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In vitro antioxidant, antibacterial and membrane stabilizing activity of plant extract from *Chrysanthemum fontanesii*

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Abstract

Antioxidant activities, antibacterial and membrane stabilizing activity of butanolic extract from leaves of *Chrysanthemum fontanesii* were investigated. The inhibition of the formation of malondialdehyde (MDA) *in vitro* and the scavenging of DPPH were assayed .The phenolic content of the extract was determined. The experimental results show that butanolic extract have antioxidant activity *in vitro.* The extracts showed a high antioxidant effect, especially scavenging of DPPH anions and inhibition of lipid peroxidation. Antioxidant activities were compared to ascorbic acid. The total content of phenolic compounds was 349 µg of gallic acid equivalents/mg extract. The butanolic extract was effective against microorganisms and against heat-as well as hypotonic solution-induced haemolysis of erythrocytes in vitro.

Keywords: *Chrysanthemum fontanesii*; Antioxidant activity; Phenolic compounds; antibacterial activity; membrane stabilizing activity.

Introduction

Lipid peroxidation is a complex process occurring in biological systems and reflects the interaction between reactive oxygen species and polyunsaturated fatty acids [1, 2], is also associated with food deterioration, aging of organisms, cancer promotion, cell death and tissue damage [3]. Therefore increasingly interest on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. Several studies have described the antioxidant properties of phenolic compounds which are widely found in the secondary metabolism of medicinal plants and in many edible plants [4, 5, 6]. Many of these antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent [7, 8, 9, 10]. In recent years there has much interest on the use of plant rich in phenolic compounds in the food industry, because they delay oxidation of lipids and thereby improve the quality and nutritive value of food [10, 11].

The present study was performed to evaluate the antioxidant activity of butanolic extract from *Chrysanthemum fontanesii* to investigate its potential as natural antioxidants *in vitro*. Furthermore, investigation in the presence of bioactive components, such phenolic content. The antibacterial and membrane stabilizing activity were also pursued.

Materials and methods

Plant material

Chrysanthemum fontanesii (Boiss. et Reut.) Q. et S., synonyms: *Leucanthemum* fontanesii (Boiss. et Reut.), Plagius virgatus (B. et T.) non DC, is a plant of 100-150 cm. It grows in the undergrowths and forests and is endemic to North Africa. C. fontanesii was collected during the flowering stage in April 2003 from the area of bijaia, north east of Algeria and authenticated by Pr. M. Kaabeche (University of Sétif, Algeria) [12].

Extraction procedure

Leaves of *Chrysanthemum fontanesii* was powdered and macerated at room temperature with EtOH–H_2O (8:2 v/v) for 48 h three times. After filtration, the filtrates were combined, concentrated under vacuum (at 35 C), diluted with 600 ml H_2O, filtered to remove chlorophyll and successively extracted with (3x400ml), chloroform, ethyl acetate and *n*-butanol. The organic layers were dried with Na_2SO_4. Removal of solvents under reduced pressure [12].

Determination of total phenolic compounds

20µl of butanolic extract from *Chrysanthemum fontanesii* (1mg/ml) was mixed with 100µl Folin–Ciocalteus reagent and 1580 µl of distilled water, followed by 300 µl 20% sodium carbonate (Na2CO3) 3 min later. The mixture was shaken for 2 h at room temperature and absorbance was measured at 765 nm. All tests

were performed in triplicate. Gallic acid was used as a standard. The standard curve was prepared using 0, 50, 100, 150, 200, 250, 500 mg /l solutions of gallic acid in methanol: water (10:90, v/v) [13].

The concentration of total phenolic compounds in butanolic extract from *Chrysanthemum fontanesii* was determined as µg of gallic acid equivalents per 1 mg of extract.

DPPH radical-scavenging activity assay

Various concentrations (1, 2.5, 5, 10, 15, 20, 30, 50, 75 µg/ml) of the extract in methanol were added to 3 ml of a solution of DPPH in methanol (0.004%), incubated during 30 min at room temperature and the absorbance was read at 517 nm. (When DPPH reacts with an antioxidant compound that can donate hydrogen, the purple colour of DPPH will turn in to yellow and the resulting decrease in absorbance).

Inhibition of free radical DPPH in percent (I%) was calculated in following way:

I% = (Ac _ As)/Ac100; Where Ac is the absorbance of the control (DPPH alone), and As is the absorbance of the test samples. The average of triplicate analysis was given as result. Ascorbic acid was used as the control [14].

Assays of lipid peroxidation using vitellose

The fresh vitellose was dissected and homogenized in ice cold PSB (20mM, pH 7.4) to produce a 10% homogenate (v/v). The homogenate was centrifuged at 4000 rpm for 20 min to remove precipitation. Different concentration of test samples (0.1 0.2, 0.3, 0.4, 0.5mg/ml) was added to 0.5ml homogenate (supernatant) and 50µl FeSO4 (0.07 M). the mixture incubated at 37 ◦C for 1 h, then 1ml trichloroacetic acid (TCA20%), and 1.5ml thiobarbituric acid (TBA, 1%,) was added in succession, and the mixture was heated in boiled water for 15 min. After centrifugation at 4000 rpm for 20min to remove precipitated protein the color of the complex was detected at 532 nm. The control group was run in parallel without sample under similar conditions, except that 1ml trichloroacetic acid (TCA, 20 %) was added before incubation. The lipid peroxidation scavenging activity (*K*, %) was calculated by the following equation: K(%) = Ac As /Ac 100%

Where *Ac* was the absorbance of the control, and *As* was the absorbance of samples. The average of triplicate analysis was given as result. Ascorbic acid was used as the control [15].

Antimicrobial activity

Microbial strains

The microorganisme strains used in this study were *Bacillus amyloquafeciens, Staphylococcus aureus, Escherichia coli,* Pseudomonas sp, *Proteus vulgaris* They were obtained from the collection of bacteriology laboratory of Ibn Badis hospital, Constantine were used.

Disc diffusion method

0.1 ml of suspension containing 10^8 CFU/ml of the tested microorganism, spread on the nutrition agar (MHA Mueller–Hinton Agar). The 100 µl of the extract (1mg/ml) was added on the discs (5 mm in diameter) and placed on the inoculated agar. The inoculated plates were incubated at 37 C for 24 h. antibacterial activity was evaluated by measuring the diameters of the inhibition zones in millimeters. The extract was screened in triplicate against each microorganisms [16].

Agar dilution method

A series of twofold dilution of extract, ranging from 0.1 to 10 mg/ml, were prepared in MHA. Plates were dried at 37 C for 30 min prior to inoculation with 1–2µl spots containing approximately 10^6 CFU of microorganism [17]. Inoculated plates were incubated at 37 C. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of each extract that completely inhibited growth of microorganism upto 24 h, whereas the minimum bactericidal concentration (MBC) was the lowest concentration at which no growth was observed after incubation upto 5 days [18].

Membrane stabilizing activity

The membrane stabilizing activity of the extract was assessed by using hypotonic solution as well as heat- induced erythrocyte haemolysis [19].

Erythrocyte suspension

To prepare the erythrocyte suspension, blood was collected from rats under chloroforme anaesthesia, heparine was used, to prevent clotting. The blood was centrifuged and blood cells were separated and washed three times with 0.9% NaCl. The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4).

Heat-induced haemolysis

The test sample comprising of stock erythrocyte suspension (30µl) mixed with 5ml of isotonic solution contained butanolic extract (0.1 mg/mL, 0.2 mg/ml) or acetyl salicylic acid (0.2 mg/mL).the experiments were carried out in duplicate pairs. The vehicle, in the same amount, was added to another tube as control. One pair of the tubes was incubated at 54 C for 20 min in a water bath. The other pair was maintained in an ice bath at 0-4 C. The mixture was centrifuged for 3 min at 1300 g. The absorbance of the supernatant was read at 540 nm.

Hypotonic solution-induced haemolysis

The test sample comprising of stock erythrocyte suspension (30µl) mixed with 5mL of hypotonic solution (50 mM NaCl) contained butanolic extract (0.1 mg/ml, 0.2 mg/ml) or acetyl salicylic acid (0.2 mg/mL). The control sample consisted of 0.5 ml of erythrocyte suspension was mixed with hypotonic-buffer saline. The mixture was incubated for 10 min at room temperature, centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either hemolysis

or membrane stabilization was calculated using the following equation: % inhibition of hemolysis = 100 x (1-OD2-OD1/OD3-OD1), where,
OD1 = optical density of test sample unheated or in isotonic solution and
OD2 = optical density of test sample heated or in hypotonic solution.
OD3= optical density of control sample heated or in hypotonic solution

Statistical analysis

Data are presented as mean ± standard deviation (SD). The student test and a one-way analysis of variance (ANOVA) were used for multiple comparisons (SPSS program, ver 13.0)

Results and discussion

Total phenolic compounds

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups [20]. The total amount of phenolic compounds in the plant extract determined as µg of gallic acid equivalents per 1 mg of extract by using an equation that was obtained from standard gallic acid graph.
Butanolic extract of *Chrysanthemum fontanesii* exhibited higher phenolic content (349 µg of gallic acid equivalents/mg extract).

DPPH radical scavenging activity

DPPH is usually used as a substrate to evaluate free radical scavenging activity of antioxidants. In this study butanolic extract of *Chrysanthemum fontanesii* presented concentration dependent hydrogen donating ability as shown in Figure. 1. These results allowed to calculated the IC50. Ascorbic acid showed an excellent scavenging activity (IC50 = 5 µg /ml). It was observed that butanolic extract of *Chrysanthemum fontanesii* have strong scavenging activity, with an IC50 value of 11.68 µg /ml. The scavenging effect of butanolic extracts and vitamin C on the DPPH radical decreased in the order of Vitamine C standard > butanolic extract of *Chrysanthemum fontanesii* at the concentration of 20 µg/ml, the resulting inhibition were 95%, 86.15% respectively.

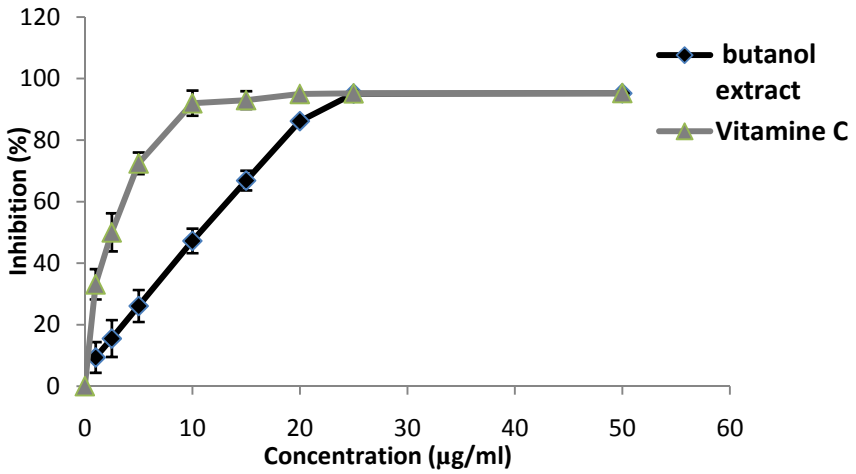

Figure1. DPPH radical scavenging activity of *Chrysanthemum fontanesii* and Vitamin C (mean±SD, n=3)

These results indicated that butanolic extract of *Chrysanthemum fontanesii* have a noticeable effect on scavenging free radicals and their scavenging effects increased with increasing concentration. It seems that this activity is mostly related to the presence role of phenolic compounds as scavengers of free radicals of the phenolic compounds such as flavonoids and phenolic acids in the extract.

Inhibition of lipid peroxidation

In biological systems, lipid peroxidation generates a number of cytotoxic products, such as MDA, which are widely used marker of the oxidation and peroxidative tissue injury [21, 22, 23]. In the present study, we measured the capacity of plant extract from *Chrysanthemum fontanesii* to inhibit non-enzymatic lipid peroxidation in egg *vitellose* homogenate, induced by the $FeSO_4$ system. The effects of butanolic extract of *Chrysanthemum fontanesii* on non-enzymatic peroxidation are shown in Figure. 2. The inhibition of lipid peroxidation of butanolic extracts of *Chrysanthemum fontanesii* was increased with increasing concentration. The percentage inhibition of lipid peroxidation by 0.3 mg/ml butanol extract of *Chrysanthemum fontanesii* was found to be 71.93%. The ratio at 0.1 mg/ml for vitamin C was found to be 86.95% inhibition of lipid peroxidation.
Decrease in lipid peroxidation by plant extract from *Chrysanthemum fontanesii* may be a result of it scavenging free radicals produced by $FeSO_4$ in the reaction system.

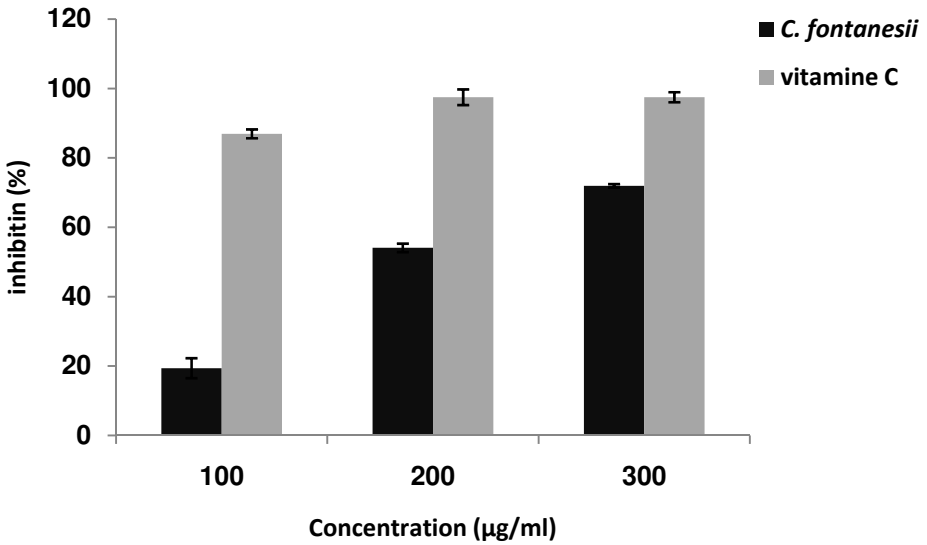

Figure 2. Inhibition of lipid peroxidation by butanolic extract of *Chrysanthemum fontanesii* and Vitamin C (mean±SD,n=3)

Membrane stabilizing activity

The butanolic extract, at concentration 0.1 and 0.2 mg/ml, significantly protected the lysis of erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.20 mg/ml) (Table 1). At a concentration of 0.1 mg/ml and 0.2 the extract produced 52.59%, 59.9% inhibition of hemolysis of erythrocyte as compared to 51.56% by acetyl salicylic acid (0.20 mg/mL), respectively. The butanolic extract also revealed significant inhibition of erythrocyte hemolysis induced by heat.

Table1. Effect of butanolic extract of leaves from *Chrysanthemum fontanesii* on heat induced and hypotonic solution induced erythrocyte haemolysis of erythrocyte membrane

treatment	concentration	Haemolysis inhibition (%)	
	(µg/ml)	Heat	Hypotonic solution
Control	-	-	-
Butanolic extract of leaves from	100	21.84±1.15	52.59±0.53
Chrysanthemum fontanesii	200	27.36±0.84	59.9±0.08
ASA	200	26.16±1.72	±1.3551.76

The effect of antiinflammatory drugs counting herbal preparations in inhibition of erythrocyte hemolysis induced by heat and hypotonic solution has been studied widely [24, 25, 26]. (The erythrocyte membrane resemble lysosomal membrane as such, the effect of drugs on the stabilization of erythrocyte could be applied to the stabilization of lysosomal membrane [27,28]. The membrane stabilizing activities of the extract are shown on Table 3. The results showed that the extracts are highly potent on erythrocyte adequately protecting it against heat and hypotonic induced lyses. The activity was comparable to that of standard. The high membrane stabilizing activity of the butanol extract of *C. fontanesii* observed in this investigation may be due to its high phenolic compounds content (flavonoids). Flavonoids enter the hydrophobic core of the membrane where they exert a membrane-stabilizing effect by modifying the lipid packing order [29]. They can penetrate the lipid bilayer, decreasing free radicals concentration or influencing antioxidant capability in biomembranes [30]. A possible explanation for the membrane stabilizing activity of butanolic extract of leaves from *C fontanesii* could be, an increase in the surface area/volume ratio of the cells which could be throught about by expansion of membrane or shrinkage of the cell, and an interaction with membrane proteins. Moreover, it has also been shown that the defomability and cell volume of erythrocytes is closely related to the intracellular content on calcium. Hence, it may be speculated that the cytoprotective effect on erythrocyte

membrane may be due to the ability of the test extract to alter the influx of calcium into the erythrocytes [19].

Antimicrobial activity

The study of antimicrobial capability of plant phenolics is well documented [2, 31, 32]. The extract of *C. fontanesii* was evaluated for their antimicrobial activity against Bacillus amyloquafeciens, *Staphylococcus aureus*, *Escherichia coli*, Pseudomonas sp, *Proteus vulgaris*. The results of disc diffusion test showed that Gram positive bacteria are sensitive than Gram negative bacteria (Table 2). The similar result was obtained by others [33]. The inhibition zones for *B. amyloquafeciens* and *S. aureus* at 100µg/disc were 7.1 and 11mm, respectively. In general, Gram negative bacteria are more resistant to polyphenols than Gram positive bacteria, possibly due to the different cell wall compositions [34].

The results of MIC and MBC about *P. vulgaris* and *S. aureus* are presented in Table 2. *S. aureus* was more susceptible microorganism with MIC of 2 mg/ml extracts, These results are very important considering that *S. aureus* can produce several types of enterotoxins that cause gastroenteritis, which is a major food-borne disease in most countries [35]. Natural products may be a particularly rich source of antiinfective agents. For example, flavonoids showed antimicrobial activity, and quercetin and other related compound acts essentially by enzyme inhibition of DNA gyrase [36].

Table 2. Antibacterial activity of butanolic extract of *Chrysanthemum fontanesii*

microorganisme	Diametre of zone of inhibition (mm) (100µg/disc)	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus	11	2	4
Bacillus amyloquafeciens Escherichia coli	7	-	-
Pseudomonas sp	6	-	-
Proteus vulgaris	0	-	-
	6	4	8

Conclusion

The results obtained in this study have shown that the extract of *Chrysanthemum fontanesii* possesses antioxidant properties, which are concentration dependent. The ability of the extract to retard lipid oxidation is attributable to the ability of its phenolic constituents to quench reactive oxygen species.

The n-butanol extract from leaves of *Chrysanthemum fontanesii* was effective against pathogens and against heat-as well as hypotonic solution-induced haemolysis of erythrocytes *in vitro.*

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