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Global chemical composition and antioxidative effect of the ethanol extracts prepared from *Globularia alypum* leaves.

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Abstract

In this paper, the phenolics content and the antioxidant activity of the ethanol extracts prepared from the leaves of *Globularia alypum* L were evaluated. These extracts were obtained by two different extraction methods.
The composition of polyphenols (198±0.52µg gallic acid equivalent/mg of dry mass) and tannins (30.65±0.11µg tannic acid equivalent/mg of dry mass) was higher in the ethanol extract obtained by soxhlet method, whereas, the macerated ethanolic extract contained high amounts of anthocyanins (35.10±0.23µg cyanidin equivalent/mg of dry mass) and flavonoids (19.26±0.22µg quercetin equivalent/mg of dry mass) which are strongly thermosensitive particles.
The extracts were also subjected to a screening for their antioxidant activity using the DPPH assay. The free radical scavenging effect of the macerated ethanol extract (IC_{50} = 1.31 ± 0.17 mg/ml) was more important than quercetin used as positive control.

Keywords: *Globularia alypum L*, chemical composition, antioxidant activity, ethanol extracts.

1. Introduction

Since recent times, there is a growing interest in the food industry and in preventive health care for the development and evaluation of natural antioxidants drugs from medicinal plant materials [1,2,3].

In the present work we have investigated the antioxidant potency of an endemic and wild species in the mediterranean area: *Globularia alypum* L., belonging to the *Globulariaceae* family and commonly used in Arab folk medicine for a wide range of conditions.

Globularia alypum L. is a perennial shrub which is found throughout the Mediterranean area. The plant is known for a variety of purposes [4,5].

In the Algerian traditional pharmacopoeia, *G. alypum* locally named "Ain Larnab" is one of the most traditional plant remedies. Its leaves are traditionally used as hypoglycaemic agent, laxative, cholagogue, stomachic, purgative and sudorific [6,7]. It also used in the treatment of cardiovascular and renal diseases as demonstrated by ethnobotanical surveys, which showed that *G. alypum* is one of the most used medicinal plant in Morocco [5,8].

The infusion of *G. alypum* exhibiting no toxicological effects was shown to produce a significant hypoglycaemic activity in rats both by oral and intraperitoneal administration [9,10].

A significant antileukemic activity of the aqueous extract of *G. alypum* was also reported [11]. Methanol and dichloromethane extracts of *G. alypum* were also shown to reduce histamine and serotonin contraction in *vitro* [12]. The methanolic extract of *G. alypum* decreases hyperglycaemia in streptozotocin – induced diabetic rats [13]. However, different extracts of *G. alypum* were significant sources of compounds with antigenotoxic and anti-tuberculosis activities [14,15,16].

In addition, *G. alypum* L. was shown to exert an anti-ulcer activity against the gastric mucosal damages caused by indomethacin [17].

Recently, the *G. alypum* aqueous extract has a beneficial effect on plasma triglycerides and gives a promising perspective for hypertriglyceridemia treatment. Moreover, in rats fed a high–fructose diet, *G. alypum* is effective by lowering lipid peroxidation and improves antioxidant enzymes [18].

The wide use of this plant for the treatment of many diseases in addition to the fact that only few studies are reported on the Algerian *G. alypum* strain [19,13], prompted us to investigate the ethanol extracts of this plant and their major compounds. So far, the most important chemical investigations of *Globularia alypum*

are those of Chaudhuri and Sticher [20], Es Safi et *al*. [21,22,23] and Boutiti et *al*. [19], where the presence of some glycosidic iridoids, phenolic acids, flavonoids and a lignan diglucoside was reported.

In the present work, we report the phenolic content and the antioxidant activity of two ethanol extracts prepared by two different protocols, from the leaves of *G. alypum* L. To our knowledge there is no report on the antioxidant effect of *G. alypum* growing wild in Algeria.

2. Material and methods

2.1. Plant material

Leaves of *G. alypum* were collected in December 2013, from Sidi moussa-Blida, Algeria.

Botanical identification of the plant was conducted at the department of Botany INA-El Harrach (Algeria) and a voucher specimen of this plant was deposited in the Herbarium of the Laboratory of Botany in the cited university.

2.2. Extraction

The fresh leaves of *G. alypum* were air – dried in shade at room temperature and powdered. In the first extraction method, fifty grams were sequentially extracted with 500ml of ethanol in a Soxhlet apparatus (AM Glassware, Aberdeen, United Kingdom) for 6 hours. The residue was filtered twice and then the ethanol was evaporated under vacuum until dryness to obtain a dry ethanol extract (EE1).

For the second method of extraction, 50g were macerated during 72 hours at room temperature with 500ml ethanol under continuous magnetic stirring.

The crude preparation was filtered twice and concentrated under reduced pressure to provide a dry extract (EE2).

2.3. Determination of total phenolic compounds by the Folin-Ciocalteu Method

The polyphenols of each extract were determined by the Folin-Ciocalteu method [24]. A diluted solution of each extract (0.5ml) was mixed with Folin Ciocalteu reagent (0.2N, 2.5ml). This mixture rest at room temperature for 5 min and then sodium carbonate solution (75g/l in water, 2ml) was added.

After one hour of incubation, the absorbance was measured at 765nm against water blank. A standard calibration curve was plotted using gallic acid (0–100 mg/l).

The results were expressed as µg Gallic acid equivalent/mg of dry mass.

2.4. Determination of tannin content

The tannin content was analyzed by the vanillin method [25]. One milliliter of each extract solution (1mg/ml) was placed in a test tube with vanillin (1% in 7M H_2SO_4, 2ml) in an ice bath**,** and then incubated at 25°C. After 15min, the absorbance of the solution was read at 500nm. The concentrations were calculated as µg Tannic acid equivalent/mg of dry mass from a calibration curve.

2.5. Determination of total flavonoids amount

The total flavonoids were estimated according to the Dowd method as adapted by Arvouet-Grand *et al.* [26]. 4ml of each extract solution were mixed with 4ml of aluminium trichloride solution ($AlCl_3$, 2%). The absorbance was read at 415nm after 15min against a blank sample consisting of methanol (4ml) and extract (4ml) without $AlCl_3$. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as µg Quercetin equivalent/mg of dry mass.

2.6. Determination of total anthocyanin content

Total anthocyanin content was measured by the pH differential method, as described by Cheng and Breen [27]. Briefly, absorbance of the extract was measured at 510 and 700nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2M) and 4.5 (acetic acid-sodium acetate, 1M). The wavelength reading was performed after 15min of incubation. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29,600 (cyanidin-3-glucoside) and absorbance was determined as follow:

$A = [(A510 - A700)_{pH\ 1.0} - (A510 - A700)_{pH\ 4.5}]$.

Results were expressed as µg cyanidin equivalent/mg of dry mass.

2.7. Evaluation of the antioxidant activity by DPPH test

Antioxidant scavenging activity was studied using the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Bors et *al*. [28]. Briefly, 50µl of various dilutions of the plant ethanol extracts were added to 5ml of a 0.004% methanol solution of DPPH. The studied extracts were tested with methanol as blank, Ascorbic acid and quercetin were used as antioxidant positive controls. The absorbance at 517nm was determined after 30min of incubation. The absorbance (A) of the controls and samples was measured, and the DPPH scavenging activity (I%) in percentage was determined as follow:

$$I\% = [(A_{control} - A_{sample})] / A_{control}] \times 100$$

The data are presented as mean of triplicate and the concentration required for a 50% reduction (IC_{50}) of DPPH radical was determined graphically.

2.8. Statistical Analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$.

3. Results and discussion

3.1. Extraction Yields

Two methods were adopted to prepare *G. alypum* extracts. In the first extraction method, the ethanol extract (EE1) was obtained using a Soxhlet apparatus.

However, the second method of extraction was based on a simple maceration in ethanol under a magnetic stirring (EE2).

The highest yield (43.50%) was recorded by the first method of extraction (EE1), for the second method the yield was less important 30.40% (table 1).

Variation in the yields of these extracts can be attributed to the method of extraction applied. So, the first is based on a long contact of the leaves powder with the solvent under heat (50-60°C), whereas, the maceration method is realised at room temperature. These results lead us to suggest that the heat could increase the yield of extraction.

There is no study in the literature which mentions the extraction yields of ethanol extracts using our methods or other methods.

3.2. Chemical composition of the extracts

The amounts of the total polyphenols**,** the tannins, the flavonoids and anthocyanins in the ethanol extracts fraction of *G. alypum* leaves are shown in Table 1

Table 1

Extraction yields and chemical composition of *G. alypum* ethanol extracts

	Chemical composition (µg eq/mg of dry mass) *				Yields (%)
	Polyphenols (Gallic acid eq)	Tannins (Tannic acid eq)	Flavonoids (Quercetin eq)	Anthocyanins (Cyanidin eq)	
EE1	198 ± 0.52	30.65 ± 0.11	10.20 ± 0.58	13.45 ± 1.32	43.50
EE2	139 ± 1.22	5.64 ± 0.16	19.26 ± 0.22	35.10 ± 0.23	30.40

*Standard derivation (SD) didn't exceed 5%.

The highest amount of polyphenols was obtained by the Soxhlet extraction method of the EE1 (198 ± 0.52 µg Gallic acid eq /mg of dry mass). For the maceration method, the EE2 exhibits 139 ± 1.22 µg Gallic acid eq /mg of dry mass**,** These results showed that, for both methods, polyphenols content was strongly dependent on the method of extraction used. Many works reported that polar fractions had more phenolics in them [16,15].

Hence, the difference in yield registered between the two extracts could be due to the impact of the heat, which has a direct action on some thermosensitive phenolics. However, the macerated extract contained a high amount of polyphenols although its lower yield.

Djeridane *et al.* [29] obtained 21.54 µg Gallic acid eq /mg of dry mass from a water-ethanol extract (30:70) of *G. alypum.* This extract was prepared according to the Soxhlet extraction method. This result showed that the content of polyphenols was six times smaller than the one we found. There are no studies in the literature investigating the content of polyphenols in others members of the genus *Globularia.*

The amount of tannins in the EE1 was the highest (30.65 ± 0.11 µg Tannic acid eq /mg of dry mass) followed by the EE2 (5.64 ± 0.16 µg Tannic acid eq /mg of dry mass). These results showed that tannin content was strongly dependent on the method of extraction and the heat has no destroying effect on tannins particles. This is the first study to record the tannin content of ethanol extracts from *G. alypum.*

For the flavonoids, the highest amount was found in the EE2 (19.26 ± 0.22 µg Quercetin eq /mg of dry mass) and 10.20 ± 0.58 µg Quercetin eq /mg of dry mass for the EE1. Djeridane *et al.* [29] reported that the water-ethanol extract (30:70) of *G. alypum* contained 4.54 µg Rutin eq /mg of dry mass. This result showed that the content of flavonoids was approximatively two times less important compared to what we found with EE1. There are no other studies in the literature that investigated the content of flavonoids in others members of the genus *Globularia.*

For the anthocyanins content, the highest amount was obtained in the EE2, equal to 35.10 ± 0.23 µg Cyanidin eq /mg of dry mass, the EE1 contained a very lower amount (13.45 ± 1.32 µg Cyanidin eq /mg of dry mass). These results showed that the anthocyanins content was strongly thermosensitive. This is the first study to record the anthocyanins content of ethanol extracts from *G. alypum.*

3.3. DPPH assay

The DPPH free radical method determines the antiradical power of antioxidants. The degree of discoloration is

attributed to the hydrogen donating ability of tested extracts.

The highest antioxidant activity was shown for the EE2 (86.798%) with an IC_{50} value of 1.31 ± 0.17 mg/ml, it was superior to that of the EE1 (52.610%) with an IC_{50} value of 4.95 ± 0.94 mg/ml (Table 2).

Table 2

Antioxidant activity of the extracts by DPPH assay

	Standards		EE1	EE2
	Ascorbic acid	Quercetin		
I%	92.620	85.562	52.610	86.798
IC_{50}(mg/ml)	0.89 ± 0.06	1.82 ± 0.28	4.95 ± 0.94	1.31 ± 0.17

We can deduce that the antioxidant activity of the EE2 ethanol extract is better than Quercetin. But the best antioxidant activity was allowed to Ascorbic acid (0.89 ± 0.06 mg/ml).

As seen on table 2, it can be concluded that the extract obtained using maceration method at room temperature was considerably more effective radical-scavenger than the extract obtained by soxhlet method using heat. Changes in temperature can alter the ability of the extract to reduce free radicals.

Khlifi et *al*. [16] reported that the water-methanol extract prepared from the leaves of *G. alypum* using Soxhlet method, has presented an IC_{50} value of 27.54mg/l. In other works, Es-Safi et *al*. [21] have isolated a phenolic compound (6-hydroxy-luteolin-7-Olaminaribioside) from the aerial parts of *G. alypum*, which displays an important antioxidant activity with IC_{50} = 1.76 mg/l; they used the BHT (butylated hydroxytoluene) as the positive control with IC_{50} = 8.81 mg/l.

Antioxidant activity has not been studied for *G. alypum* ethanol extract growing in Algeria. The difference between our results and those of Es-Safi et *al*. [21] and Khlifi et *al*. [16] can be attributed to the polarity of the solvent used in the extraction, to the difference of the extraction methods; we have used the Soxhlet and maceration, while they used a simple maceration. This variability could be also explained by the climate differences, the geographical origin, the harvesting time and the growing conditions.

4. Conclusion

G. alypum ethanol extracts were investigated for their chemical composition and antioxidant activity. This study showed that both extracts could be considered as potential natural antioxidant alternative for use in food and pharmaceutical industry for the prevention or treatment of diseases caused by free radicals. Further studies are necessary to assess the ethanolic extraction of the compounds responsible for the antioxidant activity, which could have more effective capacity than ascorbic acid and might be used as new antioxidants or alternatives to synthetic antioxidants.

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