Anti-fungal and Antibacterial Effects of *Globularia alypum* L Extracts and their Antioxidant Activities

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ABSTRACT

This study aimed to evaluate, in vitro, the antioxidant, antibacterial and antifungal activities of different extracts prepared from the leaves of *Globularia alypum* L. The quantitative assays showed that ethyl acetate extract had the highest content of phenolic and flavonoid compounds with an amount of (14.84 ± 0.07 mg GAE.g of dry mass), and (4.54 ± 0.02 mg QE.g of dry mass) respectively. Moreover, the antioxidant activity assessed by radical scavenging assay was also highest for ethyl acetate extract. Antioxidant activity was generally found to increase with total phenolic content (TPC) and total flavonoid content (TFC). The obtained results of antimicrobial activities, using the disc diffusion method from antibacterial screening and the direct contact method from the antifungal activity, indicated moderate antibacterial effect and strong antifungal activity.

Keywords: *Globularia alypum* L; Antibacterial; Antifungal; Antioxidant; Phenolic extracts

INTRODUCTION

*Globularia alypum* L, is a medicinal plant species belonging to the *Globulariaceae* family [1,2], commonly known as “Tasselgha” [3]. 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 [4]. Furthermore its leaves are often traditionally used in the treatment of diabetes, renal and cardiovascular diseases [5,6].
The use of phenolic compounds in biological control becomes very promising and considered as one of scientific interest. *Globularia alypum* L., is a rich plant in polyphenols, flavonoids, saponins, tannins, terpenoids, coumarins, and cardiac glycosides [7]. Previous studies have focused on the analysis of antioxidant molecules and the evaluation of the antioxidant activity of *Globularia alypum* L. extracts. For example, Es-Safi et al. [8], Saglam et al. [9], Djeridane et al. [10] have determined the antioxidant constituents and the α-tocopherol of *Globularia alypum* L. A significant hypoglycemic activity of the methanolic extract of *Globularia alypum* L. was also reported in rats both [11]. Another study concerning antibacterial activity of phenolic compounds of *Globularia alypum* L. has also been carried out by Krimat et al. [12]. However, no previous studies have been carried out until this day on the antifungal effect of extracts of *Globularia alypum* L. against phytopathogenic microorganisms. Therefore, the main purpose of the present study was to determine the total phenolic and the total flavonoid contents of extracts of *Globularia alypum* L. 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 *Globularia alypum* L. leaves extracts.

**MATERIALS AND METHODS**

**Plant Material**

Leaves of *Globularia alypum* L. were collected during spring 2015 from the region of Laghouat, (Algeria). The botanical identity of the plant was confirmed by Dr. Youcef Halis, and a voucher specimen (No. 0116/HBPA) was deposited at the Herbarium of Laboratory of Biomolecules and Plant Amelioration, Larbi Ben m’hidi 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 hydromethanolic extracts:** For extraction, (05 g) of fine powdered leaves of *Globularia alypum* L. 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 (05 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) [13]. 100 μL 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 [14], 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.

**Evaluation of the Antioxidant Activity by DPPH Test**

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. [15], 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:

\[
\text{DPPH Scavenging activity} \% = \left[ \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test sample}})}{\text{Abs}_{\text{control}}} \right] \times 100.
\]

Where: \( \text{Abs}_{\text{control}} \) was the absorbance without extract and \( \text{Abs}_{\text{sample}} \) was the absorbance with extract.

The antioxidant activity of the extract was expressed as an IC\(_{50}\) value defined as the concentration (in mM) of the extract that inhibited the formation of DPPH radicals by 50%. Vitamin C was used as antioxidant standard for comparison of the activity.

**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). On the other hand, fungal strains were obtained and identified by Mycology Laboratory in the University of Tlemcen (Algeria).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Reference</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 25923</td>
<td>LRVL</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 19432</td>
<td>LRVL</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
<td>LRVL</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 25922</td>
<td>LRVL</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>Isolate</td>
<td>LRVL</td>
</tr>
</tbody>
</table>
Determination of antibacterial activity: The antibacterial screening of each extract was determined by disk-diffusion method using Mueller Hinton agar (MHA) [16-20]. 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 × 10^8 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 dimethyl sulfoxide 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 [17,18]. 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 super cooled. 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 6 mm 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:

\[
PI (%) = \frac{(A - B)}{A} \times 100
\]

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 [19-23].

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 shown in Table 2. As can be noted from this table, ethyl acetate extract showed the highest total phenolic content with an amount of (14.84 ± 0.07 mg GAE. g), while the aqueous extract was the lowest one (8.17 ± 0.36 mg GAE.g). A similar level of diversity in phenolic contents was seen by Boussoualim et al. [24] who examined crude, chloroform, ethyl acetate and aqueous extracts of Globularia alypum L from the region of Setif (Algeria). Moreover, Djeridane et al. [10] obtained 21.54 μg Gallic acid eq/mg of dry mass from a water-ethanol extract (30:70) of Globularia alypum L. 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 [25]. Moreover, a previous published phytochemical study on the different parts of *Globularia alypum* L. 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 [22,23].

As regards to the total flavonoids contents, ethyl acetate extract showed the highest flavonoid content while butanol showed the lowest one with $4.54 \pm 0.02$ and $2.46 \pm 0.03$ mg QE/g, respectively. It was observed that the obtained extracts possessed higher levels of total flavonoids in comparison with the study of Krimat et al. [12] who reported that the ethyl acetate extract of *Globularia alypum* L contained an amount of $3.76 \pm 0.03$ mg QE.g. In addition a similar level of flavonoids in the aqueous extract was seen by Boussoualim et al. [24].

Generally, It has been reported that *Globularia alypum* L 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 [10].

### 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 [21]. The method is based on the reduction of alcoholic DPPH$^-$ solution in the presence of a hydrogen-donating antioxidant [14]. As shown in Table 3, ethyl acetate extract showed a notable antioxydant activity with an IC$_{50}$ value of $10.57 \pm 0.06$ μg/ml, followed by the butanol and aqueous extracts with values of $55.63 \pm 0.01$ μg/ml and $264 \pm 13.44$ μg/ml respectively. In comparison with ascorbic acid (IC$_{50}$=9.85 ± 0.06), both ethyl acetate and butanol extracts from *Globularia alypum* L 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. [10] who reported that the hydromethanolic extract *Globularia alypum* L has an antiradical activities with IC$_{50}$=8.77 ± 0.04 mg/L. This antioxidant activity could be explained by the high content of polyphenolic, flavonoid and anthocyanins in our extracts [26]. Moreover, new phenolic compounds were isolated from the hydromethanolic extract of the aerial part of *Globularia alypum* L 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 identified.
as 6-hydroxyluteolin 7-O-β-D-glucopyranoside and luteolin 7-O-sophoroside. These molecules exhibit an antioxidant activity using the DPPH assay [8]. On the other hand, the same authors reported that the hydromethanolic extract of *Globularia alypum* L 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.

**Table 3. Free radical (DPPH) scavenging activity of the different extracts of *Globularia alypum* L.**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
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<tbody>
<tr>
<td>Ethyl acetate</td>
<td>10.58 ± 0.06</td>
</tr>
<tr>
<td>Butanol</td>
<td>55.63 ± 0.01</td>
</tr>
<tr>
<td>Aqueous</td>
<td>264 ± 2.44</td>
</tr>
<tr>
<td>Acide ascorbique</td>
<td>9.85 ± 0.06</td>
</tr>
</tbody>
</table>

**Antimicrobial Study**

**Antibacterial screening:** Table 4 shows the diameters of growth inhibition zones exhibited by different extracts of *Globularia alypum* L against bacterial strains. 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 *Staphylococcus aureus* respectively (Figure 1). 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 [12]. 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 acetate 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 *Globularia alypum* L. [26-28].

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

<table>
<thead>
<tr>
<th>Inhibition zones of test microorganisms (mm)</th>
<th>Ethyl acetate extract</th>
<th>Butanol extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/ml</td>
<td>60 mg/ml</td>
<td>80 mg/ml</td>
<td>100 mg/ml</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>12.66 ± 0.1</td>
<td>15 ± 0.36</td>
<td>18 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.96</td>
<td>0.35</td>
</tr>
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<td>------</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>N±0.36</td>
<td>12.16±0.35</td>
<td>13.5±0.91</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>11±0.2</td>
<td>12.5±0.29</td>
<td>14±0.17</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>N±0.7</td>
<td>11±0.26</td>
<td>13±0.36</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>8±0.26</td>
<td>9.16±0.0</td>
<td>10.5±0.61</td>
</tr>
</tbody>
</table>

Figure 1. Growth inhibition zones exhibited by ethyl acetate extract of *Globularia alypum* L against bacterial strains. 1) Control, 2) 50 mg/ml, 3) 60 mg/ml, 4) 80 mg/ml, 5) 100 mg/ml, A) *Escherichia coli* B) *Staphylococcus aureus* C) *Pseudomonas aeruginosa* D) *Salmonella Sp.* E) *Enterococcus faecalis*

**Antifungal Activity**

The antifungal activity of the plant extracts was determined by the direct contact method. According to the results given in Table 5, the different tested extracts significantly reduced the colony diameters of the fungal pathogenic strains compared to the control. Ethyl acetate extract of *Globularia alypum* L 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* (Figures 2-4). 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.

<table>
<thead>
<tr>
<th>Table 5. Inhibition rate of mycelial growth of the fungal pathogenic strains against three different extracts</th>
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<tr>
<td><strong>Growth inhibition rate (%)</strong></td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
</tr>
<tr>
<td><em>Penicillium spp</em></td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
</tr>
</tbody>
</table>

Figure 2. Inhibition rate of mycelial growth of the three fungal strains by ethyl acetate extracts of *Globularia alypum L*. 
Figure 3. Inhibition rate of mycelial growth of the three fungal strains by butanol extracts of *Globularia alypum* L.

Figure 4. Inhibition rate of mycelial growth of the three fungal strains by aqueous extract of *Globularia alypum*

**CONCLUSION**

In conclusion, this study revealed interesting antioxidant and antimicrobial activities of the *Globularia alypum* L extracts in vitro assay. The present results showed that the ethyl acetate and butanol extracts manifested the strongest capacity to scavenge the stable DPPH free radical. More experiments in relation to this theme should be done to confirm the antioxidant activity of *Globularia alypum* L. Moreover all 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.

**Conflict of Interest**

The authors have declared no conflict of interest.

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