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In vitro investigation of the antioxidant and antimicrobial effects of hydro-alcoholic and aqueous extracts of *Globularia alypum* L.

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Abstract: *The aim of this work was to establish the antioxidant and antimicrobial activity of hydro-alcoholic and aqueous extracts prepared from the leaves of Globularia alypum L. The quantitative estimation of total polyphenols (by the Folin-Ciocalteu method) and total flavonoids (by the method of aluminium trichloride) showed that the aqueous and ethyl acetate extracts had the highest content of phenolic and flavonoid compounds. Moreover, the results of antioxidant power assessed by both method DPPH and phosphomolybdenum indicated that leaves extracts of G. alypum expressed a considerable activity. The evaluation of the antimicrobial effects, using the disc diffusion method from antibacterial screening and the direct contact method from the antifungal activity, indicated strong antibacterial and antifungal activity.*

Keywords: *Globularia alypum* L., phenolic extracts, antimicrobial activity, antioxidant activity

Introduction

Phenolic compounds are naturally occurring compounds found largely in plants and that are involved in defense against ultraviolet radiation or aggression by pathogens. They have recently aroused considerable interest because of their potential beneficial biochemical and anti-oxidant effects on human health. Commonly referred to as antioxidants, they may prevent various diseases associated with oxidative stress, such as

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cancers, cardiovascular dis-eases, inflammation and others. Nevertheless, the use of synthetic antioxidants in the food industry as additives has been reported to be dangerous because of the side effects of these compounds on human health [1, 2]. For these reasons, the development and utilization of antioxidants from natural origin for food or medical applications in replacement of synthetic antioxidants became subject of interest for many research groups (Oktay et al., 2003; Djeridane et al., 2010; Es-Safi et al., 2007; Vagi et al., 2005) [3]. Various biological activities are attributed to a great variety of phenolic compounds isolated from natural plants. Hence, the plant natural products represent promising sources to develop new antibacterial and antioxidant agents [4].

Globularia alypum L. is a medicinal plant species belonging to the Globulariaceae family [5, 6], commonly known as "Tasselgha" [1]. This specie is one of the most used medicinal plants in the Algerian traditional pharmacopoeia. The plant is known for its uses in the treatment of hypoglycemia, rheumatic, stomachic, and infectious diseases [7]. Furthermore its leaves are often traditionally used in the treatment of diabetes, renal and cardiovascular diseases [4, 8].

The use of phenolic compounds in biological control becomes very promising and considered as one of scientific interest. *G. alypum* is a riche plant in polyphenols, flavonoids, saponins, tannins, terpenoids, coumarins, and cardiac glycosides [2]. Previous studies have focused on the analysis of antioxidant molecules and the evaluation of the antioxidant activity of *G. alypum* extracts. For example, Es-Safi et al. [9], Saglam et al., [10], Djeridane et al. [11] have determined the antioxidant constituents and the α-tocopherol of *G. alypum*. A significant hypoglycemic activity of the methanolic extract of *G. alypum* was also reported in rats both [12]. Another study concerning antibacterial activity of phenolic compouds of *G. alypum* has also been carried out by Krimat et al. [13]. However, no previous studies have been carried out until this day on the antifungal effect of extracts of *G. alypum* against phytopathogenic microorganisms.

In this context, the present study aims to determine the total phenolic and the total flavonoid contents of organic and aqueous extracts of *G. alypum* leaves and to evaluate their antioxidant and antimicrobial activities. Moreover, to our knowledge, this is the first report on the study of antifungal activity of *G. alypum* leaves extracts.

Material and Methods

Plant material

Leaves of *G. alypum* were collected during spring 2015 from the region of Laghouat, Algeria. The botanical identity of the plant was confirmed by Dr. Y. Halis, and a voucher specimen (No.0116/HBPA) was deposited at the Herbarium of Laboratory of Biomolecules and Plant Amelioration, Larbi Ben m'hidi

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University of Oum El Bouaghi, Algeria. The plant leaves were dried in shade, finely powdered with an electric mill and stored in glass vials until extraction.

Preparation of plant extracts

Preparation of hydro-alcoholic extracts

For extraction (0.5 g) of fine powdered leaves of *G. alypum* were macerated with 100 ml of 80/20 (v/v) hydro-alcoholic solvent (methanol/water) at room temperature 3 times (24 hours × 3). After filtration, the methanol was removed under reduced pressure on a rotary evaporator at 40°C, and the remaining aqueous solution of the extraction was defatted with the same volume of hexane to remove lipids. Then, the aqueous phase was extracted firstly with ethyl acetate and secondly with 1-butanol. The organic fractions were dried with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 10 ml of absolute methanol and refrigerated until further use.

Preparation of aqueous extract

For the aqueous extraction (0.5 g) of powdered plant was heated in 100 ml of distilled water at 75°C, for 20 minutes. The extract was filtered and evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 10 ml of distilled water and kept at 4°C.

Determination of total phenolic compounds

The amount of total phenols in ethyl acetate, butanol and aqueous extracts was determined by using the Folin-Ciocalteu reagent as reported by Singleton and Rossi (1965) [14]. 100μl of each sample was dissolved in 500 μl of the aqueous solution of Folin-Ciocalteu reagent at 10%. After 2 minutes of incubation at room temperature, 2 ml of 2% (w/v) sodium carbonate in water was added. The reaction mixture was shaken and then incubated for 30 min in the dark at room temperature. Then, the absorbance was measured at 765 nm against water blank. Total phenolic content was expressed as gallic acid equivalent (GAE) per gram of dry weight of plant material and all the assays were carried out at least in triplicate.

Determination of flavonoids content

The flavonoids content in the extracts was determined spectrophotometrically according to Lamaison and Carnat method [15], based on the formation of a complex flavonoid–aluminium, having the maximum absorbance at 409 nm. Quercitin was used to make the calibration curve. 1 ml of the diluted sample was mixed with the same volume of 2% aluminum chloride methanolic solution. After 20 min incubation at room temperature, the absorbance of the reaction mixture was measured at 409 nm. The concentration of flavonoid compounds was expressed as milligrams of quercitin equivalents (QE) per gram of dry weight material. Triplicate measurements were taken for all samples.

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Antioxidant testing assays

DPPH radical scavenging assay

The free radical scavenging activity of our extracts against stable DPPH˙ (2-diphenyl-2 picrylhydrazyl hydrate) was determined according to the method of Brand Williams et al. [16], with slightly modification as described below.

About 1 ml of various concentrations of extracts was added to 1 ml DPPH methanolic solutions 250 µM DPPH˙. The mixture was then shaken vigorously and allowed to stand at room temperature in the dark for 30 min, and the optical density was measured at 517 nm. The capability to scavenge the DPPH radical of an antioxidant was calculated using the following equation:

DPPH radical-scavenging activity (%) = $[(Abs_{control} - Abs\ test_{sample})/ Abs_{control}] \times 100$.

where: $A_{control}$ was the absorbance without extract and A_{sample} was the absorbance with extract

The antioxidant activity of the extract was expressed as an IC_{50} value defined as the concentration (in µg/ml) of the extract that inhibited the formation of DPPH radicals by 50%.

All experiments were performed in triplicate. Gallic acid, quercitin and Vitamin C were used as antioxidants standards for comparison of the activity.

Phosphomolybdenum assay

Total antioxidant activity of the different extracts was determined by the phosphomolybdenum method which was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The antioxidant capacity of *G. alypum* extracts expressed as the concentration of vitamin C (VC) equivalent at 1 mM of the extract simple (VCEAC) [16]. 200 µl of each sample solution was mixed in duplicate with 2 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was kept for 90 min at 95°C. After cooling up to room temperature, the absorbance of samples was measured at 695 nm against blank using a UV–Vis spectrophotometer. Gallic acid, quercitin and Vitamin C were used as reference controls.

Screening for antimicrobial activity

Strains tested

The used strains for primary in vitro screening antimicrobial activity in this study are summarized in Table 1. The bacterial strains were isolated from biological fluids of patients in the Hospital Centre of Laghouat region (Algeria). Reference strains ATCC, obtained from Laboratoire vétérinaire régional de Laghouat (L.R.V.L). On the other hand, fungal strains were obtained and identified from laboratory of Mycology at Tlemcen University (L.M.U.T), Algeria.

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DOI: 10.2478/asn-2020-0006

Table 1. Pathogenic strains tested vis-à-vis *G. alypum* extracts

Strains	Reference	Origin
Staphylococcus aureus	ATCC 25923	LRVL
Enterococcus faecalis	ATCC 19432	LRVL
Pseudomonas aeruginosa	ATCC 27853	LRVL
Escherichia coli	ATCC 25922	LRVL
Salmonella typhi	Isolate	LRVL
Aspergillus flavus	Isolate	L.M.U.T
Penicillium spp.	Isolate	L.M.U.T
Fusarium oxysporum	Isolate	L.M.U.T

Determination of antibacterial activity

The antibacterial screening of each extract was determined by disk-diffusion method using Mueller Hinton agar (MHA) [17, 21]. The inoculums for the assays were prepared from overnight broth culture in physiological saline (0.8% of NaCl) adjusted to 0.5 McFarland turbidity standards (1.5 × 108 CFU/ml). About 100 ml of the inoculum was spread on sterile MHA plate, and Filter paper discs (6 mm) impregnated with 10 µL of various concentrations (50, 60, 80 and 100 mg/ml) of the extracts solutions diluted in dimethylsulfoxide DMSO (10%) were placed on the cultured plates. DMSO was used as negative control. The inoculated plates were incubated at 37°C, for 24 h. Antibacterial activities were determined by measuring the diameter of the growth inhibition zone around the discs in mm.

Determination of antifungal activity

The direct contact method was adopted to evaluate the antifungal activity [18, 19]. About 500 µl of the polyphenolic extracts at different concentrations (50, 80 and 100 mg/ml) is incorporated separately into tubes containing 20 ml of the maintained PDA medium in supercooled. Each tube was homogenized instantly by manual shaking then its contents were poured into 90 mm petri dishes in glass (20 ml/dish). After solidification of the medium, a mycelial disc of 6mm diameter was deposited aseptically on the surface of the agar medium in the center of the petri dish. The PDA without polyphenolic extracts was used as a negative control for each strain. Fungal colony diameters, in each concentration were recorded after incubation for 7 days at (25±2°C). The antifungal action was evaluated by calculating the growth inhibition rate using the following formula:

PI (%) = (A - B)/A x 100,

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DOI: 10.2478/asn-2020-0006

where PI (%) is the inhibition rate expressed as a percentage; A was mycelial growth of the control and B was mycelial growth of treated fungi [20].

Statistical analysis

All the samples were analyzed in triplicate, except those for antifungal activity which were analyzed in duplicate. Data were expressed as mean ± standard deviation (SD). Student test was used to analyze the statistical significance.

Results and Discussion

Total phenolic and total flavonoid contents

The total phenolic and total flavonoids contents of the tested extracts are illustrated in Table 2.

Table 2. Total amount of phenolic and flavonoids compounds in ethyl acetate, butanol and aqueous extracts of *G. alypum* leaves

Extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
Aqueous	14.84±0.07	3,81±0,05
Ethyl acetate	13.21±0.20	4.54±0.02
Butanol	8.23±0.12	2.46±0.03

The results obtained in this study (Table 2) showed a significant level of total phenolic content in the two extracts aqueous and ethyl acetate with an amount of (14.84±0.07 and 13.21±0.2 mg GAE/g), respectively, while the butanol extract was the lowest one (8.23±0.12 mg GAE/g). A similar level of diversity in phenolic contents was seen by Boussoualim et al. [25] who examined crude, chloroform, ethyl acetate and aqueous extracts of *G. alypum* from the region of Setif (Algeria). Moreover, Djeridane et al. [11] 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. These results showed that the polyphenols content was strongly dependent on the used method of extraction. Many works reported that polar fractions had more phenolics in them [26]. Moreover, a previous published phytochemical study on the different parts of *G. alypum* proved the richness of roots extracts in phenolic compounds, this difference may be related to the differences in the used plant tissue for extraction, the climatic and geographical factors, the harvesting time and the growing conditions [23, 24].

Corresponding author: krlamia@yahoo.fr Full Paper
DOI: 10.2478/asn-2020-0006

As regards to the total flavonoids contents, ethyl acetate and aqueous extract showed the highest flavonoid content with 4.54±0.02 and 3,81±0,05 mg QE/g, respectively. It was observed that the obtained extracts possessed higher levels of total flavonoids in comparison with the studies of Krimat et al. [13] who reported that the ethyl acetate extract of *G. alypum* contained an amount of 3.76±0.03 mg QE/g. In addition, Boussoualim et al. [25] saw a similar level of flavonoids in the aqueous extract. Generally, it has been reported that *G. alypum* was among the plants showing the highest total phenolic contents. However, the large discrepancy between its phenolic and flavonoid contents could be explained by its predominance by hydroxycinnamic and hydrobenzoic derivatives [11].

Antioxidant activities

Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative. Phenolic compounds are a major group of compounds acting as primary antioxidants or free radical scavengers [3, 7].

There are several methods for the determination of antioxidant activities. The chemical complexity of extracts, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed [15]. In this study DPPH, radical scavenging activity and phosphomolybdenum assay were used for evaluation of antioxidant properties of *G. alypum*.

Antioxidant activity by the DPPH method

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, phenolic acids and flavonoids [22]. The method is based on the reduction of alcoholic DPPH˙ solution in the presence of a hydrogen-donating antioxidant [15]. As shown in Table 3, butanol extract showed a notable antioxydant activity with an IC_{50} value of 10.54±0.02 µg/ml, followed by the ethyl acetate and aqueous extracts with values of 10.58±0.08 µg/ml and 60.24±0.33 µg/ml respectively. The synthetic antioxidants ascorbic acid, galic acid and quecétine had a value of 9.85±0.06, 4.21±0.01 and 2.18±0.03 µg/ml respectively.

The results showed that both ethyl acetate and butanol extracts from *G. alypum* manifested the strongest capacity for neutralization of DPPH radical. But the aqueous extract showed lower antioxidant capacity. Our results are in agreement with those of Djeridane et al. [11], who reported that the hydromethanolic extract *G. alypum* has an antiradical activities with IC_{50}=8.77±0.04 mg/ml. This antioxidant activity could be explained by the high content of polyphenolic, flavonoid and anthocyanins in our extracts [3]. Moreover, new phenolic compounds were isolated from the hydromethanolic extract of the aerial part of *G. alypum* named as (6-hydroxyluteolin 7-O-laminaribioside, eriodictyol 7-O-sophoroside and 6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl)ethyl]-β-D-glucopyranoside), as well as two flavonoid glycosides

Corresponding author: krlamia@yahoo.fr Full Paper
DOI: 10.2478/asn-2020-0006

identified as 6-hydroxyluteolin 7-O-β-D-glucopyranoside and luteolin 7-O-sophoroside. These molecules exhibit an antioxidant activity using the DPPH assay [9]. On the other hand, the same authors reported that the leaves of *G. alypum* could be used as a potential source of natural antioxidants and bioactive molecules in pharmaceutical industry.

Table 3. Free radical (DPPH) scavenging and total antioxidant capacity of the different extracts of *G. alypum*

Extracts	DPPH IC_{50} (µg/ml)	Phosphomolybdenum (VCEAC)
Ethyl acetate	10.58±0.08	17.014±0.07
Butanol	10.54±0.02	37.61±0.04
Aqueous	60.24±0.33	12.29±0.49
Standard antioxidants		
Gallic acid	4.21±0.01	0.59±0.05
Quercetin	2.18±0.03	0.42±0.03
Vitamin C	9.85±0.06	-

Antioxidant capacity by phosphomolybdenum method

The phosphomolybdenum assay is based on the reduction of phosphate-Mo (VI) and its transformation to phosphate Mo (V) in the sample, which is a bluish green colored phosphate that is complex at acid pH. The antioxidant capacity of plant extracts expressed as the number of equivalents ascorbic acid [16]. According to authors' knowledge, there is no study on total antioxidant capacity of *G. alypum* by phosphomolybdenum assay. The results obtained showed that all *G. alypum* extracts had a strong total antioxidant activity. Whereas the hydroalcoholic extracts had maximum values compared to aqueous extract. Overall, the butanol extract was the best antioxidant as demonstrated by the highest value compared to other extracts and standards used.

As seen on Table 3, it can be concluded that the antioxidant activity obtained using phosphomolybdenum assay was significantly higher than those obtained from the DPPH method.

Moreover, the aqueous extract containing high levels of polyphenols have the lowest antioxidant effect for both methods. This low value could be explained by the fact that the antioxidant activity not only depends on the concentration of the total phenolics content which was present in the extract, but also on the structure and the interaction between the antioxidants. It is probable that the aqueous extract has lost some active ingredients after heat [31]. In conclusion, the results from this study show that the hydroalcoholic extract of *G. alypum* could thus be considered as a source of potential antioxidants and will promote the reasonable usage of this plant in food technology and processing as well as for medical use.

Corresponding author: krlamia@yahoo.fr Full Paper
DOI: 10.2478/asn-2020-0006

Antimicrobial study

Antibacterial Screening activity

The diameters of growth inhibition zones exhibited by different extracts of *G. alypum* against bacterial strains shows in Table 4. As can be noted from this table, the ethyl acetate extract showed significantly the highest antibacterial activity against all pathogens bacteria, with a maximum inhibition zone of 15.5±0.2 and 18±0.46 mm against *Pseudomonas aeruginosa* and *Staphyloccocus aureus* respectively. However, clinical pathogens strains were less susceptible to butanol extract. On the other hand, no significant results were recorded in aqueous extract, which was active only against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The solvent used as control (DMSO) exerted no effect against all the tested microbial strains. In general, the gram-positive bacteria were found to have more susceptibility as compared to gram-negative bacteria species.

This result may be explained by the variation in chemical composition and structure of cell wall of both types of microorganisms [13]. Thus, it is clear that the effectiveness of the extracts largely depends on the type of used solvent. It can be speculated that the ethyl actetate extracts contained compounds with greater polarity than that of their counterparts present in butanol or aqueous extracts. This may be the likely explanation for significant differences in the bacteriostatic activity between the different compound extracts of *G. alypum* [28].

Table 4. Growth inhibition zones (mm) exhibited by *G. alypum* extracts against bacterial strains

	Inhibition zones of test microorganisms (mm)											
	Ethyl acetate extract				**Butanol extract**				**Aqueous extract**			
	50 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml	50 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml	50 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml
Staphylococcus aureus	12.66 ± 0.15	13.3 ±0.1	15 ±0.36	18 ±0.46	11.83 ±1.15	12.16 ±0.96	13.66 ±0.85	15.33 ±0.76	10.16 ±0.35	10.33 ±0.47	11.83 ±0.40	12.5 ±0.5
Enterococcus faecalis	N	9 ±0.36	12.16 ±0.35	13.5 ±0.91	N	8 ±0.2	11.16 ±0.35	11.5 ±0.35	N	N	7.66 ±0.40	9.66 ±0.76
Pseudomonas aeruginosa	11 ±0.2	12.5 ±0.29	14 ±0.17	15.5 ±0.2	9.66 ±0.72	10.66 ±0.29	11.83 ±0.57	12.16 ±0.61	8.16 ±0.85	9.66 ±0.76	9.83 ±0.35	10.66 ±0.76
Escherichia coli	N	9.5 ±0.7	11 ±0.26	13 ±0.36	N	N	8.5 ±0.65	9.33 ±0.23	N	N	N	N

Corresponding author: krlamia@yahoo.fr Full Paper
DOI: 10.2478/asn-2020-0006

Salmonella typhi	8 ±0.26	9.16 ±0	10.5 ±0.61	11.5 ±0.3	N	N	9.5 ±0.62	10.66 ±0.47	N	N	8 ± 0.2	10 ± 0.4

Antifungal activity

The antifungal activity of the plant extracts was determined by the direct contact method. According to the results given in Table 5.

Table 5. Inhibition rate of mycelial growth of the fungal pathogenic strains against three different extracts

	Growth inhibition rate (%)								
	Ethyl acetate extract			**Butanol extract**			**Aqueous extract**		
	50 mg/ml	80 mg/ml	100 mg/ml	50 mg/ml	80 mg/ml	100 mg/ml	50 mg/ml	80 mg/ml	100 mg/ml
Aspergillus flavus	31.81%	39.39%	43.93%	28.78%	34.84%	37.87%	0%	0%	0%
Penicillium spp.	43.47%	52.17%	58.69%	36.95%	45.65%	47.82%	0%	0%	0%
Fusarium oxysporum	47.22%	58.33%	66.66%	37.5%	44.44%	51.38%	12.12%	16.66%	19.69%

The different tested extracts significantly reduced the colony diameters of the fungal pathogenic strains compared to the control. Ethyl acetate extract of *G. alypum* showed significant antifungal activity against all strains mycelial tested, with a percentage inhibition varied by 43.93% on *Aspergillus flavus* up to 66.66% on *Fusarium oxysporum* at the concentration of 100 mg/ml. However, the mechanism of the action of these plant constituents is not yet fully known it is clear that the effectiveness of the extracts largely depends on the type of the used solvent. Ethyl acetate and butanol extracts exerts a more powerful inhibitory activity as compared to aqueous extract, which was active only against *Fusarium oxysporum*. This observation clearly indicates that the existence of non-polar residues in the extracts which have higher fungal abilities. According to several authors [29, 30], methanol extraction was more effective at antifungal activity than at water. This difference can be attributed to the origin of the different chemical composition between extracts; many studies have revealed a relationship between the chemical structure of phenolic compounds and their antimicrobial activity [27]. However, we can note that percent inhibition of plant extracts on pathogen growth also varied with increasing concentrations. Therefore, the use of higher concentrations or other extraction method in order to obtain a more potent effect against all strains mycelial could be recherched.

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DOI: 10.2478/asn-2020-0006

Conclusion

This study revealed interesting antioxidant and antimicrobial activities of the *G. alypum* extracts *in vitro* assays. The present results showed that the butanol and ethyl acetate extracts are the most effective antioxidants as determined through DPPH radical scavenging activity and phosphomolybdenum method. More experiments in relation to this theme should be done to confirm the antioxidant activity of *G. alypum*. Morever the extracts remarkably inhibited the growth of all tested gram positive and gram-negative bacteria and proved to be an effective antifungal agent against tested fungi. In addition, we note that no previous studies on the antifungal activity of the plant extracts. These data confirm the great potential of this plant for the production of bioactive compounds, which can be suggested as a natural additive in food and pharmaceutical industries. However, further studies are needed to identify the compounds responsible for these beneficial properties.

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The authors confirm that this article content has no conflict of interest.

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