ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of antioxidant activity of flavonoids Extracted from *Galium tunetanum* Poiret

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ABSTRACT

The antioxidant properties of flavonoids of *Galium tunetanum* Poiret, Rubiaceae herb from Megriss (Setif, Algeria) were evaluated**.** The study was carried out by different methods such as radical scavenging activity (DPPH), the β-carotene bleaching assay and the reducing power, tested on different interphases of flavonoids extract. Results of this study exhibit a high antioxidant activity which makes the flavonoids from this plant better than BHT.

Keywords: *Galium tunetanum*, antioxydant, flavonoids, Megriss region.

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ISSN: 0975-8585

INTRODUCTION

Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, isoflavanoids, anthocyanidins, and catechins[1, 2]. Flavonoids are universal within the plant kingdom; they are the most common pigments next to chlorophyll and carotenoids. They generally occur in plants as glycosylated derivatives and their physiological roles in the ecology of plants are diverse. Due to their attractive colors, flavones, flavonols and anthocyanidins may act as visual signals for pollinating insects. In consideration of their astringency, catechins and other flavanols can represent a defense system against insects harmful to the plants[3,4]. Some beneficial bioactivities of flavonoids have been proved, such as antibacterial, anticarcinogenic, antioxidant, antimutagenic, anti-inflammatory activities and immunomodulatory activities [5-7]. One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals [8, 9]. They resemble in their regulatory properties most of the lipid-soluble vitamins, but serve in addition, due to their color and odor, as communicators with the environment[10].

The remarkable diversity of form and function of flavonoids in present-day, plants have provided a rich foundation for research in areas ranging from genetics and biochemistry to chemical ecology and evolution to human health and nutrition. To date, more than 6,400 different flavonoids compounds have been described in the literature [11-13].

In this study, the optimal conditions to extract flavonoids from *Galium tunetanum* Poiret were investigated systematically in order to explore a proper process to utilize *Galium tunetanum* Poiret in the area of healthcare and to investigate the secret of this endemic species for the first time.

MATERIALS AND METHODS

Flavonoids extraction

The dried aerial plant matrix was milled into coarse powder, then (100g) were defatted three times with petroleum ether (each 400 mL) for 3 hours, at 50 °C [14]. The powder was taking up again three times with 70% ethanol (raw material: solvent ratio was 1: 10) for 90 min at 100°C. The extracts were pooled and concentrated in vacuum to collect the aqueous residue (100 mL), which was extracted with chloroform, and then acidified with 20% H_2SO_4 (pH = 5) and extracted with ethyl acetate. The appearance of an interphase precipitate was observed upon extraction with ethyl acetate. The ethyl acetate fraction and the interphase precipitate were taken as a two flavonoids fraction for our experiment [15].

Determination of Total Phenolic Content

For total polyphenol determination, the Foline-Ciocalteu method was used [16]. The sample (0.2 mL) is mixed with 1 mL of the Folin-Ciocalteu reagent previously diluted with 10 mL of deionized water. The solution is allowed to stand for 4 min at 25 °C before 0.2 mL of a

saturated sodium carbonate solution (75 mg/mL) is added. The mixed solution is allowed to stand for another 180 min before the absorbance at 765 nm is measured. Gallic acid is used as a standard for the calibration curve. The total phenolics content is expressed as mg equivalent gallic acid per gram of extract(mg EAG/GE)

Determination of total flavonoids contents

The flavonoids contents in the extracts were estimated by the Aluminum chloride solution according to the method described by Bahorun*et al.,*[17]. Briefly, 1 mL of the methanol solution of each extract was added to 1 mL of 2% $AlCl_3$ in methanol. After 10 min, the absorbance was determined at 430 nm. Quercetin and rutin (0 – 40 µg/mL) were used as a standard. Results were expressed as mg equivalent Quercetin and rutin per gram of extract (mg EQ/GE)

DPPH assay

The donation capacity of the obtained phases was measured by bleaching of the purple-colored solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato*et al*., [18]. One milliliter of the extract at different concentrations was added to 0.5 mL of a DPPH-methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm. The antiradical activity was expressed as IC50 (micrograms per milliliter), the antiradical dose required to cause a 50% inhibition. A lower IC50 value corresponds to a higher antioxidant activity of plant extract. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/ A_0] \times 100$$

Where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. BHT was used as a positive control. Samples were analyzed in triplicate [19].

β-Carotene linoleic acid bleaching assay

The β-Carotene linoleic acid bleaching assay was first described by Miller [20], is one of the antioxidant assays suitable for plant samples. In this assay, the antioxidant capacity is determined by measuring the inhibition of the production of volatile organic compounds and the formation of conjugated dienehydroperoxides arising from linoleic acid oxidation, which results in the discoloration of β-Carotene.

β-Carotene (0.5 mg) in 1 mL of chloroform is added to 25 µL of linoleic acid and 200 mg of the Tween 40 emulsifier mixture. After evaporation of the chloroform under vacuum, 100 mL of oxygen-saturated distilled water is added with vigorous shaking. Next, 4mL of this mixture is transferred into test tubes containing different concentrations of the sample. As soon as the emulsion is added to each tube, the zero time point absorbance is measured at 470nm using a spectrophotometer. The emulsion is incubated for 2 hours at 50 $^{\circ}$C. A blank, devoid of β-carotene, is prepared for background subtraction [21]. BHT is used as standard.

The antioxidant activity of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula:

$$\text{Inhibition (\%)} = [(At - Ct)] / [(C_0 - Ct)] \times 100$$

Where At and Ct are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min, and C_0 is the absorbance value for the control measured at zero time during the incubation. The results are expressed as IC50 (micrograms per milliliter), the concentration required to cause a 50% β-carotene bleaching inhibition [19].

Reducing power

The reducing power was determined according to the method of Oyaizu [22]. Each extract (0.5–10 mg/mL) in methanol (2.5 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide, and the mixture was incubated at 50 C° for 20 min. After 2.5 mL of 100 mg/mL trichloroacetic acid were added, the mixture was centrifuged at 200g for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1mL of 1 mg/mL ferric chloride. Then the absorbance was measured at 700 nm against a blank.

A higher absorbance indicates a higher reducing power. EC50 value (mg extract/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA was used for comparison [23].

Statistical analysis

Results were expressed as the mean ± standard deviation. Statistical differences were assessed using one-way ANOVA to determine whether there were any significant ($P < 0.05$) differences between flavonoids from *Galium tunetanum* Poiret and controls. Data were subjected to analysis using the Microsoft Excel 2010 and Graphpad prism 5 Demo.

RESULTS

Polyphenols and flavonoids contents

Two phases are determined, the ethyl acetate with a percentage of 4.4 % and the aqueous interphase with 17.2 %, which mean 21,6% of total flavonoids extracted from *Galium tunetanum* Poiret. However the quantities of polyphenols determined in the flavonoids phases are 111,56±2,82 mg EAG/GE in ethyl acetate phase and 65.26±0,99 mg EAG/GE in the aqueous interphase. The quantity of flavonoids was reach in ethyl acetate phase rather than the aqueous phase.

The scavenging effects of flavonoids from *Galium tunetanum* Poiret on the DPPH radical were related to the amounts of substance added. The scavenging effects of three phases on the DPPH radical increased in the order, acetate phase <total flavonoids

ISSN: 0975-8585

<aqueous interphase with IC 50 equal to 22, 17±2,49 µg/mL , 39,10±5,04 µg/mL and 53,44±7,70 µg/mL respectively (Figure 1). Statistical comparison indicates that there is a significant difference between both ethyl acetate phase, aqueous interphase and the BHT, but there is no difference between the total flavonoids and BHT.

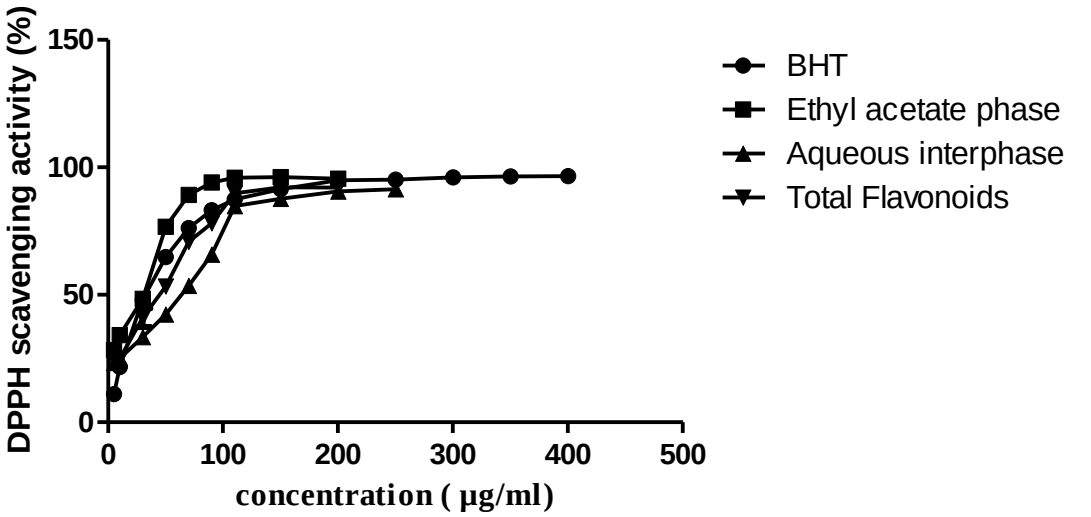

Figure 1: Scavenging effects of flavonoids of *Galium tunetanum* Poiret on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

β-Carotene linoleic acid bleaching assay

As shown in Figure 2, the absorbance of the control at 470 nm gave a value of 1,107±0,039 after 120 min, while the extracts decreased to a range of 0,396±0,055 for the ethyl acetate phase, 0 for the aqueous interphase and 0,512±0,043 for total flavonoids. These results indicate that all the phases can weakly or cannot inhibit oxidation of linoleic acid.

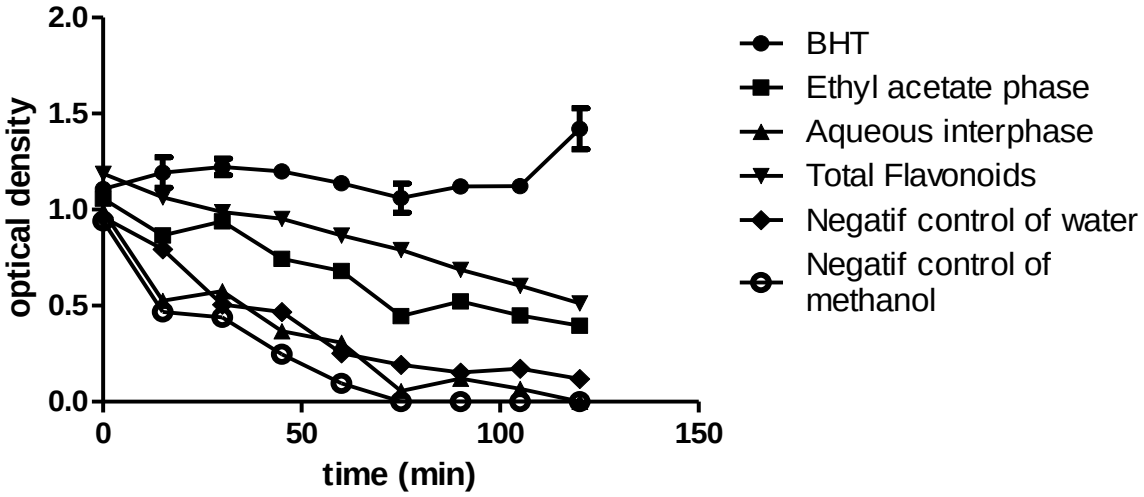

Figure 2: Antioxidant activities of flavonoids of *Galium tunetanum* Poiret by the β-carotene bleaching method

ISSN: 0975-8585

The calculated inhibition capacities percentage of the extracts are given in table 1 and show a high concentration to inhibit oxidation of linoleic acid for all the flavonoids phases in comparison with methanolic extract of this species (141,58±22.55 µg/mL). Statistical analysis indicates that there is a significant difference between all phases and BHT.

Table 1: Inhibition of linoleic acid oxidation (%)

	(%)	$\pm SD$
Ethyl acetate phase	32,58	7,97
Aqueous interphase	0	0
Total flavonoids	46,40	5,79
BHT	107.48	16.14

Reducing power

In the present study, ethyl acetate phase and aqueous interphase exhibited the highest reducing power followed by the total flavonoids but not satisfactory in comparison with BHT and ascorbic acid. An important capacities of flavonoids in reducing ferric cyanide complex to the ferrous is shown in table 2 and Figure 3.

Table 2: EC50 value for reducing power

	EC50 (µg/ml)	$\pm SD$
BHT	16,06	0,18
Ascorbic acid	14,47	0,28
Ethylacetate phase	46,24	0,42
Aqueous interphase	53,12	1,02
Total flavonoids	56,97	0,67

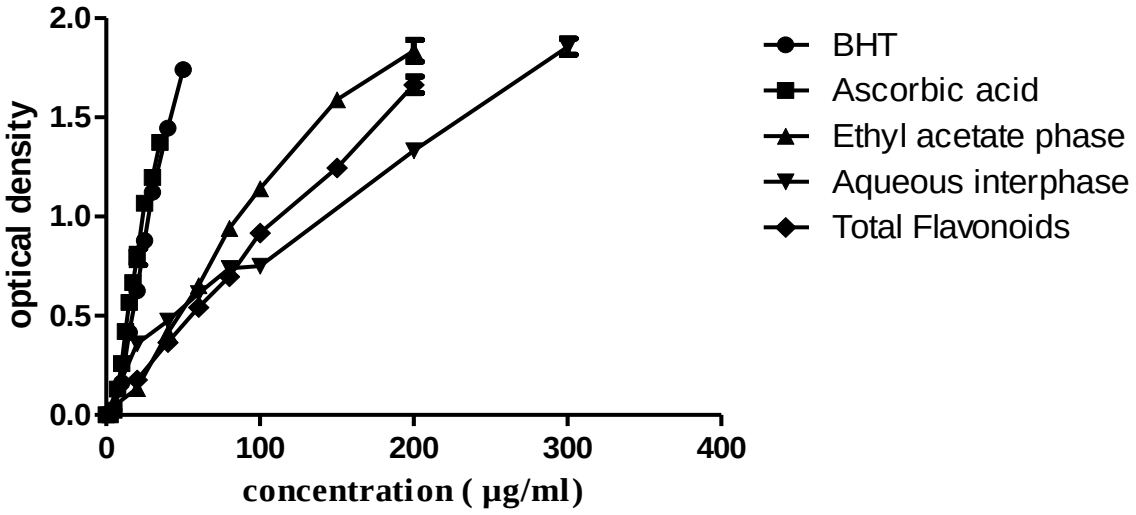

Figure 3: Antioxidant activities of flavonoids of *Galium tunetanum* Poiret by reducing power test.

ISSN: 0975-8585

DISCUSSION

Scavenging of DPPH free radical determines the free radical scavenging capacity or antioxidant potential of the test sample, which shows its effectiveness, prevention, interception and repair mechanism against injury in a biological system [24]. Lower EC50 value indicates stronger ability of the extract to act as DPPH scavenger while the higher EC50 value indicates the lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction.

The ethyl acetate phase exhibit a low value of EC50, lower than BHT which makes this phase better than positive control that's why this phase present a significant difference due to the high continence of flavonoids . Nevertheless, the aqueous interphase marks a high value of EC50 because of the low concentration of flavonoids, which indicates the lower scavenging activity. Total flavonoids which are the mixture of the two phases exhibit a low antagonism with a benefic effect by a similar value of EC50 with the BHT.

In the β-carotene bleaching assay, the linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As a result, β-carotene molecules lose their double bonds by oxidation in this model system. In the absence of an antioxidant, the β-carotene molecule loses its chromophore and undergoes rapid discoloration, which can be monitored spectrophotometrically. [25, 26].

All phases of flavonoids of *Galium tunetanum* Poiret cannot accomplish the reaction as BHT with a high concentration of substance expressed as IC50 and the mixture of the two phases present a little synergic effect.

In the reducing power assay, the presence of antioxidants in the samples result in the reduction of the ferric cyanide complex to the ferrous form which can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. The increased absorbance at 700 nm indicates an increase in reducing power of samples [27]. The extracts that showed comparable absorbance readings with ascorbic acid and BHA are considered to have high reducing power. On the other side the flavonoids of *Galium tunetanum* Poiret show low capacities to reducing Fe^{3+} to Fe^{2+} in comparison with BHT and ascorbic acid but an acceptable value of EC50.

CONCLUSION

On the basis of the results of this study, it is clearly demonstrated that ethyl acetate phase and the mixture of the two phases are novel natural antioxidants, it possessed excellent antioxidant properties, including scavenging abilities against DPPH and reducing power.In conclusion, this study suggested that *Galium tunetanum* Poiret is a potential source of natural antioxidants. However, further investigations on *in vivo* antioxidant activities are highly recommended.

ISSN: 0975-8585

ACKNOWLEDGMENT

This work was supported by the Directorate General for Scientific Research and Technological Development, the Algerian Ministry of Higher Education and Scientific Research (MESRS).

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