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In vivo and *in vitro* antioxidant and anti-hemolytic effect of Algerian *Centaurea calcitrapa* L. extracts

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ABSTRACT

In the present study, aerial part of *Centaurea calcitrapa* L. were extracted with solvent of varying polarity allowed their separation into three main subfractions, the analysis of methanol crud (CrE), chloroform (ChE) and ethyl acetate (EaE) extracts, showed that the EaE contains the highest amount of flavonoids (50,71 ± 0,65 mg Eq / Quercetin g dry extract and 31,96 ± 0,39 mg Eq Rutin / g dry extract). Using DPPH assay, the highest activity was observed with EaE (IC_{50} = 0,037 ± 0,0006 mg / ml). The β-carotene / linoleic acid bleaching assay revealed that the extracts have a very important antioxidant activity. The results showed that CrE has the highest antioxidant activity. The antioxidant activity of the CrE is confirmed by an *in vivo* assay in mice, using two doses: CrD_1 (50 mg/kg/day) and CrD_2 (100 mg/kg/day) during 21 days. Total antioxidant capacity of plasma and red blood cells was measured. The half-life (HT_{50}), which corresponds to 50% of cell lysis was calculated, the results showed that both groups treated with plant extract had a protective effect against erythrocytes hemolysis (CrD_2: HT_{50}= 167,3 ± 3,72 min). The CrD_2 group showed a strong scavenging activity using DPPH assay (51,64 ± 5,24 %), higher than that of Vit C group (38,92 ± 1,72 %). All results confirmed that the extracts have a dose dependent effect on the growth of overall antioxidant defenses. These results support the use of this plant against anti-inflammatory diseases in traditional medicine.

Keywords: *Centaurea calcitrapa* L., *in vivo*-antioxidant activity, DPPH, hemolysis, flavonoids.

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INTRODUCTION

In recent studies, it has been repeatedly asserted that oxidative stress is correlated with over 100 diseases, it was defined as the lack of balance between the occurrence of reactive species (free radicals) and the organism's capacity to counteract their action by the antioxidative protection systems[1]. Free radicals are small, diffusible molecules that differ from most biological molecules in that they have an unpaired electron[2]. Free radicals generated in aerobic metabolism are involved in a series of regulatory processes. When generated in excess, free radicals can counteract the defense capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA, and enzymes[3].

An antioxidant is defined as a molecule capable of slowing or preventing the oxidation of other molecules, whereas a biological antioxidant has been defined as; any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate[4].

Use of medicinal plants to treat common ailments has been prevalent since ancient times and different parts of the plants were used for public health. The use of natural treatments is cost-effective. Since ancient times, plants have been important in reducing pain and today the focus is on their role and ability in healing and their treatment properties for various diseases[5].

The diversity of natural compounds in herbs and their different functions in preventing and treating different diseases on the one hand and their property of being natural and comfortable with the body and not having adverse effects providing their proper usage induces people toward their consumption; educated public and health care professionals have enormous interests concentrating studies on these herbs and diagnosing their therapeutic properties[6].

Recently, research on natural antioxidants has become increasingly active in various fields. Accordingly, numerous

articles on natural antioxidants, including polyphenols, flavonoids, vitamins, and volatile chemicals, have been published. Assays developed to evaluate the antioxidant activity of plants.

The genus *Centaurea* comprises 300-350 species. Several species have been applied in traditional medicine, however, the rationale of their application has been analyzed only in few studies[7]. *Centaurea calcitrapa*, a plant widely distributed in Hungary[7], Portugal[8], Italy[9] and Algeria[10]. *C. calcitrapa* may be used, in a similar way to make artisanal cheese, a traditional dairy product[7]. Its healing properties have been known since ancient times in the traditional medicine of different nations; antipyretic, digestive, for constipation and diarrhea[9].

In current work, we investigated reducing antioxidant potential and flavonoids contents of plant sample. To the best of our knowledge this plant has not been investigated for these features. We evaluated two *in vitro* methods; DPPH and β-carotene techniques for determining the antioxidant potential. Finally, an *in vivo* model was successfully used for analyzing total antioxidant capacity of plasma and red blood cells of methanolic extract of the plant.

MATERIALS AND METHODS

Centaurea calcitrapa L. was collected from N'Gaous, Algeria in Mai 2015 and identified by Pr. Oudjehih B. (Institut of nutrition and agronomy, university of Batna, Algeria). A voucher specimen (N°: I.A.B./993) was kept in the herbarium of the same department for future reference. All other reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolab.

Adult male mice (weighting 25 - 30 g) were purchased from the Pasteur Institute of Algeria and were kept under standardized conditions (21 - 24 °C and a 12 h light/dark cycle) and fed a normal laboratory diet. The animal experiments were performed in accordance with international guidelines for the use and care of laboratory animals[11]. Ethical approval for the animal experimentation was obtained from Ethics Review Committee of Faculty of Exact Sciences and Nature and Life Sciences, University of Biskra, Biskra, Algeria (approval ref no. 307/V.D.P.G.). The animals were transferred to the laboratory at least 1 week before the experiments.

Flavonoids extraction

The extractions were carried out using the powdered plant material (100 g), the powder was extracted with methanol, at room temperature for overnight. CrE was successively extracted with different solvents of increasing polarity: hexane for defatting, chloroform for aglycone flavonoids extraction and ethyl acetate for glycoside flavonoids extraction, each fraction was evaporated to dryness under reduced pressure to give methanol (CrE), chloroform (ChE) and ethyl acetate (EaE) extracts[12].

Determination of total flavonoid contents

The total flavonoid content of each extract was determined by a colorimetric method[13]. Each sample (1 ml) was mixed with 1ml of aluminum chloride ($AlCl_3$) solution (2%) and allowed to stand for 15 min. Absorbance of the mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as equivalent quercetin and rutin (mg quercetin or rutin /g dried extract).

In vitro antioxidant activity

DPPH radical scavenging activity

The stable free radical scavenging activity was determined bythe1,1-diphenyl-2-picryl-hydracyl (DPPH) method[14]. Fifty microliters of various concentrations of the samples in methanol were added to 1250 µl of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of DPPH free radical in percent (I%) was calculated in the following way:

$$I\% = \frac{A_c - A_e}{A_c} X100$$

Where A_c is the absorbance of the control reaction (containing all reagents except the test compound), and A_e is the absorbance of the test compound (in the presence of extracts). Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate.

Beta-carotene bleaching test

The antioxidant capacity was determined by measuring the inhibition of *C. calcitrapa* extracts compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation[15]. Briefly, oxygenated water was mixed with b-carotene, Tween-40 and linoleic acid. The mixture was incubated with or without 250 µl of each extract (2 mg/ml), the emulsion system was incubated up to 48h in dark at room temperature and the absorbance was read at 490 nm. Antioxidant activity (AA%) was calculated using the equation:

$$AA\% = \frac{A_e}{A_c} X100$$

Where A_c: control absorbance (BHT), and A_e: absorbance in the presence of extracts.

In vivo antioxidant activity

Adult male mice were divided into four groups of 8-9 animals, the extract was dissolved in normal saline. Groups 1 and 2 received CrE dose 1 ($GCrD_1$: 50 mg/kg/day), CrE dose 2 ($GCrD_2$: 100 mg/kg/day) expressed as mg dry extract per kg body mass, via intra-peritoneal injection for 21 days. The 3rd group or native control received only saline (GT) and the 4rd group was treated with vitamin C (GVit C: 50 mg/kg/day). In the 21th day, about 1 mL of blood was collected through direct heart puncture from anesthetized mice. An aliquot of 100 µl of whole blood is immediately transferred to tube containing 2.4 ml of phosphate buffer (300 mOsm; , pH 7.4) to obtain a dilution of 1:25 (v:v). The rest of blood was centrifuged at 2000× g for 10 minutes to separate plasma. The plasma was kept at –20°C for subsequent determination of antioxidant status.

Total antioxidant capacity of plasma and red blood cells

Resistance to free radical damage was tested and measured as the capacity of red blood cells (RBCs) to withstand free radical induced hemolysis[17], with slight modifications. Briefly, the mice's blood diluted 1:25, then 50 µL of RBC suspension or whole blood were assayed using a 96-well microplate coated with a *t*-BH as a free radical generator. The kinetics of RBC resistance to hemolysis was determined at 37°C by continuous monitoring of changes in 630-nm absorbance. The time to reach 50% of total hemolysis (HT_{50}) was retained, and hemolysis inhibition (HI): (HT_{50} sample-HT_{50} temoin)/HT_{50}temoin.

DPPH radical scavenging activity of serum

In this test, the serum ability to inhibit DPPH radical was measured [18], DPPH is one of the few stable organic nitrogen radicals and has a maximum absorption at 517 nm. 25 µL of mice's serum was added to 625 µL of DPPH solution (4 mg / 100 ml methanol) and the reaction mixture was shaken vigorously. After incubation at room temperature for 30 min, the absorbance of the solution was determined at 517 nm. The results were compared with the DPPH solutions in the absence of the serum.

Statistical Analysis

The results are expressed as mean ± SD or SEM. Where applicable, the data were subjected to one-way analysis of variance (ANOVA), where the differences between extracts were determined by Tukey's multiple comparison test, Dunnett's and multiple comparison test for comparison between extracts and standards, using GraphPad program. $P \leq 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Determination of total flavonoid contents

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. There has been increasing interest in the research on flavonoids from plant sources because of their versatile health benefits reported in various epidemiological studies. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties[19].

The flavonoids content of *C. calcitrapa* was estimated using $AlCl_3$ method, and calculated as mg QE and RE/g of extract. According to the results shown in Table 1, the content of flavonoids decrease in the order: EAE, ChE and CrE.

Table 1: Flavonoids content of *C. calcitrapa* extracts.

Extracts	Flavonoids content	
	mg QE/g extract	mg RE/g extract
EAE	50,71 ± 0,65	31,96 ± 0,39
ChE	30,96 ± 0,55	19,38± 0,33
CrE	27,29 ± 0,18	17,16 ± 0,11

Denni and mammen (2012) showed that any blockage of the hydroxyl groups by glycosylation in carbons of positions 3, 5, 3' or 4' prevents chelation with $AlCl_3$[20]. Which explains, in our studie, the strong reactivity of quercetrin with $AlCl_3$ compared to rutin (quercetin-3-O-rutinosid).

***In vitro* antioxidant activity**

DPPH radical scavenging activity

The DPPH radical scavenging method is a standard procedure applied to evaluate the general antioxidant activity of plant extracts, especially which assigned to phenolic compounds, i.e. phenolic acids and flavonoids. DPPH radical scavenging activity of extracts increased with concentration (**Figure 1**).

DPPH assay was an easy and accurate method with regard to measuring the antioxidant capacity. However, the method has its limitations, particularly since the N-radical portion at the center of the DPPH structure is more accessible only to small molecules. Larger molecules may have limited access due to steric hindrances. Small molecules that have better contact with the N-radical centre site show higher antioxidant activity[21].

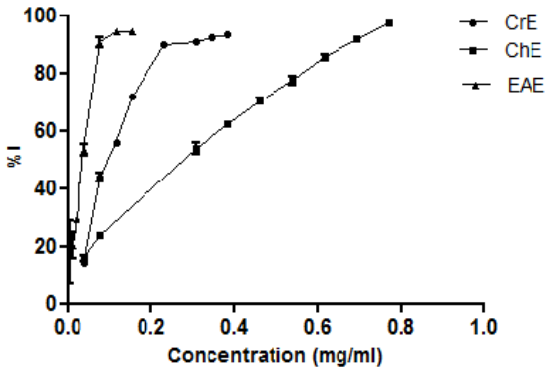

Figure 1: DPPH scavenging activity of *C. calcitrapa* extracts. Values were expressed as the mean ± SD of triplicate.

The EAE was found to exhibit the greatest scavenger activity with IC_{50} of (0.037 ± 0,0006 mg/ml), which is has the same activity of butylated hydroxytoluene (BHT: 0,032 ± 0,0186 mg/ml), followed by CrE and ChE (**Table 2**). BHT is a most commonly used antioxidant recognized as safe for use in foods containing fats, pharmaceuticals, petroleum products, rubber and oil industries[22]. The results showed that quercitin have a stronger scavenger activity compared to rutin, this decrease in activity of rutin may be caused by the blockage of the hydroxyl groups by glycosylation (Rutinose) in carbons of positions 3.

Table 2: DPPH scavenging activity of *C. calcitrapa* extracts and standards.

Standards	IC_{50} (mg/ml)	Extracts	IC_{50} (mg/ml)
BHT	0,032 ± 0,018	CrE	0,109 ± 0,001
Gallic acid	1.267 ± 0.001#	ChE	0,290 ± 0,005
Quercetin	2.565 ± 0.000#	EAE	0,037 ± 0,006
Rutin	5,586 ± 0.000#	Vit C	5,103 ± 0,021#

#: µg/ml.

Boumerfeg and their collaborators (2011), reported that the radical scavenging activity of plant extracts depends on the amount of polyphenolic compounds in the extracts. The extract, which had a strong antioxidant activity, contain high flavonoid contents[23]. However, in our study, the order of decreasing scavenging activity among the *C. calcitrapa* extracts was found to be EAE > CrE > ChE, which is not in accordance with the amount of phenolic compounds present in these extracts. Also steric accessibility of an antioxidant compound, to the N-radical portion at the center of the DPPH, can determine the type of reaction mechanism. EAE exhibit the highest activity, this activity could be attributed to its highest contents of phenols compounds as well as to the compound's nature (glycosidic polyphenols).

In recent years, the antioxidant activity of plant polysaccharide has become a hot spot in the field of polysaccharide research. Which has the functions of

increasing antioxidant enzyme activity, eliminating free radicals, inhibiting lipid peroxidation and anti-oxidation[24].

Beta-carotene bleaching test

Peroxidation of fatty acid chains is the basic cause of rancidity in fats and oils, and can occur in biological cells during oxidative stress. Indeed, breakdown products of fatty acid peroxides are often used as oxidative stress markers[25].

Beta-carotene bleaching method is widely used to measure antioxidant activity of plant extracts. It is an *in vitro* assay that measures the inhibition of coupled auto-oxidation of linoleic acid and b-carotene. This method is based on the fact that lipid radicals as auto-oxidation products of linoleic acid attack double bonds of β-carotene, but the presence of an antioxidative substance can prevent the attack and retain the yellowish-orange colour of β-carotene and thus reduce their bleaching activity[26].

All *C. calcitrapa* extracts reduced b-carotene bleaching by at least 70% (Figure 2), while EAE, CrE and ChE inhibited β-carotene bleaching by 84,32 ± 2,07%, 84,05 ± 4,99% and 78,48 ± 0,34%, respectively. The EAE present the highest contents, and nature (glycosidic) of polyphenoland the highest antioxidant activity, which was near to that of BHT (96.32± 0.99%) used as positive control. The present study found no direct correlation between phenol levels and β-carotene bleaching inhibition

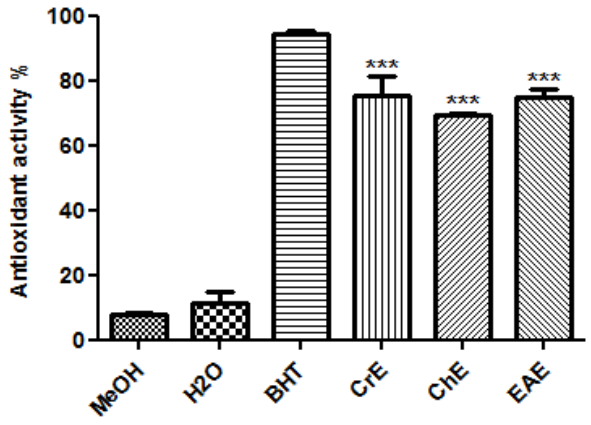

Figure 2: Antioxidant activity of *C. calcitrapa* extracts compared with BHT, methanol and water. Comparison was realized against positive control (BHT);***: $p \leq 0.001$.

***In vivo* antioxidant activity**

Total antioxidant capacity of plasma and red blood cells

The capacity of flavonoids to act as antioxidants *in vitro* has been the subject of several studies in the past years, and important structure-activity relationships of the antioxidant activity have been established. The antioxidant efficacy of flavonoids *in vivo* is less documented, presumably because of the limited knowledge on their uptake in humans. During absorption of across the intestinal membrane, flavonoids are partly transformed in their conjugates. However, the major part of ingested flavonoids is not absorbed and is largely degraded by the intestinal microflora[27]. For that reason intra-peritoneal injection was used.

In the case of the group received only saline (GT), half-hemolysis (HT_{50}) was reached at about 140 min after *t*-BH addition, whereas under Vit C no hemolysis occurred during at least 160 min (Figure 3). The CrD_2 showed the most effective inhibition capacity compared to the antioxidant reference (Vit C).

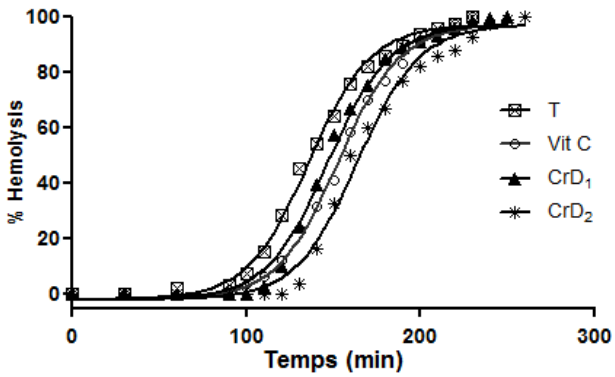

Figure 3: Hemolysis curve of fresh mice blood, in the presence of *t*-BHP for different groups, diluted in phosphate buffer (pH 7.4) in isotonic conditions from optical density reading.

Hemolysis, or the abnormal breakdown of circulating red blood cells (RBCs), is a major clinical problem that can be a disease process unto itself, or manifest as a component of another disorder. Because of the critical role that the lab plays in assessment of hemolytic disorders, and because there are a very wide variety of assays available for hemolysis[28].

Exposure of red cells to t-butyl hydroperoxide resulted in crosslinking of hemoglobin subunits and probable crosslinking of cytoskeletal proteinsas suggested by the diminution in intensity of theprotein bands and the parallel increase in high molecular weight material. Membrane glycoproteins were not crosslinked. Cytoplasmic and membrane protein changes were not dependent on *t*-butyl hydroperoxide-induced lipid peroxidation[29].

Hemolysis inhibition was noticed for thethree groups (Vit C, CrD_1 and CrD_2), from the kinetics of hemolysis obtained, the D_1 group mice treated with methanol extract (50 mg / kg), the increase in HT_{50} (HT_{50} = 156,5 ± 5,31 min) is not statistically significant compared with the control group (HT_{50} = 147,7 ± 0,40 min). The administration of higher doses of methanol extract (100 mg / kg) in mice group D_2 caused a delay of hemolysis (HT_{50} = 167,3 ± 3,72 min) ($p > 0.05$), which confirmed that the extracts have a dose dependent effect on the growth of overall antioxidant defenses (Figure 4). The mice's group treated with vitamin C (100 mg/kg) didn't significantly increase the HT_{50}.

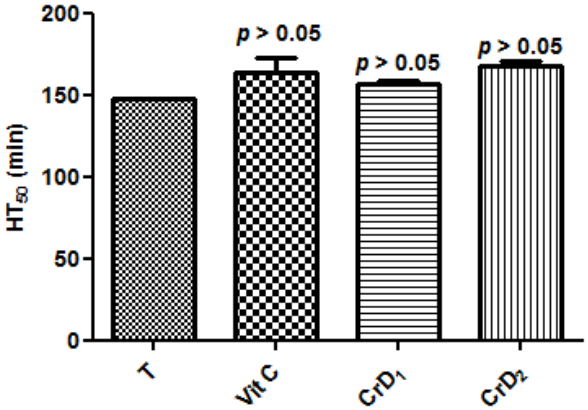

Figure 4: The half-life (HT_{50}) for the different groups. Values are means ± SEM (n = 8-9). Comparisons are made with respect to the temoin group (control group treated with normal saline), Vit C (groups treated with a solution of Vit C 100 mg / kg) D_1, D_2 (groups treated with CrE 50 and 100 mg/kg, respectively).

The CrD_2 showed the most effective inhibition capacity, higher than that of Vit C (Table 3). The results showed that both groups treated with plant extract had a protective effect against erythrocytes hemolysis comparable to GVitC and largely higher than the native control.

Table 3: Percentage of hemolysis inhibition.

Groups	Hemolysis inhibition (%)
Vit C	10,62 ± 0,86
CrD_1	5,95 ± 0,32
CrD_2	13,27 ± 0,67

DPPH radical scavenging activity

The results showed that all the doses for *C. calcitrapa* extracts CrD_1 (45,95 ± 4,26%, 51,64 ± 5,24 %) and VitC (38,92 ± 1,72 %) improved the serum DPPH scavenging potential (Figure 5).

The antioxidative potential of samples can be assayed by different *in vitro* and *in vivo* methods. Several antioxidants of plant origin are experimentally proved and used as effective protective agents against oxidative stress[30]. Vit C present a very strong activity *in vitro* (Table 2), compared to the CrE, but the intra-peritoneal administration of Vit C and CrE showed that the CrE decrease the capacity of plasma better than the Vit C at the same dose (50 mg/kg/day).

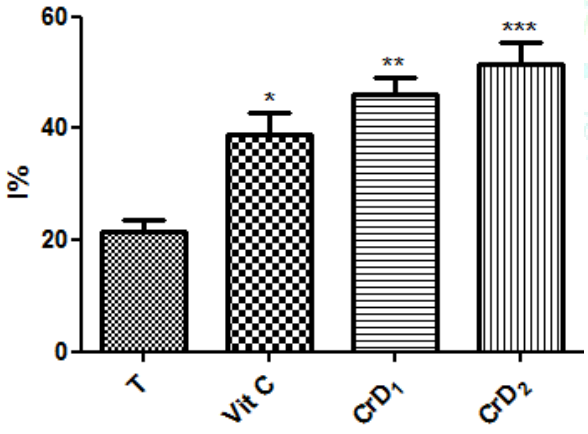

Figure 5: Antioxidant potential of *C. calcitrapa* in mice blood by using DPPH assay: T (control group treated with normal saline), Vit C (groups treated with a solution of Vit C 50 mg / kg) CrD_1, CrD_2 (groups treated with methanol extracts; 50 and 100mg/kg, respectively). Data are mean ± SEM of 8-9 animals in each group, comparisons are made with respect to the control group,*: $p \leq 0.05$, **: $p \leq 0.01$,***: $p \leq 0.001$.

CONCLUSION

It is known that antioxidant activities increase proportionally with the flavonoids content. No significant correlation was recorded between flavonoids content of *Centauria calcitrapa*, DPPH scavenging and the reduction of β-carotene oxidation activities. However, the extracts showed a strong activity. From the *in vitro* results, the CrE seem to be of real and potential interest. To confirm the antioxidant potential of the CrE, *in vivo* antioxidant properties were investigated. The analysis of results revealed that the CrE administration increased significantly the antihemolytic and free radical scavenging capacity of plasma. It can therefore be suggested that *Centauria calcitrapa* had potent antioxidant activities both *in vitro* and *in vivo*.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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