture was vigorously shaken and then allowed to stand at a room temperature for 30 min in the dark. The absorbance of the mixture measured at 517 nm by using a double-beam UV–vis Camspec M550 spectrophotometer. A mixture of 100 µl of ethanol and 10 ml of DPPH solution is used as control. The scavenging activity was expressed as percentage of inhibition using the following equation:

$$\%\ Inhibition = \frac{[AB\ (DPPH) - ABS]}{AB\ (DPPH)} \times 100$$

Where AB is the absorbance of the control reaction (containing all reagents except the extract), and ABS is the absorbance of the extract. BHT (butylated hydroxytolune) used as positive control. All the tests are carried out in triplicate.

Table 1: Effect of *Cedrus*, *Basilicum* and *Linum* flavonoids extract on DPPH. Comparative study to the % FRS (Free-Radical Scavenging percentage) values. Free radical scavenging ability

Dose mg/ml	% FRS(AA)
	BHT
0.25	87.998 ± 1.695
0.125	86.860 ± 1.082
0.0625	86.517 ± 1.397
0.03125	81.377 ± 1.767
	*Cedrus*Extract
2	87.875 ± 0.1778
1	86.291 ± 0.095a*
0.5	59.579 ± 0.733
0.25	37.743 ± 0.785
	*Basilicum*Extract
1	75.699 ± 3.132a*
0.0625	40.077 ± 4.575
0.03125	26.815 ± 6.281
	*Linum*Extract
2	71.425 ± 2.243
1	46.142 ± 1.406b
0.5	30.785 ± 1.168
0.25	19.644 ± 0.808

Each value represents the mean ± SD.Values followed by different letters (a,b) within the same column are significantly different at
$p \leq 0.05$. a* statistically Significant ($p \leq 0.05$)

The extract concentration providing 50% of inhibition (*IC50*) is calculated from the graph of inhibition percentage (Fig.1) plotted against extract concentrations C (1, 0.5 and 0.25 mg/ml), L (2, 1, 0.5 and 0.25 mg/ml) and B (1, 0.0625 and 0.03125 mg/ml) (Table.1).

DPPH radical-scavenging activity

The DPPH* test is largely used in plant or food biochemistry to evaluate the free radical-scavenging effect of specific compounds or extracts. This stable free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In its radical form, DPPH* has a broad absorption band with a maximum at 517 nm, while if it is protonated by an antiradical compound, it loses this property (Lo Scalzo 2008). The extract concentration providing 50% inhibition (EC50) was calculated from the curve of FRS inhibition percentage against extract concentration.

In this assay, all test extracts effectively reduced the stable radical DPPH* to the yellow color and their scavenging effect was dose-dependent (Fig. 1).

It was also found that *Cedrus* extract possesses the most potent DPPH radical-scavenging activity, with an *IC50* value of 0.40 mg/ml, followed by a *Basilicum* extract with 0.41 mg/ml, and *Linum* extract with 1.21 mg/ml, while to BHT (0.003 mg/ml) used as positive control. Based on the *IC50* values, the potency of DPPH free radical-scavenging activity of the tested flavonoids extract was in the order of Cedrus (0.40 mg/ml) > Basilicum (0.41 mg/ml) > Linum (1.21 mg/ml). The effect is more significant at higher doses with 2 and 1 mg/ml which show maximum inhibition effects of 86 ± 0.09, 75 ± 3.13, 46 ± 1.40% FRS (= %IE) at a concentration of1 mg/ml, respectively. The thin layer chromatography with ethyl acetate/methanol/H_2O revealed in the *Basilic* seeds extract (BE), and the *Cedar* seeds extract (CE) the same spot with R_f 0.43.

Four spot were obtained with ethyl acetate/Methanol/ H2O, and two spots were obtained with the same mixture with DPPH spray with BE. One spot were obtained with *Linum* extract (LE). Four spots with the mixture, no spots with DPPH spray were obtained with CE. These preliminary finding indicate the presence of flavonols subclass of flavonoids, the flavonol substrate, may be present in the *Basilic* and *Cedar* extract.

The mechanism of reaction between antioxidant and DPPH* depends on the structural conformation of the antioxidant. Some compounds react very quickly with DPPH, reducing a number of DPPH molecules equal to the number of the hydroxyl groups (Bondet *et al.,* 1997).

Our results on the efficiency of flavonoids extract in inhibiting DPPH free radical are generally consistent with these criteria. Hence, *Linum* flavonoids extract, showing the lowest potency.

Finally, although a large number of antioxidant assays are available, the DPPH free radical is very stable and thus allows for easy handling and manipulation. Furthermore, its stability implies that a potential antioxidant will react with other well-known free radical entities, which are more unstable, and therefore more reactive(Frum *et al.,* 2007). Thus, an antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective studies.

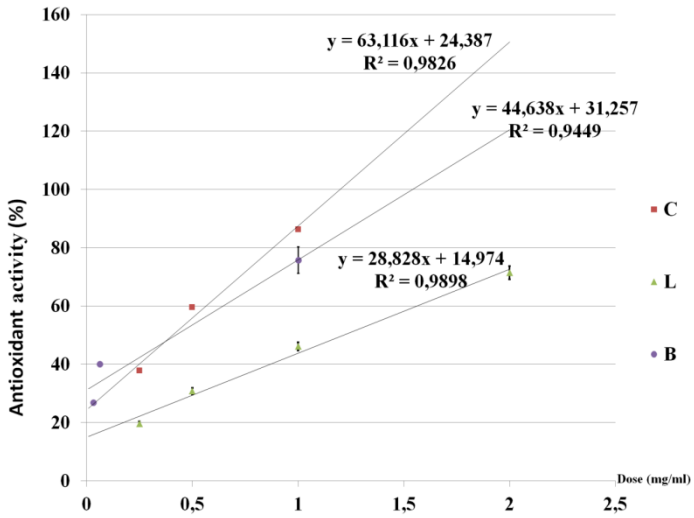

Fig. 1: Antioxidant activity of extracts *Cedrus* (C), *Basilicum* (B) and *Linum* (L) expressed as percentages of inhibition (%), results show a statistical significance from control ($p < 0.05$).

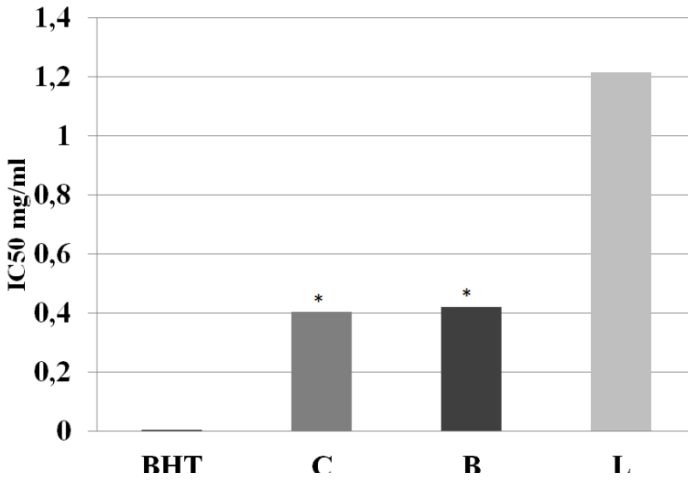

Fig. 2: DPPH radical scavenging activity of extracts, *Cedrus* (C), *Basilicum* (B) and *Linum* (L) expressed as *IC50*, versus the positive control (BHT). (*) Symbols represent statistical significance from control ($p < 0.05$).

CONCLUSION

In this study, the antioxidant potential of flavonoids extracted from *Cedrus*, *Basilicum* and *Linum* species seeds was evaluated by using DPPH assay. Our study provides evidence that the tested flavonoids extract exhibit interesting antioxidant properties, expressed either by their capacity to scavenge free radicals. Briefly, the information presented here could be used as preliminary data and biologically more relevant experiments that will examine the therapeutic potential of the *Cedrus*, *Basilicum*, and *Linum* seeds flavonoids extract will be designed. This study submit that *Cedrus atlantica Manetti ,Ocimum basilicum and Linum usitatissimum;* providing antioxidant properties and suggest potential functional protection from free radicalsmay act as a chemopreventive agent. Then, it's necessary to identify and isolate the compounds that are responsible to the antioxidant effects.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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