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RESEARCH ARTICLE

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Phenolic composition and multi-target bioactivities of *Globularia alypum* extracts across seasons

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Abstract

Background: This study investigates the influence of seasonal variation on the phytochemical profile and biofunctional activities of ethanolic leaf extracts from *Globularia alypum*. Leaves collected across four seasons were extracted with a hydroethanolic solvent (80:20, v/v) and analyzed by LC-ESI-MS/MS. Total phenolic and flavonoid contents were determined using Folin–Ciocalteu and aluminum chloride assays, while antioxidant capacity was evaluated using five complementary methods (DPPH, ABTS, ADS, FRAP, and SNPAC). Anti-inflammatory, analgesic, enzyme-inhibitory, antimicrobial, photoprotective, and acute oral toxicity (OECD 423) activities were also assessed. Trans-cinnamic acid and trans-ferulic acid were identified as major constituents across all seasons.

Results: Spring and winter extracts showed the highest phenolic and flavonoid levels and exhibited stronger antioxidant activity, particularly in ABTS, ADS, and SNPAC assays, together with significant anti-inflammatory and antinociceptive effects. The spring extract produced 76.81% inhibition of nociceptive behavior at 500 mg/kg and 74.90% inhibition of carrageenan-induced edema, comparable to reference drugs. All extracts displayed high in vitro photoprotective potential (SPF 31.25–34.90), while antimicrobial activity was negligible. Notably, the summer extract showed strong urease-inhibitory activity ($IC_{50} = 15.25$ µg/mL), comparable to thiourea ($IC_{50} = 11.57$ µg/mL), despite having the lowest phenolic content. No acute toxicity was observed up to 2000 mg/kg.

Conclusion: These findings demonstrate that harvest season significantly influences the phytochemical composition and biological properties of *G. alypum*, highlighting its potential as a source of bioactive compounds for further pharmacological investigation.

KEYWORDS

acute toxicity, anti-inflammatory, antinociceptive, enzyme inhibition, phenolic content

[Correction added on 28 May 2026, after first online publication: The spell error in the family name of the co-author "Khalil Guelift" has been corrected.]

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INTRODUCTION

Despite the significant advances achieved in the pharmaceutical industry, which have enabled modern medicine to combat numerous diseases once considered fatal, medicinal plants and natural remedies continue to hold a prominent position in both traditional practices and contemporary scientific research.[1] Historically, these plants served as the primary therapeutic agents across various cultures, and to this day they attract increasing attention due to their rich ethnobotanical heritage and content of bioactive natural compounds with diverse pharmacological properties.[2,3] This renewed interest has led to a growing body of pharmacological and biochemical studies investigating their therapeutic potential.[4]

Among the most important bioactive constituents that contribute to the therapeutic value of medicinal plants are phenolic compounds. These secondary metabolites play key physiological roles in protecting plants against environmental stressors such as ultraviolet radiation and microbial pathogens.[5] Phenolics are known for their ability to counteract oxidative stress, a major factor in the pathogenesis of various chronic diseases, including cancer,[6] cardiovascular disorders,[7] and chronic inflammation.[8] Recent studies indicate that phenolic-rich plant extracts exhibit diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer effects, highlighting their potential as promising natural sources for the development of novel therapeutic agents.[9]

However, the production of phenolic compounds in plants is not constant; it is influenced by multiple complex environmental and physiological factors.[10] These include temperature, sunlight intensity, water availability, soil composition, and biotic stresses such as microbial infections or interspecific competition. In addition, the phenological stage of the plant plays a crucial role in determining the type and quantity of phenolic compounds synthesized. Several studies have demonstrated that certain phenolics accumulate in greater quantities during early growth stages, whereas others increase during maturation or under environmental stress. These dynamic changes are directly reflected in the biological activity of plant extracts, highlighting the importance of understanding seasonal and ecological influences on chemical composition to optimize their use in therapeutic applications.[11,12]

In this context, *Globularia alypum* L. represents an ideal model among Mediterranean medicinal plants,[13,14] as its phytochemical profile and biological properties are likely affected by seasonal and environmental factors. This species is widely used in traditional medicine across North Africa, particularly in Algeria, where it is locally known as "Tasselgha." Belonging to the family Plantaginaceae (formerly Globulariaceae),[15] *G. alypum* is a perennial shrub traditionally employed for the treatment of a wide range of ailments, including hypoglycemia, rheumatic conditions, gastric disorders, and infectious diseases.[6] Its leaves are commonly used to manage diabetes, kidney diseases, and cardiovascular conditions.[16]

Although previous studies have reported promising biological activities of *G. alypum* extracts,[17–21] data on the impact of seasonal variation on its chemical composition and bioactivity remain scarce, particularly under Algerian ecological conditions, where the plant is naturally abundant. Understanding how seasonal changes influence the production of total phenolics and flavonoids is essential, given the central role these compounds play in determining the biological efficacy of plant extracts.

Accordingly, the aim of this study is to evaluate the effect of seasonal variation on the total phenolic content (TPC) and flavonoid content (TFC) of *G. alypum* extracts, and to investigate the relationship between these contents and multiple biological activities, including antioxidant potential, inhibition of α-amylase and urease enzymes, antimicrobial efficacy, anti-inflammatory activity, and UV protection capacity. Particular emphasis was placed on the spring extract, through assessment of its antinociceptive and anti-inflammatory effects in vivo, along with acute toxicity evaluation. This integrated approach represents a key step toward deepening the scientific understanding of the phytochemical and biological properties of *G. alypum* and paves the way for its valorization as an effective and safe natural therapeutic alternative.

MATERIALS AND METHODS

Chemicals and reagents

All solvents used in this study were of analytical grade and sourced from Honeywell. The following reagents were utilized: 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, aluminum chloride hexahydrate ($AlCl_3{\cdot}6H_2O$), sodium carbonate (Na_2CO_3), ferrozine, potassium ferricyanide, ammonium molybdate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$), sodium molybdate (Na_2MoO_4), and sodium acetate ($C_2H_3NaO_2$), all purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Reference compounds were obtained from EXTRASYNTHESE (Genay Cedex, France), including quercetin ($C_{15}H_{10}O_7$), butylated hydroxytoluene (BHT, $C_{15}H_{24}O$), gallic acid ($C_{11}H_{16}O_2$), butylated hydroxyanisole (BHA, $C_{11}H_{16}O_2$) ascorbic acid ($C_6H_8O_6$), EDTA, and α-tocopherol.

Sample collection and preparation of extracts

During the 2022–2023 period, leaves of *G. alypum* (Figure 1) were collected at the midpoint of each season—autumn, winter, spring, and summer. Botanical identification was confirmed by the Department of Chemistry at the University of El Oued, Algeria (Figure 1). A reference specimen (voucher number Glo-alyp-002-1-2022) was archived in the Herbarium of the Laboratory of Biomass, ENS-Kouba, Algiers. The plant material was harvested from the El Kef Lakhdar site in Medea province, situated at 1164 m elevation (coordinates: 35°57′33″ N, 3°12′16″ E). Only mature, healthy plants growing under comparable environmental conditions were selected. Upon collection, samples were placed in clean, sealed containers to avoid any degradation or contamination. They were then thoroughly washed with

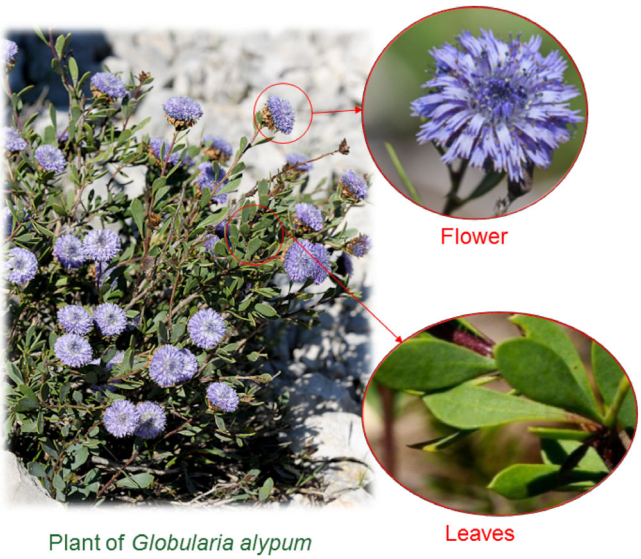


FIGURE 1 Image of *Globularia alypum* plant with flowers and leaves.

deionized water to remove any adhering impurities, spread out on clean trays, and air-dried in the shade at 20–25°C for 15 days to prevent photodegradation of sensitive compounds. Once dried, the material was ground into a fine powder (<200 µm) using either an agate mortar and pestle or an electric grinder. The powdered samples were stored in airtight containers, kept in a cool and dry environment until further use.

Plant samples collected across different seasons were thoroughly washed and air-dried at room temperature in a well-ventilated environment for approximately 3 weeks. Upon complete drying, the plant materials were ground into a fine powder, transferred into labeled paper bags, and stored in sealed, opaque containers at ambient temperature to prevent degradation. For the extraction procedure, 15 grams of the powdered leaves of *G. alypum* were macerated in 500 mL of an ethanol–distilled water mixture (80:20, v/v) at room temperature for 24 h under continuous mechanical stirring. The resulting mixture was filtered, and the maceration step was repeated twice to ensure exhaustive extraction. All filtrates were combined and concentrated under reduced pressure using a rotary evaporator set at 40°C. The crude extracts obtained were stored in amber glass bottles at 4°C until further analysis. The extraction yield (%) was calculated using the following formula:

$$\%\text{Yield} = \frac{(\text{Weight of dried crude extracts(g)}/(\text{g}))}{\text{Weight of dried plant sample taken(g)}} \times 100. \quad (1)$$

Extraction of phenolic compounds

Extraction was performed using 80% ethanol as a solvent. Ten grams of powdered plant material were macerated in 100 mL of ethanol with continuous stirring for 48 h at room temperature. The mixture was then filtered, and the solvent was evaporated under reduced pressure to obtain the crude extract.

Determination of total bioactive compounds

Determination of TPC

The total phenolic content was determined using the Folin–Ciocalteu method described in reference 22 with a microplate technique. The absorbance was measured using a Thermo Scientific Multiskan™ FC microplate photometer (Thermo Fisher Scientific, USA) set at 765 nm. The equipment was operated at room temperature with 96-well polystyrene microplates. For all spectrophotometric measurements in this study (TPC, TFC, antioxidant assays), the same microplate reader was used. Briefly, 20 µL of extract was mixed with 100 µL of diluted Folin–Ciocalteu reagent (1:10) and 75 µL of 7.5% sodium carbonate. After incubation in the dark for 2 h, absorbance was measured at 765 nm. Gallic acid was used as a standard, and the results were expressed as µg gallic acid equivalents (GAEs) per mg of extract.

Determination of TFC

TFC was measured by a colorimetric method as described in reference 23. A mixture of 50 µL of extract, 130 µL of methanol, 10 µL of 1 M potassium acetate, and 10 µL of 10% aluminum nitrate was incubated for 40 min at room temperature in the dark. Absorbance was measured at 415 nm. Quercetin was used as a reference standard, and results were expressed as µg quercetin equivalents (QE) per mg of extract.

LC-ESI-MS/MS analysis

Seasonal variations in the phenolic composition of *G. alypum* extracts collected during spring, summer, autumn, and winter were investigated using LC-ESI-MS/MS technique. A total of 34 phenolic standards were used for identification and quantification. The analysis was conducted on a HPLC (High Performance Liquid Chromatography)system coupled with a mass spectrometer, with ionization carried out via ESI. Chromatographic separation was achieved using a Poroshell 120 EC-C18 column (100 mm × 4.6 mm, 2.7 µm). For sample preparation, 50 mg of each extract was weighed into an Eppendorf tube and dissolved in 1 mL of a solvent mixture consisting of acetonitrile, methanol, and water (1:1:1, v/v/v). The solution was vortexed to ensure complete dissolution. If undissolved residues remained, the mixture was sonicated in an ultrasonic bath. Next, 0.8 mL of hexane was added for phase separation, followed by centrifugation at 7000 rpm for 5 min. The lower polar phase was collected, diluted at a 1:4 ratio with the same solvent mixture, and filtered through a 0.25µm syringe filter prior to injection. The prepared samples were

injected in a volume of 4 µL using full-loop injection mode. Chromatographic analysis was performed at a flow rate of 0.4 mL/min under gradient elution. The mobile phase consisted of ultrapure water and methanol, both containing 0.1% formic acid and 5 mM ammonium. The gradient program initiated with 15.12% methanol for 1 min, ramped to 50% over 30 min, increased to 90% over the subsequent 30 min, and then returned to 10% at 32 min to allow for column re-equilibration.

Evaluation of antioxidant activity

DPPH radical scavenging assay

The DPPH free radical scavenging activity was evaluated according to the method described in reference 24, using a 0.1 mM DPPH solution in methanol. A total of 160 µL of the DPPH solution was mixed with 40 µL of extract and incubated in the dark for 30 min. Absorbance was read at 517 nm, and IC_{50} values were calculated to express antioxidant activity.

ABTS radical scavenging assay

The ABTS radical scavenging capacity was assessed following the method described in reference 25. $ABTS^{+}$ radicals were generated by reacting ABTS with potassium persulfate and incubating the mixture in the dark for 16 h. Then, 40 µL of the extract was added to 160 µL of the $ABTS^{+}$ solution, and absorbance was measured at 734 nm. $IC_5$50 values were calculated.

Ferric reducing antioxidant power (FRAP)

The reducing power was measured using the method described in reference 26. The extract was mixed with potassium ferricyanide and phosphate buffer and incubated at 50°C for 20 min. Trichloroacetic acid was then added, and the absorbance was measured at 700 nm. $A_{0.5}$ values were determined.

Superoxide radical scavenging (ADS) assay

The ability of the extracts to neutralize superoxide radicals was evaluated using the alkaline DMSO method as outlined by Kunchandy and Rao.[27] The reaction mixture included 30 µL of nitro blue tetrazolium (NBT, 1 mg/mL), 40 µL of the sample extract, and 130 µL of alkaline DMSO composed of dimethyl sulfoxide (DMSO), 5 mM sodium hydroxide, and distilled water. Absorbance was recorded at 560 nm, and the IC_{50} values were determined to quantify the superoxide scavenging potential of the extracts.

Silver nanoparticle antioxidant capacity (SNPAC) assay

To further assess antioxidant capacity, the SNPAC assay was performed following the procedure described by Özyürek et al. The assay was performed following the procedure described by Özyürek et al.[28] A colloidal silver nanoparticle (SNP) solution was prepared by heating 50 mL of 1 mM silver nitrate ($AgNO_3$) for 10 min, followed by the gradual addition of 5 mL of 1% sodium tri-citrate until a pale-yellow color developed, indicating nanoparticle formation. For the assay, 130 µL of the AgNP solution and 50 µL of distilled water were combined with 20 µL of the extract. The mixture was incubated at 25°C for 30 min, after which the absorbance was measured at 423 nm. Antioxidant efficacy was expressed as the concentration required to reach an absorbance of 0.5 ($A_{0.5}$).

Enzyme inhibitory assays

α-amylase inhibition

The inhibitory activity against α-amylase was evaluated using the colorimetric iodine-potassium iodide method as described in reference 29. The extract was mixed with α-amylase and starch solution. The reaction was stopped using hydrochloric acid, and iodine reagent was added. Absorbance was measured at 630 nm.

Urease inhibition

Urease inhibition was assessed using the phenol–hypochlorite method as described in reference 30. The extract was mixed with a urea solution and urease enzyme, followed by the addition of phenol and hypochlorite reagents after incubation. Absorbance was measured at 630 nm, and IC_{50} values were calculated.

Determination of sun protection factor

The sun protection factor (SPF) was determined by UV spectrophotometry, following the method in reference 31. The absorbance of an ethanolic solution of the extract (2 mg/mL) was recorded in the UVB range (290–320 nm), and the SPF value was calculated using the standard Mansur equation.

$$\mathrm{SPF} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}(\lambda) \times I(\lambda) \times \mathrm{Abs}(\lambda), \quad (2)$$

where CF is the correction factor (10), EE (λ) is the erythemal effect spectrum, $I(\lambda)$ is the solar intensity spectrum, and Abs(λ) is the sample absorbance at each wavelength.

Antimicrobial activity

The antimicrobial potential was evaluated using the disc diffusion method on Mueller–Hinton agar, as described in reference 12. Filter paper discs loaded with 35 µg of extract were placed on agar plates inoculated with standard microbial strains. Plates were incubated at 37°C for 48 h, and inhibition zone diameters were measured. The antimicrobial activity was evaluated against a panel of standard microbial strains, including *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and the yeast *Candida albicans* (ATCC 10231). Standard antibiotics—fosfomycin, carbenicillin, erythromycin, and cephalexin—were used as positive controls to validate the assay.

Anti-inflammatory activity

In vitro anti-inflammatory activity (bovine serum albumin denaturation assay)

The anti-inflammatory activity in vitro was assessed by inhibiting heat-induced denaturation of bovine serum albumin (BSA), following the method described in reference 32. The extract was mixed with a BSA solution, incubated at 37°C, and then heated at 72°C. After cooling, absorbance was recorded at 660 nm.

In vivo anti-inflammatory activity

All animal experiments performed in this study were conducted following approval from the Ethical Committee of Animal Experimentation (CEEA) of the University of Sciences and Technology Houari Boumediene (USTHB), Algeria (Approval No. CEEA-USTHB-08-2023/11118). The procedures complied with institutional regulations and internationally accepted guidelines for the care and use of laboratory animals.

In this study, the in vivo anti-inflammatory effects were investigated using the carrageenan-induced paw edema model in Swiss albino mice weighing between 20 and 25 g, following standard protocols according to reference 33. The animals were divided into three groups of five: a control group receiving 0.5 mL of physiological saline, a standard group treated with 10 mg/kg diclofenac sodium, and a treatment group administered 0.5 mL of 10% *G. alypum* extract. Thirty minutes after these treatments, each mouse received a subplantar injection of 0.025 mL of a 1% carrageenan solution into the left hind paw. After 4 h, the mice were sacrificed, the paw weights were recorded, and the degree of edema inhibition was calculated as a percentage relative to the control. The animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection before being sacrificed by cervical dislocation.

Evaluation of analgesic activity

The analgesic potential of the plant extract was evaluated using the acetic acid-induced writhing assay in mice, as described in reference 34, a validated model for assessing peripheral nociceptive responses. Experimental animals were randomly assigned to different groups and received either the test extract, paracetamol (as a reference drug), or normal saline (control group). After administration, acetic acid was intraperitoneally injected to induce a pain response characterized by abdominal constrictions (writhes), which were recorded over a fixed observation period of 20–30 min. The analgesic effect was determined by calculating the percentage inhibition of writhing using the formula: Protection % = ((Mean number of writhes in control group − Mean number of writhes in treated group)/Mean number of writhes in control group) × 100. A higher percentage of inhibition reflects a greater analgesic effect of the tested substance.

Acute toxicity evaluation

The acute oral toxicity of the ethanolic extract was evaluated in compliance with OECD Guideline 423,[35] to determine its safety profile. Groups of fasted female mice ($n = 5$ per group) received a single oral administration of the extract at graded dose levels (275, 1562, and up to 2000 mg/kg). Following treatment, the animals were closely observed during the initial hours and subsequently monitored daily over a 14-day period for any signs of toxicity, including behavioral changes, clinical symptoms, or mortality. This assessment facilitated the estimation of the median lethal dose (LD_{50}) and provided preliminary toxicological data supporting the extract's potential for further pharmacological investigation.

Statistical analysis

All results were expressed as the mean ± standard deviation (SD) based on three independent, parallel experiments. Statistical analyses were performed using STATISTICA software version 10.0 (StatSoft Inc., USA). One-way analysis of variance (ANOVA) was conducted to assess overall differences among groups, followed by Tukey's Honest Significant Difference (HSD) post hoc test to determine statistically significant differences between individual group means. A p-value of less than 0.05 was considered indicative of statistical significance.

RESULTS AND DISCUSSION

TPC and TFC estimation

The total phenolic and TFC of the four seasonal extracts of *G. alypum* were determined using the previously described methods. The results, summarized in the Table 1, demonstrate notable variations in the

TABLE 1 Yield and total phenol and flavonoid amounts of *G. alypum* extracts.

Specification		Yield of extraction (%)	TPC (μg GAE/mg)	TFC (μg QE/mg)
Ethanolic extract	CSpring	60 ± 2.3	380.07 ± 2.06	217.11 ± 0.47
	CWinter	55.5 ± 2.3	380.66 ± 2.20	225.88 ± 2.12
	CAutumn	3.3 ± 53.4	264.98 ± 3.48	170.11 ± 4.24
	Csummer	5.7 ± 3.4	200.96 ± 2.66	138.11 ± 2.12

Abbreviations: TFC, total flavonoid content; TPC, total phenolic content.

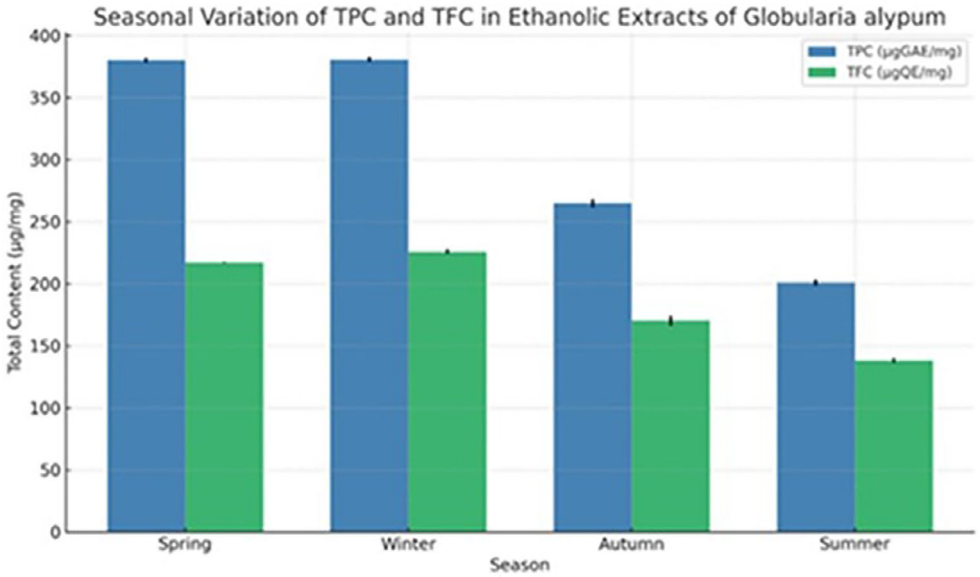


FIGURE 2 Seasonal variation of total phenolic content (TPC) and total flavonoid content (TFC) in ethanolic extracts of *Globularia alypum*.

levels of phenolic and flavonoid compounds depending on the season in which the plant samples were collected. These findings highlight the influence of seasonal dynamics on the phytochemical composition of the plant (Figure 2).

The analysis of ethanolic extracts from *G. alypum* leaves across different seasons revealed significant variations in TPC and TFC, reflecting the influence of seasonal factors on the plant's secondary metabolism. TPC ranged from 200.96 to 380.66 μg GAEs/mg of extract, with the highest values recorded in winter (380.66 ± 2.20 μg GAE/mg), followed closely by spring (380.07 ± 2.06 μg GAE/mg). In contrast, a notable decline was observed during autumn (264.98 ± 3.48 μg GAE/mg) and summer (200.96 ± 2.66 μg GAE/mg).

A similar pattern was observed for TFC, with values ranging from 138.11 to 225.88 μg quercetin equivalents (QE)/mg of extract. The highest concentration was found in winter (225.88 ± 2.12 μg QE/mg), followed by spring (217.11 ± 0.47 μg QE/mg), autumn (170.11 ± 4.24 μg QE/mg), and the lowest in summer (138.11 ± 2.12 μg QE/mg). These results highlight the role of environmental and seasonal factors in triggering secondary metabolic pathways. The elevated accumulation of phenolic and flavonoid compounds during winter is likely related to defense mechanisms activated in response to climatic stressors, such as low temperatures and reduced solar radiation.

Compared to previous studies, our results substantially exceed the values reported by Nefzi et al.,[36] who indicated a total phenolic content of 25.33 mg GAE/g dry weight (DW) and a flavonoid content below 6.52 mg catechin equivalents (CE)/g DW in ethanolic extracts of *G. alypum* leaves. Similarly, our findings surpassed those reported by Asraoui et al.[37] for ethyl acetate and chloroform extracts, which contained markedly lower amounts of phenolics and flavonoids (56.5 ± 0.61 μg GAE/mg and 30.2 ± 0.55 μg CE/mg, respectively).

On the other hand, our results partially align with the findings of Massinissa et al.,[4] who reported a relatively high TPC (240 ± 6.20 mg GAE/g DW) in 80% methanol extracts. However, our study recorded even higher values during specific seasons, particularly in winter, further emphasizing the significant influence of climatic conditions and harvest period on the phytochemical composition of plant extracts. Such variability supports earlier findings by studies such as those by,[8,38] which pointed out the influence of factors like geographical location, solvent type, and extraction method.

Moreover, the results reported by Ouffai et al.[39] on *G. alypum* further emphasize the importance of solvent selection, showing that ethanolic extracts exhibited higher levels of phenolics and flavonoids compared to aqueous and butanolic extracts.

In light of these comparisons, it is evident that the phenolic and flavonoid contents in *G. alypum* extracts are highly variable and influenced by multiple environmental and technical factors. These findings underscore the importance of considering seasonal variation when determining the optimal harvest time to maximize the plant's biological and medicinal value. Additionally, there is a clear need for further research aimed at optimizing and standardizing extraction protocols to ensure the quality and consistency of phytopharmaceutical preparations derived from *G. alypum*, thereby enhancing its potential for pharmaceutical and industrial applications.

Chemical composition using LC-ESI-MS/MS

The phenolic composition of *G. alypum* extracts collected across the four seasons spring, summer, autumn, and winter was investigated using LC-ESI-MS/MS. A set of 34 phenolic standards was employed for the identification of target compounds. Seasonal corresponding compound data, including concentrations and retention times, are presented in Table 2 and Figure S1.

Table 2 presents the compounds identified in the seasonal extracts of *G. alypum*. Eleven compounds were detected in both the

TABLE 2 Seasonal chemical composition of *Globularia alypum* extracts.

No.	Compounds	Rt (min)	Autumn (μg/g) (A)	Winter (μg/g) (B)	Spring (μg/g) (C)	Summer (μg/g) (D)
1.	Gallic acid	2.47	-	-	-	-
2.	Epigallocatechin	2.52	-	-	-	-
3.	Chlorogenic acid	2.53	-	-	-	-
4.	Catechin	2.73	-	-	-	-
5.	Gentisic acid	2.71	-	-	10.48	-
6.	Caffeic acid	3.22	0.82	3.97	39.04	-
7.	Syringic acid	3.31	3.05	2.38	4.26	2.17
8.	Vanillic acid	3.78	-	-	-	-
9.	Rutin	2.95	-	-	-	-
10.	Isoquercitrin	4.80	-	-	-	-
11.	Polydatin	4.44	-	-	-	-
12.	4-hydroxybenzaldehyde	4.55	-	-	-	-
13.	*p*-coumaric acid	4.45	1.52	4.79	83.28	-
14.	Sinapic acid	5.10	1.92	3.33	2.69	0.85
15.	Vanillin	5.20	9.83	6.82	7.30	7.20
16.	*Trans*-ferulic acid	4.93	32.68	76.76	392.17	24.63
17.	Taxifolin	5.11	-	-	-	-
18.	Salicylic acid	7.21	2.87	3.82	3.95	-
19.	*o*-coumaric acid	8.22	-	-	-	-
20.	Baicalin	7.95	-	-	-	-
21.	Protocatechuic ethyl ester	8.48	-	-	-	-
22.	Protocatechuic acid	8.50	-	-	-	-
23.	Kaempferol	9.80	52.59	35.81	27.86	5.81
24.	Resveratrol	10.51	-	-	-	-
25.	*Trans*-cinnamic acid	11.17	961.52	906.22	530.61	417.44
26.	Naringenin	11.45	-	-	-	-
27.	Morin	11.84	-	-	-	-
28.	Quercetin	11.84	-	-	-	-
29.	7-hydroxyflavone	11.97	-	-	-	-
30.	Chrysin	13.58	-	-	-	-
31.	Luteolin	13.63	0.44	-	-	0.93
32.	Biochanin A	13.75	1.37	1.24	1.10	2.16
33.	5-hydroxyflavone	15.27	-	-	-	-
34.	Diosgenin	20.28	-	-	-	-

Abbreviations: -, not detected; Rt, retention time.

autumn and winter extracts, 10 in the spring extract, and eight in the summer extract.

The results indicate a notable diversity of phenolic compounds in the seasonal extracts of *G. alypum*, with phenolic acids and flavonoids being the predominant classes. Among these, *trans*-cinnamic acid and *trans*-ferulic acid were consistently identified as the major constituents across all seasons, although their concentrations varied significantly depending on the season. In the autumn extract, *trans*-cinnamic acid (961.52 μg/g) was the most abundant compound, followed by kaempferol (52.59 μg/g) and *trans*-ferulic acid (32.68 μg/g). The winter extract also exhibited high levels of *trans*-cinnamic acid (906.22 μg/g), *trans*-ferulic acid (76.76 μg/g), and kaempferol (35.81 μg/g), along with moderate amounts of vanillin (6.82 μg/g) and sinapic acid (3.33 μg/g).

The spring extract showed the highest overall phenolic content, particularly *trans*-cinnamic acid (530.61 μg/g), *trans*-ferulic acid (392.17 μg/g), and *p*-coumaric acid (83.28 μg/g). Spring also contained elevated levels of caffeic acid (39.04 μg/g), kaempferol (27.86 μg/g), and vanillin (7.30 μg/g), suggesting enhanced phenolic biosynthesis during this active growth period. In contrast, the summer extract was the least rich in phenolics, though it still contained notable amounts of *trans*-cinnamic acid (417.44 μg/g), *trans*-ferulic acid (24.63 μg/g), and kaempferol (5.81 μg/g), along with biochanin A (2.16 μg/g) and vanillin (7.20 μg/g).

These findings demonstrate the consistent presence of key phenolic compounds, especially *trans*-cinnamic acid, *trans*-ferulic acid, kaempferol, and vanillin, across all seasonal extracts, with quantitative variations likely influenced by environmental factors such as temperature, light exposure, and the plant's developmental stage. Among all extracts, the spring extract stands out for its richness in bioactive phenolics, making it potentially the most suitable season for harvesting *G. alypum* for therapeutic or nutraceutical applications.

Previous studies have characterized the chemical profile of *G. alypum* extracts, revealing a high content of secondary metabolites. It has been reported that *G. alypum* diethyl ether and ethyl acetate extracts contain phenolic acids and flavonoids. Among the identified compounds were gallic acid, *p*-coumaric acid, rutin, naringenin, and quercetin.[36] A comparison with our extracts indicated the presence of *p*-coumaric acid as one of the main compounds in the spring extract.

It also has been reported the detection of 19 phenolic compounds, including *trans*-cinnamic acid and luteolin-7-*O*-glucoside.[37] In comparison with our seasonal extracts, we observed *trans*-cinnamic acid as one of the main compounds present in all seasons.

The infusion extracts contain phenolic acids and flavonoids, including caffeic acid, *trans*-ferulic acid, verbascoside, nepetin-7-glucoside, and isorhamnetin-3-*O*-rutinoside.[4] In comparison with our seasonal extracts, we observed the presence of *trans*-ferulic acid in all seasons.

Based on the comparison between our results and previous studies, we observed similarities in some identified compounds, such as *trans*-cinnamic acid and *trans*-ferulic acid. Phenolic acids are known to exhibit various biological activities, including antioxidant, anti-inflammatory, and anticancer effects.[38,39]

Antioxidant activity

The antioxidant activity of seasonal *G. alypum* extracts was evaluated using five standard analytical methods: DPPH, ABTS, ADS, FRAP, and SNPAC assay. These assays aimed to assess the extracts' capacity to scavenge various types of free radicals. The results revealed a clear variation in antioxidant efficacy depending on the season, which may reflect the influence of environmental conditions on the accumulation of bioactive compounds, including phenolics and flavonoids. Comparable studies on phenolic-rich plant extracts have demonstrated significant correlations between chromatographically identified phenolic and flavonoid constituents and measured antioxidant activity across multiple assay systems, including DPPH, ABTS, and metal chelation methods.[40,41]

Table 3 presents the results of the analysis of the antioxidant activity of the samples depending on the season of harvest (Spring, Winter, Autumn, Summer), expressed as IC_{50} or $A_{0.5}$ values (μg/mL) for different methods. These values (IC_{50} and $A_{0.5}$) indicate the sample concentration required to achieve 50% or 0.5% (depending on the method, usually IC_{50}) of the maximum effect, which means that lower values indicate higher antioxidant activity. The Table 3 includes the activity measured using the DPPH, ABTS, ADS, FRAP and SNPAC methods. Additionally, for comparative purposes, the results for synthetic antioxidant standards: BHA, tannic acid and ascorbic acid are presented. The letters (a, b, c, d) next to the values indicate statistically significant differences between the means in the groups for a given parameter, according to the least significant difference (LSD) test at $p < 0.05$.

DPPH activity

The highest antioxidant activity (lowest IC_{50}) using the DPPH method was demonstrated by samples from Summer (48.63 μg/mL), significantly different from samples from Spring (69.94 μg/mL) and Autumn (77.86 μg/mL). Samples from Winter (52.82 μg/mL) were also characterized by high activity. The activity of the samples was lower than for the BHA standard (6.89 μg/mL) and ascorbic acid (22.11 μg/mL).

These results are partially consistent with the findings of Khlifi et al.,[8] who reported IC_{50} values for aqueous extracts of *G. alypum* ranging between 223.04 ± 0.73 and 85.37 ± 1.48 μg/mL values weaker than those obtained in the present study (Table 3). This discrepancy may be due to the difference in extraction solvents, as more polar and efficient solvents (ethanol) were employed in our study, in addition to variations in climatic and geographical conditions. Similarly, Ouffai et al.[39] reported higher antioxidant activity in ethanolic extracts ($IC_{50} = 15.58 \pm 0.17$ μg/mL) compared to acetone extracts ($IC_{50} = 20.33 \pm 0.61$ μg/mL), whereas extracts prepared with petroleum ether or dichloromethane displayed very weak activity ($IC_{50} > 285$ μg/mL). These findings emphasize the crucial role of solvent polarity in extracting bioactive compounds, Similar observations have been widely reported, where solvent polarity significantly influences the extraction efficiency of phenolic antioxidants from plant

TABLE 3 The influence of the season (vegetation season) on the antioxidant activity of samples, expressed by IC_{50} and $A_{0.5}$ values (μg/mL), compared to standards.

	IC_{50} (μg/mL)			$A_{0.5}$ (μg/mL)	
Specification	**DPPH**	**ABTS**	**ADS**	**FRAP**	**SNPAC**
G Spring	$69.94^{b} \pm 3.09$	$14.60^{bc} \pm 0.95$	$20.60^{b} \pm 0.08$	$23.70^{a} \pm 2.00$	$39.31^{b} \pm 3.41$
G Winter	$52.82^{c} \pm 1.44$	$16.14^{b} \pm 2.06$	$19.98^{bc} \pm 0.07$	$11.22^{c} \pm 1.42$	$20.87^{d} \pm 0.16$
G Autumn	$77.86^{a} \pm 1.97$	$15.57^{b} \pm 0.94$	$20.30^{b} \pm 0.59$	$5.86^{d} \pm 0.05$	$34.61^{c} \pm 1.26$
G Summer	$48.63^{cd} \pm 0.90$	$31.23^{a} \pm 2.71$	$22.31^{a} \pm 0.93$	$21.63^{b} \pm 0.49$	$189.44^{a} \pm 4.29$
Mean	62.31	19.38	20.80	15.60	71.06
$LSDp_{0.05}$	3.61	1.12	1.23	0.90	4.20
BHA	6.89 ± 0.12	1.91 ± 0.09	nt	nt	nt
Tannic acid	nt	nt	3.125 ± 0.03	nt	nt
Ascorbic acid	22.11 ± 0.78	14.15 ± 1.1	nt	6.77 ± 1.15	7.14 ± 0.05

Note: Different letters indicate statistically significant differences.
Abbreviations: BHA, butylated hydroxyanisole; nt, not tested.

matrices.[42] According to Taghzouti et al.,[43] the highest antioxidant activity was observed in the ethanolic root extract ($IC_{50} = 10.01$ μg/mL), followed by the ethyl acetate root extract ($IC_{50} = 18.85$ μg/mL), demonstrating that root extracts exhibited stronger activity than those from leaves and stems, regardless of the solvent used. Furthermore, a strong correlation was reported between total phenolic content and antioxidant activity ($\rho = -0.87$), although the author acknowledged that interactions between compounds within the extract may influence the final antioxidant capacity. Optimizing extraction parameters is therefore considered essential for maximizing the recovery of antioxidant phenolics from plant materials.[44] In line with this, Nefzi et al.[36] also reported that the ethanolic extract of *G. alypum* showed notable antioxidant activity in the DPPH assay, consistent with its high phenolic and flavonoid content. Similarly, Chen et al.[45] reported that total flavonoid extracts of *Artemisia princeps* demonstrated significant DPPH and ABTS radical scavenging activity alongside lipid peroxidation inhibition, further supporting the contribution of flavonoid constituents to the overall antioxidant capacity of phenolic-rich plant extracts.

ABTS activity

In the case of ABTS activity, the lowest value (highest activity) was recorded for samples from Spring (14.60 μg/mL), Winter (16.14 μg/mL), and Autumn (15.57 μg/mL), which did not differ significantly from each other. A significantly lower activity (higher $A_{0.5}$ value) was observed for samples from Summer (31.23 μg/mL), indicating that they were the least active by the ABTS method. ABTS activity for all seasons was lower than for the BHA standard (1.91 μg/mL). In the study by Ben et al.,[13] the hydroethanolic leaf extract of *G. alypum* (HEGA) demonstrated an IC_{50} value of 3.02 ± 0.09 μg/mL in the ABTS assay, reflecting a higher antioxidant activity compared to our extracts. This discrepancy may be attributed to differences in solvent type and extraction methodology, which can influence the concentration of phenolic constituents. Conversely, the researcher Nefzi et al.[36] reported a moderate antioxidant activity for *G. alypum* extracts in the ABTS assay, with an IC_{50} value of 37.0 ± 2.45 μg/mL, which is lower than the values observed in our study. This difference likely results from variations in extraction protocols, environmental conditions, and solvent types used.

ADS activity

Samples from Summer (22.31 μg/mL) showed the lowest activity (highest ADS value), significantly different from the other seasons. Samples from Winter (19.98 μg/mL), Spring (20.60 μg/mL), and Autumn (20.30 μg/mL) showed similar, higher activity, without significant statistical differences between each other. In the case of the ADS method, tannic acid (<3.125 μg/mL) showed significantly higher antioxidant activity than the tested samples.

FRAP activity

The highest reducing activity (lowest FRAP) was recorded for samples from Autumn (5.86 μg/mL), which made them the most effective in reducing iron ions. Samples from Winter (11.22 μg/mL) were also characterized by high activity. Samples from Summer (21.63 μg/mL) and Spring (23.70 μg/mL) showed significantly lower reducing capacity. Ascorbic acid (6.77 μg/mL) showed activity similar to autumn samples. Comparing these results with previous studies, Asraoui et al.,[37] reported that the ethyl acetate extract of *G. alypum* demonstrated a high reductive power of 531.1 ± 1.08 mg AAE/g DW, reflecting strong antioxidant activity. Despite differences in measurement methods and units, the overall trend aligns with our findings, and higher antioxidant efficacy may be associated with elevated phenolic content. In the study by Massinissa,[4] the ethanolic extract showed a reductive capacity of 106.95 μg EAA/mg extract at 1 mg/mL

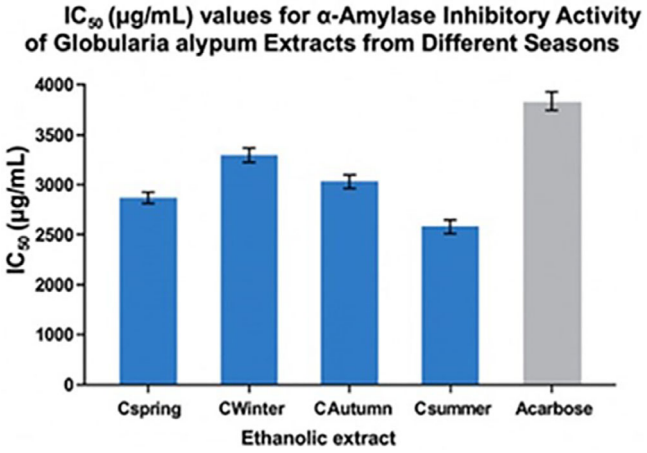


FIGURE 3 IC_{50} (µg/mL) values for alpha-amylase inhibitory of *Globularia alypum* extracts from different seasons.

concentration, representing notable activity but still lower than the autumn extract in our study, highlighting the influence of both the harvesting season and solvent type on bioactivity. Furthermore, Oufai et al.[39] reported IC_{50} values for aqueous extracts of *G. alypum* in the FRAP assay ranging from 0.31 to 0.68 mg/mL (310–680 µg/mL), significantly higher than those recorded for the autumn extract in our study, indicating weaker reductive activity of aqueous extracts compared to alcoholic or phenol-rich seasonal extracts.

SNPAC activity

The highest activity in inhibiting nitric oxide production (lowest SNPAC) was shown by samples from Winter (20.87 µg/mL). Samples from Autumn (34.61 µg/mL) and Spring (39.31 µg/mL) also had significant activity, but lower than those from Winter. The definitely lowest activity (highest SNPAC) was observed for samples from Summer (189.44 µg/mL), indicating their poor ability to neutralize nitric oxide compared to other seasons. Ascorbic acid (7.14 µg/mL) showed significantly higher activity in the SNPAC assay than all samples tested.

The analysis indicates a significant effect of the harvest season on the antioxidant activity of the samples, which is confirmed by statistically significant differences between seasons for all methods tested. There is no single season that consistently showed the highest activity in all tests, which highlights the complexity of the bioactive profile. This complexity is consistent with observations in other plant-based systems, where total phenolic content and antioxidant activity do not always follow a linear relationship, as changes in phenolic composition can result in increased antioxidant capacity even when total phenolic levels decrease.[46] For example, summer samples were characterized by the highest DPPH activity but the lowest ABTS and SNPAC activity. In contrast, autumn samples were characterized by the highest FRAP activity and winter by SNPAC activity. These observations emphasize the importance of environmental conditions during the growing season for the synthesis of bioactive compounds. Comparison with synthetic standards confirms that the tested samples show antioxidant activity, although usually lower than pure reference compounds at these concentrations.

Enzymatic inhibition: α-amylase and urease and its relationship with seasonal variation in phenolic content

α-amylase with seasonal variation in phenolic content

The α-amylase inhibitory activity of *G. alypum* ethanolic extracts showed marked variation across different seasons, suggesting a strong influence of seasonal changes on the bioactive phytochemical composition of the plant (Figure 3). As presented in Table 4, the IC_{50} values ranged from 491.40 µg/mL in summer to 1751.31 µg/mL in winter, indicating the highest inhibitory potency during the summer season and the lowest in winter. Interestingly, the summer extract exhibited significantly higher activity (lower IC_{50}) compared to the standard drug acarbose ($IC_{50} = 3650.93$ µg/mL), highlighting the potential of *G. alypum* as a natural source of α-amylase inhibitors.

These findings are consistent with previous studies suggesting that phenolic compounds may contribute to α-amylase inhibitory activity. It is possible that variations in phenolic content during summer, potentially influenced by environmental stressors such as high temperature and solar radiation, may contribute to the observed inhibitory activity. Conversely, the relatively poor activity observed in winter may reflect reduced phenolic accumulation under less stressful environmental conditions.

TABLE 4 IC_{50} (µg/mL) values for α-amylase and urease inhibitory of *Globularia alypum* extracts from different seasons.

Specification		IC_{50} (µg/mL) anti-urease	C_{50} (µg/mL) for *Amylase*
Ethanolic extract	CSpring	NA	1519.75 ± 2.21
	CWinter	NA	1751.31 ± 2.17
	CAutumn	NA	1240.69 ± 2.15
	Csummer	15.25 ± 0.15	491.40 ± 1.25
Standard IC_{50} (µg/mL)	Thiourea	11.57 ± 0.68	-
	Acarbose	-	3650.93 ± 10.70

Abbreviation: NA, no activity.

The data also reveal moderate activity in autumn (IC_{50} = 1240.69 μg/mL) and spring (IC_{50} = 1519.75 μg/mL), suggesting a transitional phytochemical profile during these periods. This seasonal fluctuation in enzyme inhibition supports the importance of harvest timing when optimizing plant-based therapeutics for antidiabetic applications. The superior α-amylase inhibition observed in summer-harvested extracts, surpassing even the standard acarbose, underlines the significance of seasonal variation in determining the pharmacological efficacy of *G. alypum*. Further phytochemical profiling during each season is warranted to identify specific compounds responsible for this activity and to validate these results through in vivo models.

Urease and its relationship with seasonal variation in phenolic content

The urease-inhibitory activity of *G. alypum* ethanolic extracts exhibited a distinct seasonal dependence, with significant inhibition observed exclusively in the summer extract (IC_{50} = 15.25 ± 0.15 μg/mL) (Table 4), while no activity (NA) was detected in the extracts obtained during spring, autumn, or winter. This pattern may indicate that bioactive constituents with anti-urease properties are more abundant during the summer season. Compared to the reference inhibitor thiourea (IC_{50} = 11.57 ± 0.68 μg/mL), the summer extract demonstrated a promising level of activity, albeit slightly less potent. This finding emphasizes the potential of *G. alypum* as a natural urease inhibitor, particularly when harvested in the summer. Urease, an enzyme implicated in the pathogenesis of Helicobacter pylori infections and urinary tract disorders, is a relevant pharmacological target; thus, identifying plant-derived inhibitors with seasonal optimization may offer an effective and safer alternative to synthetic compounds.

The absence of detectable activity in other seasonal extracts could be attributed to the lower concentration or absence of specific secondary metabolites during those periods. Environmental factors such as increased temperature and UV radiation during summer likely stimulate the biosynthesis of certain phenolic or flavonoid compounds known for their urease-inhibitory potential.

These results highlight the crucial role of harvest timing in maximizing the therapeutic potential of *G. alypum*. Future studies should focus on the phytochemical characterization of the summer extract to isolate and identify the active constituents responsible for urease inhibition. Additionally, mechanistic studies and in vivo validation are necessary to support the application of this plant in treating urease-related pathologies.

Photoprotective potential of seasonal *G. alypum* extracts as natural sunscreen agents

The SPF is a widely accepted indicator used to evaluate the effectiveness of compounds against ultraviolet (UV) radiation. It quantitatively reflects the ability of a substance to reduce UV-induced skin damage, with higher values indicating stronger protection.

TABLE 5 Sun protection factor (SPF) values of seasonal *Globularia alypum* extracts (±standard deviation).

Season	SPF value (±SD)	Protection level
G Spring	31.25^{bc} ± 0.25	High protection
G Summer	32.52^{b} ± 0.67	High protection
G Autumn	32.35^{b} ± 0.44	High protection
G Winter	34.90^{a} ± 0.43	High protection
Mean	32.76	
$LSDp_{0.05}$	1.97	

Note: Different letters indicate statistically significant differences.

Table 5 shows the mean values of the SPF of *G. alypum* extracts collected in four different seasons: Spring (G Spring), Summer (G Summer), Autumn (G Autumn), and Winter (G Winter). The SPF values are given with the SD. Additionally, for each season, the assigned level of sun protection was determined. The letters (a, b, c) next to the SPF values indicate statistically significant differences between the means for the individual seasons, determined using the least significant difference (LSD) test at a significance level of $p < 0.05$. The row "Mean" shows the overall mean SPF value for all seasons, and "$LSDp_{0.05}$" indicates the LSD value for this parameter.

Analysis of data on SPF values of *G. alypum* extracts reveals significant differences depending on the season of collection.

The highest SPF value was recorded for extracts collected in Winter (34.90 ± 0.43). This value was statistically significantly higher compared to all other seasons.

Extracts collected in Summer (32.52 ± 0.67) and Autumn (32.35 ± 0.44) showed similar, high SPF values, which did not differ statistically significantly but were significantly higher than for Spring.

The lowest SPF value was observed for extracts from Spring (31.25 ± 0.25).

Regardless of the season of collection, all tested *G. alypum* extracts showed SPF values above 30, which qualifies them as providing a high level of sun protection according to the applicable classifications. The average overall SPF value for all seasons was 32.76.

According to the SPF classification, these values fall within the "high protection" range (30–50), compared to other categories: low (2–12), moderate (12–30), high (30–50), and very high (>50).[47] This photoprotective activity may be associated with the high levels of total phenolic and total flavonoid contents in the extracts, as shown in Table 1.

Although the summer extract had a lower total phenolic content, it exhibited the highest SPF value. This may be attributed to the biochemical adaptations developed by the plant in response to intense sunlight exposure during the summer season, leading to the accumulation of biologically active compounds with strong UV-absorbing properties. Previous literature highlights the crucial role of phenolic and flavonoid compounds in photoprotection. These molecules act as

potent antioxidants, reducing DNA damage through the modulation of oxidative stress signaling pathways, inhibiting lipid peroxidation, and neutralizing UV-induced free radicals.[48,49] Several studies have demonstrated that flavonoids, due to their multiple hydroxyl groups, possess strong UV-absorbing capabilities and play a key role in mitigating UV-related cytotoxic effects.[48]

In the conclusions from these data, clearly indicate that the season of collection has a significant effect on the photoprotective potential of *G. alypum* extracts. Extracts collected in the winter season were distinguished by the highest SPF value. Despite these differences, all extracts, regardless of the season, present a high level of sun protection, which suggests their potential use in photoprotective products. This seasonal variation in SPF may be related to the variable concentration of bioactive compounds, such as polyphenols and flavonoids, which are known for their UV-absorbing properties and whose seasonal variation has been previously observed. In this context, the photoprotective activity of seasonal *G. alypum* extracts (spring, summer, autumn, winter) was evaluated.

Study of the antimicrobial activity of ethanolic extracts of the *Teasel* plant

The antimicrobial activity of the seasonal extracts was evaluated at different concentrations, and the detailed results are provided in Table S1.

The antimicrobial efficacy test of the ethanolic extracts from the four seasons of the *Teasel* plant was conducted against the same bacterial strains and yeast previously mentioned, namely *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25932, *B. subtilis* ATCC 25973, and *C. albicans* ATCC 10231.

The ethanolic extracts of *G. alypum*, obtained from samples collected during the four seasons, exhibited negligible antimicrobial activity under the tested conditions (10–80 mg/mL) against the tested bacterial strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25932, *B. subtilis* ATCC 25973) or the yeast *C. albicans* ATCC 10231 at all examined concentrations (10, 20, 40, and 80 mg/mL). All results were negative (no inhibition, NI), except for the winter extract, which showed a weak inhibitory effect against *S. aureus* ATCC 25932 at the highest concentration tested (80 mg/mL), with an inhibition zone diameter of only 9 mm.

In contrast, the standard antibiotic gentamicin (GNT) exhibited markedly superior activity, with inhibition zones ranging from 22 to 32 mm, confirming assay sensitivity and excluding methodological failure.

The HPLC polyphenol profile provides a mechanistic explanation for these results. The dominant compounds identified across all seasons were trans-cinnamic acid (417.44–961.52 µg/g), trans-ferulic acid (24.63–392.17 µg/g), and kaempferol (5.81–52.59 µg/g). While phenolic acids such as ferulic acid have documented antibacterial activity as isolated compounds, their effective concentration within the crude extract is substantially lower than the MIC values reported for pure standards.[50,51] The antimicrobial potential of cinnamic acid derivatives is highly structure-dependent, with the free acid generally exhibiting weaker activity than corresponding aldehyde or ester derivatives.[52] Notably, flavonoids commonly associated with strong antibacterial mechanisms, including quercetin, catechin, rutin, and luteolin, were absent or present only in trace amounts across all seasonal extracts.[53] This qualitative deficiency in bioactive flavonoid composition, rather than overall phenolic content, constitutes the principal reason for the observed inactivity.

The complete inactivity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) is consistent with the permeability barrier imposed by their lipopolysaccharide-rich outer membranes, which restrict the diffusion of hydrophobic natural compounds to intracellular targets.[54,55] Even for Gram-positive *S. aureus*, only marginal inhibition was detected at the highest concentration, suggesting insufficient accumulation of active constituents at the site of action. Additionally, the agar disc diffusion assay primarily detects compounds capable of passive radial diffusion; poorly soluble or high-molecular-weight phenolics may exhibit limited diffusion, underestimating intrinsic antimicrobial potential. Therefore, complementary methods such as broth microdilution are recommended for complex plant extracts.[56,57]

Comparative literature indicates substantial variability in antimicrobial outcomes for *G. alypum*, influenced by geographic origin, collection season, extraction solvent, and assay methodology. Nefzi et al.[36] reported an inhibition zone of 14 mm against *S. aureus* at an MIC of 6.25 mg/mL in Tunisia, whereas Abderraouf et al.[58] observed 20.22 ± 0.18 mm at 25 mg/mL in Algeria. Conversely, Nouir et al.[14] found no antibacterial activity of ethanolic extracts against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) in Tunisia, corroborating the findings of the present study.

Future studies aiming to evaluate the antimicrobial potential of *G. alypum* should employ selective fractionation strategies to concentrate minor bioactive constituents and complement disc diffusion screening with broth microdilution assays to account for the limited diffusibility of certain phytochemicals.

Evaluation of anti-inflammatory activity

In vitro anti-inflammatory activity of extracts of *G. alypum*

The anti-inflammatory activity of seasonal extracts of *G. alypum* was evaluated using the heat-induced albumin denaturation inhibition assay by calculating the IC_{50} values (µg/mL) and comparing them with the reference drug Diclofenac (Figure 4). The results, shown in Table 6 demonstrated a significant variation in biological activity according to the season.

Table 6 shows the anti-inflammatory activity of *G. alypum* extracts collected in four different seasons: Spring (G Spring), Winter (G Winter), Autumn (G Autumn), and Summer (G Summer). Anti-inflammatory activity is expressed as IC_{50} value (µg/mL), which means the concentration of the extract necessary to inhibit the inflammatory

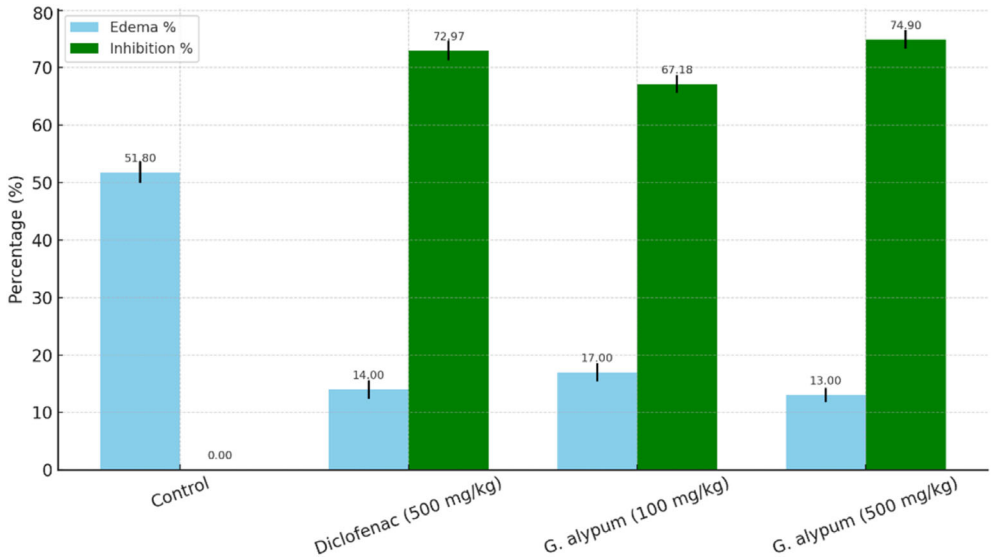


FIGURE 4 In vivo anti-inflammatory effect of spring extract of *Globularia alypum* in carrageenan-induced paw edema in rats.

TABLE 6 IC_{50} (μg/mL) values for in vitro anti-inflammatory activity of *Globularia alypum* extracts from different seasons.

Specification	IC_{50} (μg/mL)
G Spring	$49.89^{d} \pm 6.22$
G Winter	$73.05^{c} \pm 2.00$
G Autumn	$127.48^{a} \pm 9.54$
G Summer	$107.78^{b} \pm 7.21$
Diclofenac	$40.90^{e} \pm 0.89$
Mean	87.30
$LSDp_{0.05}$	5.37

Note: Different letters indicate statistically significant differences.

reaction by 50%. Therefore, lower IC_{50} values indicate higher anti-inflammatory activity. Results are given as mean ± SD. Additionally, for comparative purposes, the activity of the standard anti-inflammatory drug—diclofenac is presented. The letters (a, b, c, d, e) next to the IC_{50} value indicate statistically significant differences between the means, according to the LSD test (least significant difference) at the significance level of $p < 0.05$. The "Mean" row shows the overall mean IC_{50} value for all seasonal extracts tested and "$LSDp_{0.05}$" indicates the LSD value for this parameter.

The spring extract (GSpring) exhibited the highest anti-inflammatory activity with an IC_{50} value of 56.26 ± 6.22 μg/mL, closely approaching the efficacy of the reference drug diclofenac (40.90 ± 0.89 μg/mL), followed by the winter extract (GWinter) with an IC_{50} of 73.05 ± 2.00 μg/mL. In contrast, a marked decrease in bioactivity was observed in the autumn (IC_{50} = 127.47 ± 9.54 μg/mL) and summer extracts (IC_{50} = 107.78 ± 4.46 μg/mL). This variation in anti-inflammatory effectiveness corresponds with the recorded changes in the total phenolic and flavonoid contents,[43] where the spring extract showed the highest phenolic content (TPC = 380.07 ± 2.06 μg GAE/mg) and flavonoid content (TFC = 217.11 ± 0.47 μg QE/mg), followed by the winter extract with a comparable level. This supports the hypothesis that these phenolic compounds significantly contribute to the anti-inflammatory activity.

Phenolic compounds are known for their ability to inhibit inflammatory mediators such as prostaglandins and histamine, as well as to suppress pro-inflammatory enzymes like COX and LOX. These observations suggest that higher TPC and TFC levels may be associated with enhanced anti-inflammatory activity, which is consistent with previous reports highlighting the potential contribution of phenolic compounds to anti-inflammatory effects.[59,60]

The poor performance of the summer extract, which showed the lowest phenolic content (TPC = 200.96 ± 2.66 μg GAE/mg), reflects the negative impact of reduced active compounds on the extract's biological efficacy, likely due to degradation or decreased biosynthesis caused by high temperatures and sun exposure.

Comparing these findings with those reported by researcher Massinissa et al.,[4] reveals a notable agreement regarding the anti-inflammatory potential of *G. alypum* extracts. While Massinissa's methanol extract demonstrated 85.45% inhibition of albumin denaturation at 800 μg/mL, our results show a comparable activity in the spring extract with an IC_{50} close to that of the reference diclofenac. These results confirm the key role of phenolic and flavonoid compounds in mitigating inflammation.

However, Massinissa et al.'s study,[4] did not address the seasonal influence on chemical composition or bioactivity, which distinguishes our study by highlighting seasonal variation in anti-inflammatory efficacy, with the summer extract showing the lowest activity concurrent with reduced phenolic content.

Accordingly, these results emphasize the importance of environmental and seasonal factors in determining the biological efficacy of the plant and support the potential exploitation of *G. alypum* as a promising natural source of anti-inflammatory compounds. The study recommends focusing on optimal harvesting timing and extraction

TABLE 7 Anti-inflammatory activity results of the spring extract of *Globularia alypum*.

Treatment	Dose (mg/kg)	Percentage of edema (%)	Percentage of inhibition (%)
Control	-	51.80 ± 1.92[d]	-
Diclofenac	500	14.00 ± 1.58[a]	72.97
G. alypum	100	17.00 ± 1.58	67.18
	500	13.00 ± 1.22	74.900

Note: Different letters indicate statistically significant differences.

TABLE 8 Analgesic activity of the spring extract of *Globularia alypum*.

Treatment	Dose (mg/kg)	Number of cramps	Percentage of protection (%)
Control	0.0	69.00[a] ± 3.16	0.0
Paracetamol	500	20.40[c] ± 1.94	70.43
G. alypum	100	26.00[b] ± 1.22	62.31
	500	16.00[d] ± 1.58	76.81

Note: Different letters indicate statistically significant differences.

conditions, alongside further research to isolate active phenolic compounds and elucidate their molecular mechanisms of action.

In vivo anti-inflammatory activity of the spring extract of *G. alypum*

Inflammation is a fundamental biological response triggered by the immune system to combat injuries or harmful agents. However, chronic inflammation can lead to various diseases, necessitating the search for effective and safe treatments. Medicinal plants emerge as a rich source of bioactive compounds with anti-inflammatory properties, acting by inhibiting multiple inflammatory pathways through complex biochemical mechanisms. After evaluating the extract's activity in in vitro studies, its efficacy was further assessed in vivo using the carrageenan-induced paw edema model in rats to confirm the bioactivity of the extract under conditions closer to physiological reality.

The results of the carrageenan-induced edema test demonstrated a clear efficacy of the spring extract in reducing paw swelling in rats. The dose of 500 mg/kg recorded an inhibition percentage of 74.90%, surpassing the effect produced by diclofenac at the same dose (72.97%) (Table 7). Even at a lower dose (100 mg/kg), the extract showed significant efficacy with an inhibition percentage of 67.18%.

This anti-inflammatory effect can also be attributed to the rich content of phenolic compounds, which play a key role in suppressing inflammatory mediators such as prostaglandins and interleukins, in addition to their antioxidant activity, contributing to the inhibition of the cascade of reactions involved in the inflammatory response.

Analgesic activity of the spring extract of *G. alypum*

As part of the comprehensive evaluation of the seasonal biological activities of *G. alypum* extracts, the acetic acid-induced writhing test was conducted in mice to assess the analgesic potential of the spring extract. This experimental model is widely used to evaluate peripheral inflammatory pain, as it reflects the stimulation of prostaglandin synthesis and is thus considered a reliable method for assessing the pain-relieving properties of natural compounds.

In this study, the spring extract of *G. alypum* demonstrated a remarkable analgesic effect. At a dose of 100 mg/kg, it reduced the number of acetic acid-induced abdominal writhes by 62.31% (see Table 8), a value comparable to that of paracetamol at a higher dose of 500 mg/kg (70.43%). Notably, the 500 mg/kg dose of the extract exhibited even greater efficacy, achieving a 76.81% inhibition, thereby surpassing the standard drug. This potent activity is likely related to the high levels of phenolic (TPC: 380.07 ± 2.06 µg GAE/mg) and flavonoid compounds (TFC: 217.11 ± 0.47 µg QE/mg) in the spring extract, which are known to exert antinociceptive and anti-inflammatory effects through the inhibition of prostaglandin synthesis and modulation of nociceptive neurotransmission.

When compared to the findings of Boutemak et al.,[18] who evaluated the anti-inflammatory activity of the butanolic fraction of *G. alypum* against carrageenan-induced paw oedema in mice, our results show a higher or comparable biological efficacy. In that study, the butanolic extract achieved 8.55%, 27.15%, and 33.01% inhibition at concentrations of 0.1, 0.2, and 0.3 g/mL, respectively, while the reference drug acetylsalicylic acid produced 39.76% inhibition. These findings, together with ours, highlight the therapeutic relevance of *G. alypum* and support its traditional use as a source of natural anti-inflammatory and analgesic agents.

Preliminary evaluation of acute toxicity

The spring extract of *G. alypum* demonstrated high biological safety, as no mortality or significant behavioral or clinical changes were observed in mice even at increased doses up to 2000 mg/kg. No signs of toxicity, such as hyperactivity, ataxia, convulsions, or lethargy, were reported. These findings are consistent with those of Mohamed

et al.,[61] who reported no signs of acute toxicity or mortality at a dose of 400 mg/kg in a study conducted in Tunisia.

These results reinforce the potential of this extract as a natural source of safe anti-inflammatory and analgesic compounds, supporting the development of plant-based alternatives to conventional pharmaceutical drugs that are often associated with adverse side effects.

Descriptive statistics for the analyzed qualitative features/biological activity of *G. alypum*

For each feature, such as: TFC, TPC, antioxidant activity measured by DPPH and ABTS methods, and FRAP reduction capacity, key measures of central tendency and variability are presented. The table includes: arithmetic mean, median, SD, kurtosis, skewness, range of variability, minimum and maximum value, and coefficient of variation expressed in percentage (V%). These statistics allow assessment of data distribution, dispersion, and potential outliers for each investigated parameter (Table S2).

Analysis of the data from the table reveals diverse phenotypic variability of individual traits. The highest mean values were recorded for TPC (306.67) and TFC (187.56), which indicates a significant presence of polyphenols and flavonoids in the tested samples. Their high maximum values (383.43 and 227.83, respectively) and wide range (184.26 and 91.66) suggest significant diversity between samples in terms of the content of these compounds. Among the methods of assessing antioxidant activity, DPPH (average 62.31) and ABTS (average 19.38) showed significant variability, with a high coefficient of variation V% of 20.32% and 37.84%, respectively. It is worth noting FRAP (average 15.60), where the coefficient of variation V% was as high as 49.66%, which indicates a very high heterogeneity of data in relation to the reducing capacity. The particularly high coefficient of variation V% 101.01% for the SNPAC method highlights the extreme variability in the ability to inhibit nitric oxide production, which may indicate significant differences in the antioxidant and anti-inflammatory activity of the tested materials.

The "anti-inflammatory" parameter (mean 89.55) showed a variability V% at the level of. Low values of kurtosis and skewness for most traits, especially TFC, TPC, DPPH and FRAP, suggest distributions close to normal, however, positive skewness for ABTS, ADS, SNPAC, and SPF and negative for TFC, TPC, FRAP and anti-inflammatory may indicate asymmetry of the data distribution.

In summary, the wide range of minimum and maximum values and the varied coefficients of variation (especially high for SNP, FRAP and anti-inflammatory) emphasize the significant phenotypic variability of the tested traits. This observation is crucial for further analysis, as it suggests that factors such as genotype, environment, and interactions between them may play an important role in shaping these properties. The particularly high variability for single nucleotide polymorphisms (SNPs) and FRAPs indicates potentially large differences in antioxidant and anti-inflammatory activity, which may have important implications for the applications of the materials studied.

The Pearson correlation matrix illustrating the strength and direction of linear relationships among the nine analyzed variables is presented in Table S3. The variables include TFC, TPC, antioxidant activity (DPPH, ABTS, ADS, FRAP, and SNPAC assays), anti-inflammatory activity, and Sun Protection Factor (SPF). Values are reported as Pearson correlation coefficients.

Analysis of the correlation matrix allows for the identification of relationships between individual parameters:

Strong positive correlations (close to 1) indicate that an increase in the value of one feature is associated with an increase in the value of the other. An example is the almost perfect correlation between TFC and TPC (0.99), which suggests that flavonoids constitute a significant part of the total polyphenols in the samples studied, or that both measures are closely related.

Strong negative correlations (close to −1) indicate that an increase in the value of one feature is associated with a decrease in the value of the other. For example, we observe strong negative correlations of TFC with ABTS (−0.78) and TPC with ABTS (−0.78) and TFC with SNPAC (−0.82) and TPC with SNPAC (−0.80). This result is often observed in antioxidant studies, where lower values in tests such as ABTS or SNPAC indicate higher antioxidant activity (since they measure the degree of free radical deactivation), which would be consistent with a positive effect of high polyphenol/flavonoid content.

Weak or no correlation (close to 0): Suggests no or very weak linear relationship. An example is the correlation of TFC with FRAP (−0.06) and TPC with FRAP (0.02), which indicates that the reducing capacity measured by FRAP in these samples may be independent of the total phenol/flavonoid content, or that other, unmeasured components are responsible for this activity.

CONCLUSION

This study demonstrates that the season of harvest is a decisive and statistically significant factor influencing the phenotypic variability, phytochemical profile, and biological activities of *G. alypum* extracts. Analysis of variance revealed that cultivation system, variety, year, and their interactions (G × E and Y × G × E) significantly shaped yield characteristics, chemical composition, and bioactivity.

TPC and TFC were markedly higher in extracts collected during colder seasons (spring and winter), indicating these as optimal periods for obtaining bioactive secondary metabolites. Antioxidant capacity, assessed using DPPH, ABTS, ADS, FRAP, and SNPAC assays, exhibited pronounced seasonal variation, with no single season maximizing all antioxidant endpoints. The highest FRAP activity was recorded in autumn, whereas the strongest SNPACinhibition occurred in winter, reflecting the diversity of antioxidant mechanisms present.

All extracts displayed high photoprotective potential (SPF > 30) throughout the year, with the highest value in winter, highlighting their relevance for cosmeceutical applications. Anti-inflammatory activity peaked in spring, with IC_{50} values approaching those of diclofenac. Correlation analysis revealed strong positive associations between TPC/TFC and certain antioxidant assays, alongside negative correlations with others (e.g., ABTS, SNPAC), suggesting the involvement of both phenolic-dependent and phenolic-independent mechanisms.

In conclusion, optimizing harvest season is essential for maximizing the desired bioactive properties of *G. alypum*, with clear

implications for its pharmaceutical, cosmetic, and nutraceutical applications. Future research should aim to isolate and characterize the compounds responsible for the observed effects and assess their efficacy in vivo.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

No data were used for the research described in the article.

ETHICS STATEMENT

All experimental procedures involving animals were reviewed and approved by the Ethical Committee of Animal Experimentation (CEEA) of the University of Sciences and Technology Houari Boumediene (USTHB), Algeria, under reference number: CEEA-USTHB-08-2023/11118.

INFORMED CONSENT

There are no human subjects in this article and informed consent is not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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