



***In vitro* evaluation of the antioxidant and antimicrobial effects of *Globularia alypum* L. extracts**

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Abstract

In the present study, extracts obtained from different parts of *Globularia alypum* are subjected to a phytochemical screening and investigated for their antioxidant and antimicrobial activities. The results revealed the presence of saponins, polyphenols, flavonoids, tannins, terpenoids, coumarins, and cardiac glycosides. Furthermore, quantitative assays showed that methanolic extract of roots was the richest in phenolic compounds with an amount of 131.48 µg equivalent of gallic acid/mg of extract. While, the methanolic extract of stems was the richest in flavonoids with an amount of 39.14 µg equivalent of quercetin/mg of extract. Moreover, the antioxidant activity assessed by radical scavenging assay showed that roots extracts exhibited strong antioxidant activity. A linear relationship has been found between the total phenolic content and the antioxidant activity of the studied extracts. In addition, all plant extracts were active against Gram-positive bacteria, notably those of roots which were the most efficient against *Bacillus subtilis* and *Staphylococcus aureus*.

Keywords: *Globularia alypum* L, phytochemical screening, antioxidant; antimicrobial, phenolic, flavonoid, plant extracts.

1. Introduction

Globularia alypum L. is a medicinal plant species belonging to the *Globulariaceae* family. It is known in Morocco as "Ein larneb" [1] and is often used for a variety of purposes such as hypoglycemic agent, laxative, cholagogue, stomachic, purgative, sudorific and for cardiovascular and renal diseases' treatments [2].

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 [3]. Moreover, the excessive use of the antibiotics agents has led to the emergence of multidrug resistant bacterial strains [4]. Hence, the major challenge is the development of new bioactive agents to fight against multidrug-resistant bacteria and the lipid oxidation of foods. For this reason, the plant natural products represent promising sources to develop new antibacterial and antioxidant agents.

In this context, the present study aims to determine the total phenolic and the total flavonoid contents of leaves, stems and roots of *G. alypum* extracts and to evaluate their antimicrobial and antioxidant activities. Moreover, to our knowledge, this is the first report on the phytochemical profile, the antimicrobial and antioxidant effects of *G. alypum* roots extract.

2. Experimental

2.1. Plant material and extraction method

The whole plant of *Globularia alypum* was harvested from Taounate region (Morocco) in March 2014. Voucher specimens served for the botanical identification were deposited at the herbarium of National Agency of Medicinal and Aromatic Plants. Then, the whole plant was separated to three samples (leaves, stems and roots), dried at room temperature and powdered. Then, 100 g of each powdered sample was sequentially extracted with hexane (1 L), ethyl acetate (1 L) and methanol (1 L) during 24 h under stirring conditions at room temperature. Hexane was used for lipids elimination. After filtration, mixtures were evaporated under vacuum and the obtained extracts were weighted and stored at 4 °C for further analyses.

2.2. Phytochemical screening

The ethyl acetate and methanol extracts of the tested plant were screened for phytochemical constituents.

2.2.1. Test for phenolic compounds (Ferric chloride test)

A small amount of each extract was dissolved in 5 mL of distilled water, and a few drops of 1% (w/v) ferric chloride were added [5]. The appearance of a dark green color indicates the presence of phenolic compounds.

2.2.2. Test for flavonoids (Alkaline reagent test)

A few drops of sodium hydroxide were added to extracts dissolved in distilled water, the appearance of an intense yellow color indicates the presence of flavonoids [5]. Disappearance of the color after the addition of diluted hydrochloric acid confirms the presence of flavonoids.

2.2.3. Test for tannins

To each extract dissolved in distilled water, a solution of ferric chloride 1 % (w/v) was added [6]. The appearance of green color indicates the presence of tannins.

2.2.4. Test for terpenoids

To each crude extract dissolved in chloroform (2 mL), a volume of concentrated sulfuric acid (2 mL) was added [7]. The appearance of red color at the interface indicates the presence of terpenoids.

2.2.5. Test for coumarins

An amount of each crude extract was dissolved in distilled water. Then, 3 mL of NaOH 10 % (w/v) were added [8]. The appearance of yellow color indicates the presence of coumarins.

2.2.6. Test for saponins

A solution of each sample in distilled water (10 mL) was stirred vigorously until the formation of foam. A few drops of olive oil were added, then the mixture was stirred vigorously for a few minutes [7]. The formation of an emulsion confirms the presence of saponins.

2.2.7. Test for alkaloids

Crude extracts were stirred with a few drops of diluted hydrochloric acid and filtered. Then, each filtrate was used for the following tests [5]. Mayer's test: To each filtrate, one or two drops of Mayer's reagent were added [5]. The appearance of white or creamy precipitate indicates the presence of alkaloids.

Wagner's test: Similarly, few drops of Wagner's reagent were added to each filtrate [5]. The appearance of reddish brown precipitate confirms the presence of alkaloids.

2.2.8. Test for cardiac glycosides (Keller-Kiliani test)

Each extract was dissolved in 2 mL of glacial acetic acid solution containing one drop of ferric chloride solution 0.1 % (w/v). The mixture was then treated with 1 mL of concentrated sulfuric acid [7]. The formation of brown ring at the interface or greenish ring in the acetic acid layer confirms the presence of cardiac glycosides.

2.2.9. Test for amino acids and proteins

Each extract dissolved in distilled water and filtered was used for the following tests to detect the presence of proteins and amino acids [5].

To each mixture, a drop of copper sulfate solution 2 % (w/v) and 2 mL of ethanol (95 %) were added, followed by the addition of an excess of solid potassium hydroxide pellets. The appearance of pink color in the ethanol layer indicates the presence of proteins [5].

Separately, an equal volume of sodium hydroxide solution 5 % (w/v) and copper sulfate solution 1 % (w/v) were added to each extract solution previously prepared. The appearance of violet coloration indicates the presence of amino acids [9].

2.3. Total phenolic contents

Total phenolic content of each extract was determined using Folin–Ciocalteu method [10]. Briefly, 1 mL of each extract solution (1 mg/mL) was mixed with Folin–Ciocalteu's reagent (2.5 mL). After 5 min, 2 mL of sodium carbonate solution 7.5 % (w/v) was added and the reaction mixture was allowed to stand for 2 h at room temperature. Then, the optical density at 765 nm was measured. Gallic acid was used to establish the standard calibration curve (0–300 µg/mL). The results were expressed as µg of gallic acid equivalent (GAE)/mg of extract.

2.4. Total flavonoids contents

Total flavonoid content was determined using Dowd method [11]. Briefly, 1 mL of diluted solution of each extract (1 mg/mL) was mixed with 2 mL of aluminum trichloride (AlCl₃) solution 2 % (w/v) prepared in methanol. The optical density at 415 nm was measured against blank sample consisting of methanol (2 mL) and extract (1 mL) without AlCl₃. Quercetin was used to perform the standard calibration curve (0–50 µg/mL). The results were expressed as µg of quercetin equivalent (QE)/mg of extract.

2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging activity assay

The ability of each extract to scavenge free radical was assayed using the synthetic radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH), according to the method employed by [12]. Briefly, extracts were serially diluted in methanol. A solution of DPPH 0.004 % (w/v) was prepared in the same solvent. Then 2 mL of each dilution were mixed with 2 mL of DPPH solution. The mixtures were kept in the dark for 30 min and the optical density was measured at 517 nm. Butylhydroxytoluene (BHT) was used as positive control. Each test was performed in triplicate.

The antioxidant activity was calculated as follow:

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

The half maximal inhibitory concentrations (IC₅₀) were determined graphically.

2.6. Antimicrobial activity

2.6.1. Disk-diffusion method

The antimicrobial screening of each extract was determined by disk-diffusion method [13]. Five bacterial strains and one yeast strain were used: *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 3366, *Micrococcus luteus* ATCC 10240, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. Bacterial strains were grown on Luria-Bertani agar (LB) and malt extract-agar for *C. albicans*. From 24 h-old microbial culture, the microbial inoculums were prepared in sterile saline and adjusted to 0.5 McFarland scale. Afterwards, sterile agar plates were inoculated with the microbial suspension, and sterile filter paper discs (5 mm in diameter) were deposited onto the agar plate surfaces. Each disk was impregnated with 10 µL of the extract solution prepared at the concentration of 500 mg/mL in DMSO 2 % (v/v).

After incubation at 37° C during 24 h for bacteria and 30 °C during 48 h for *C. albicans*, diameters of growth inhibition zones were measured. Each experiment was performed in duplicate.

2.6.2. *Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)* The determination of MIC against *S. aureus* and *B. subtilis* was performed in 96 well-microplate using broth microdilution assay according to the protocol previously described [14], with slight modifications. Firstly, each extract was serially diluted in Mueller Hinton broth (MHB) supplemented with agar at 0.15% (w/v), used as emulsifier. The 12th well was considered as growth control. Then, 50 µL of bacterial inoculum were added to each well at the final concentration of 10⁶ CFU/mL. After inoculation, the final concentrations of the extract ranged from 16 to 0.00391 mg/mL. After incubation at 37° C for 18-20 hours, 10 µL of resazurin were added to each well as bacterial growth indicator. Then, after further incubation at 37 °C for 2 h, the bacterial growth was revealed by the change of coloration from purple to pink. The MIC value was determined as the lowest concentration that prevented the change of resazurin's color. Experiments were carried out in duplicate. The minimum bactericidal concentration (MBC) corresponds to the lowest concentration of the extract yielding negative subcultures after incubation at 37°C for 24 h. It was determined by spotting 2 µL from negative wells on LB plates. Experiments were conducted in triplicate.

2.7. Statistical analysis

Data were expressed as the mean ± standard deviation of at least three measurements. Data were analyzed using ANOVA and the correlational analysis was performed with Pearson test, by IBM SPSS Statistics 21. Differences were considered significant at the p < 0.05 level of probability.

3. Results and Discussion

3.1. Phytochemical screening

The phytochemical screening of all extracts obtained by both solvents revealed the presence of phenolic compounds, flavonoids, tannins, terpenoids, coumarins and cardiac glycosides in leaves, stems and roots of *G. alypum*. However, saponins are detected only in the methanolic extracts (Table 1). This finding corroborates with previous study on the *G. alypum* leaves extracts obtained with petroleum ether using sequential Soxhlet extraction [2]. However, coumarins were not detected in all extracts investigated by the same authors and tannins were not detected in the ethyl acetate and petroleum ether extracts [2].

Table 1. Phytochemical screening of *G. alypum* leaves, stems and roots extracts.

Compounds family	Methanol extract			Ethyl acetate extract		
	Leaf	Stem	Roots	Leaf	Stem	Roots
Phenolic	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+
Saponins	+	+	+	-	-	-
Alkaloids	-	-	-	-	-	-
Cardiac Glycosides	+	+	+	+	+	+
Amino Acids	-	-	-	-	-	-
Proteins	-	-	-	-	-	-

+: presence / - : absence

3.2 Total phenolic and total flavonoid contents

The total phenolic and total flavonoids contents of the tested extracts were presented in Table 2. As can be noted from this table, methanolic extract of roots showed the highest total phenolic content (131.48 µg GAE/mg of extract). Generally, methanolic extracts showed significantly higher phenolic contents compared to ethyl acetate

extracts ($p < 0.05$). Moreover, the roots extracts showed the highest phenolic contents compared to those of leaves and stems, indicating the richness of *G. alypum* roots in phenolic compounds. As regards to the total flavonoids contents, the methanolic extract of stems showed the highest total flavonoids content ($39.14 \mu\text{g QE/mg}$ of extract), which was significantly higher than those of the other extracts ($p < 0.05$).

It has been reported that *G. alypum* was among the plants showing the highest total phenolic contents [15]. However, the large discrepancy between its phenolic and flavonoid contents could be explained by its predominance by hydroxycinnamic and hydrobenzoic derivatives [15]. Moreover, the methanol and aqueous extracts obtained from *G. alypum* leaves showed the highest flavonoids contents compared to ethyl acetate and petroleum ether extracts [2].

A previous published phytochemical study on the aerial part of *G. alypum* led to the isolation of three new phenolic compounds (6-hydroxyluteolin 7-O-laminaribioside, eriodictyol 7-O-sophoroside, and 6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl)ethyl]- β -D-glucopyranoside), three phenylethanoid glycosides (acteoside, isoacteoside and forsythiaside) and two flavonoid glycosides (6-hydroxyluteolin 7-O- β -D-glucopyranoside and luteolin 7-O-sophoroside) [16]. Moreover, the same authors have isolated iridoid glucosides from the aerial part of *G. alypum*, in which they found a new chlorinated iridoid (Globularioside) [17].

Table 2. Total phenolic and total flavonoids contents of *G. alypum* extracts.

Extract		Total phenolic content ($\mu\text{g GAE/mg}$ of extract)	Total flavonoid content ($\mu\text{g QE/mg}$ of extract)
Methanol	Leaves	67.55 ± 2.26^a	25.90 ± 0.18^c
	Stems	94.83 ± 1.27^b	39.14 ± 0.48^f
	Roots	131.48 ± 8.5^c	14.48 ± 0.22^g
Ethyl acetate	Leaves	50.99 ± 1.39^d	8.96 ± 0.42^h
	Stems	51.42 ± 1.86^d	18.75 ± 0.42^i
	Roots	83.70 ± 5.79^b	13.85 ± 0.16^g

Means in same row that do not share a letter are significantly different ($p < 0.05$).

3.3. DPPH radical scavenging activity

Several methods based on different kinds of defense systems have been developed to measure the antioxidant efficacy. In this study, the DPPH radical scavenging capacity of the *G. alypum* extracts has been studied. The half maximal inhibitory concentrations (IC_{50}) of all studied extracts and the BHT were determined graphically at the concentration-dependent part of each plot. Results were presented in Figure 1.

All *G. alypum* extracts were able to reduce the stable DPPH radical, with IC_{50} values for the methanol and ethyl acetate extracts ranged from 10.01 to 48.28 $\mu\text{g/mL}$ (Table 3). The methanolic extract of roots showed the highest antioxidant activity ($IC_{50} = 10.01 \mu\text{g/mL}$) followed by the ethyl acetate extract of roots ($IC_{50} = 18.85 \mu\text{g/mL}$). Moreover, the antioxidant activity of roots extract was significantly higher than those obtained from leaves and stems by comparing the extracts of each plant's part ($p < 0.05$). This finding is valid for either methanol and ethyl acetate extracts. Likewise, the methanolic extracts always showed the highest antioxidant capacity than those of the ethyl acetate extracts for each plant part (Figure 1). This finding corroborates with previously published work [2], which showed that aqueous and methanol extracts of *G. alypum* exhibited the highest antioxidant effect compared to the ethyl acetate and petroleum ether extracts. Furthermore, it has been showed that *G. alypum* exhibited remarkable antioxidant effect tested using ABTS assay [15].

A linear relationship was found between the total phenolic contents and the antioxidant activity expressed with IC_{50} values ($\rho = -0.87$, R-square = 0.75, $p < 0.05$). Similar findings had been reported in numerous studies [15,18,19]. This low value of R-square could be explained by the fact that the total phenolic content does not include all the antioxidants present in the extract and also to the interaction between the antioxidants in the mixture, which makes the antioxidant activity dependent not only on the concentration of the phenolic compounds, but also on their structures and their interactions [15]. In our study the contribution of the non-

flavonoids phenolic compounds can be unveiled by comparing the IC_{50} of both roots extracts showing the same flavonoid contents, where methanol extract of roots with higher total phenolic contents exhibited the higher antioxidant effect. It has been found that phenolic compounds (6-hydroxyluteolin 7-O-laminaribioside, eriodictyol 7-O-sophoroside, and 6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl) ethyl]- β -D-glucopyranoside) and phenylethanoid glycosides (acteoside, isoacteoside and forsythiaside) isolated from *G. alypum* exhibited strong DPPH scavenging ability more efficient than that of synthetic BHT [16]. Furthermore, it has been also reported that phenylethanoids showed more efficient antioxidant activity than iridoids and syringin [17,20].

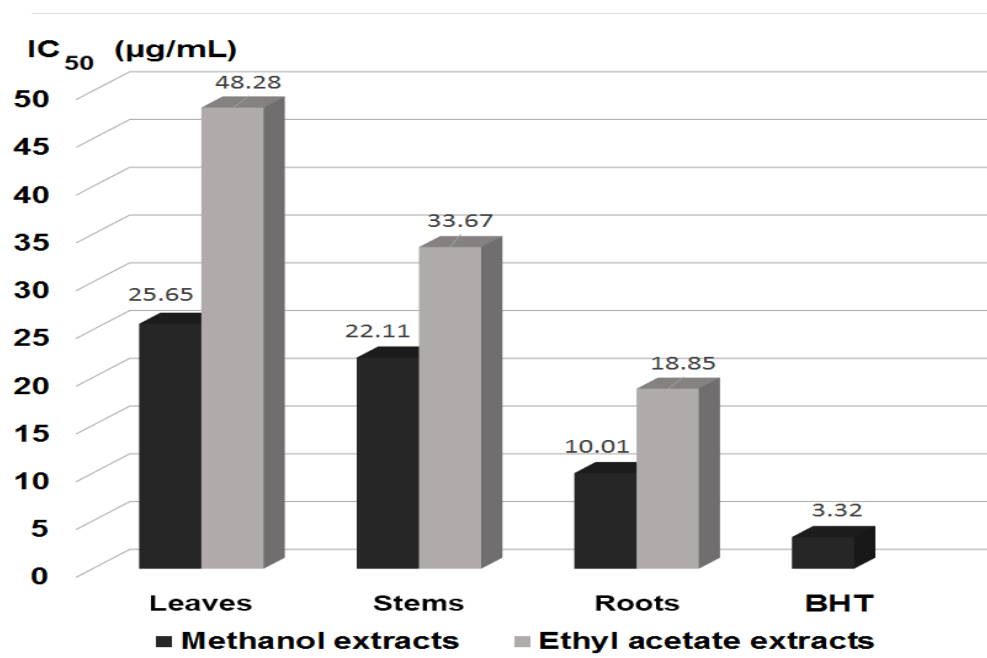


Figure 1. Antioxidant activity of *G. alypum* extract expressed by IC_{50} values.

Nevertheless, no correlation has been found between total flavonoid contents of the studied extracts and their antioxidant effects, same finding has been previously reported [15]. In fact, it is known that the flavonoids exhibited important antioxidant effect, which is evident in this study by comparing the IC_{50} of the ethyl acetate extracts of leaves and stems showing the same total phenolic content but different total flavonoids contents, where stems extract was more efficient. Moreover, it has been reported that 6-hydroxyluteolin 7-O- β -D-glucopyranoside and luteolin 7-O-sophoroside, two flavonoids glycosides of the aerial part of *G. alypum*, exhibited strong antioxidant activity compared to BHT [16].

3.4. Antimicrobial activity

Table 3 shows the diameters of growth inhibition zones exhibited by methanol and ethyl acetate extracts of *G. alypum* against several bacteria and one yeast. As can be noted from this table, all extracts were not active against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and *C. albicans*. However, they were able to inhibit the growth of *S. aureus* and *B. subtilis*. Moreover, only ethyl acetate extracts showed inhibitory effect against *M. luteus*. The negative control (DMSO 2%) had no inhibitory effect against all the tested microbial strains.

The MIC and MBC values were investigated to quantitatively determine the antibacterial effect of *G. alypum* extracts against *S. aureus* and *B. subtilis*. As can be seen from table 4, all extracts depicted antibacterial activity with some differences depending upon the plant's part used and the target microbial strain. Particularly, ethyl acetate extract of roots was the most active with a MIC value of 0.5 mg/mL against both bacterial strains, followed by stems' and leaves' extracts with MIC values of 2 and 4 mg/mL respectively against both strains.

The MBC values of these extracts were equal to the corresponding MIC values indicating that extracts of *G. alypum* act by a bactericidal effect. Moreover, both bacterial strains showed the same susceptibility against *G. alypum* extracts, except for the methanolic ones, where *B. subtilis* was more susceptible to roots and stems extracts respectively. Conversely, *S. aureus* was more susceptible to the methanolic extract of leaves than *B. subtilis*. It has been reported that *G. alypum* extracts exhibited remarkable antibacterial activity particularly against Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *B. subtilis* and *B. cereus*) with MIC values ranging from 2 to 16 mg/mL depending upon the solvent used for the extraction [21]. However, poor antimicrobial activity of *G. alypum* leaves aqueous extract, which was active only against *Agrobacterium tumefaciens*, has been reported [22]. For the first time, the investigation of the antimicrobial activity of *G. alypum* roots extracts showed that they are the most active compared to those of leaves and stems.

Table 3. Growth inhibition zones (mm) exhibited by *G. alypum* extracts against microbial strains.

Microorganism test	Methanol extract			Ethyl acetate extract		
	Leaves	Stems	Roots	Leaves	Stems	Roots
<i>S. aureus</i> ATCC 29213	6.67±0.33	8.33±0.67	9.00±0.00	7.00±0.00	10.83±0.17	12.17±0.17
<i>B. subtilis</i> ATCC 3366	10.67±0.33	12.00±0.29	13.67±0.33	10.67±0.33	16.66±0.33	17.67±0.33
<i>M. luteus</i> ATCC 10240	-	-	-	7.00±0.00	10.50±0.10	10.00±0.00
<i>P. aeruginosa</i> ATCC 27853	-	-	-	-	-	-
<i>E.coli</i> ATCC 25922	-	-	-	-	-	-
<i>C. albicans</i> ATCC 10231	-	-	-	-	-	-

Diameter of paper disk included (6 mm)

Table 4. MIC/MBC values (mg/mL) exhibited by *G. alypum* extracts against *S. aureus* and *B. subtilis*.

Microorganisms test	Methanol extract			Ethyl acetate extract		
	Leaves	Stems	Roots	Leaves	Stems	Roots
<i>S. aureus</i> ATCC 29213	2/2	>16/>16	>16/>16	4/4	2/2	0.5/0.5
<i>B. subtilis</i> ATCC 3366	4/4	4/4	2/2	4/4	2/2	0.5/0.5

Conclusions

Overall, this study revealed promising antioxidant and antimicrobial activities of the *G. alypum* extracts, notably for those obtained from roots. The plant parts can be classified according to their antioxidant capacity in the following descending order: root, stems and leaves. Moreover, linear relationship has been found between the antioxidant activity and the phenolic contents of the extracts. Thus, *G. alypum* extracts (especially roots extract) can be a source of new potential antioxidant and antimicrobial agents.

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