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Phytochemical composition and biological activities of *Caryopteris grata* and *Caryopteris odorata* from the Nakyal mountains, Azad Jammu, and Kashmir

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Abstract: Medicinal plants *Caryopteris grata* and *Caryopteris odorata* from Azad Jammu and Kashmir (AJK), a biodiversity-rich region of the western Himalayas shaped by varied topography and climate, were examined. The Nakyal mountainous zone of the Kotli district represents a distinct ecological niche where plants experience environmental pressures that could influence their phytochemical profiles. Leaf extracts of *C. grata* and *C. odorata* were prepared using solvents of varying polarities. We hypothesized that polar extracts would show stronger antioxidant and anti-inflammatory effects. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that *C. grata* was dominated by 7-methylcyclopenta[c]pyran-4-carboxylic acid methyl ester (61.93%), while *C. odorata* exhibited a more balanced profile rich in coumarin (30.59%), phytol (10.39%), and n-hexadecanoic acid (7.02%), with both species sharing three compounds but differing markedly in their relative abundance. Dose-response curves revealed that *C. odorata* exhibited significantly stronger cyclooxygenase (COX) inhibitory activity than *C. grata*, with methanol and ethyl acetate fractions showing the highest potency. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, and ferric thiocyanate (FTC) assays showed clear dose-dependent inhibition by all extracts, with *C. odorata*, particularly its methanol and ethyl acetate extracts, achieving higher activity at lower concentrations than *C. grata*. ANOVA with repeated measures for FTC inhibition (24-72 h) exhibited significant effects of species, extract type, and their interaction ($P<0.05$), with *C. odorata* methanol and ethyl acetate extracts consistently outperforming other treatments. *C. odorata* extracts show greater antioxidant and anti-inflammatory potential than *C. grata*, as indicated by their stronger bioactive profile.

Keywords: chromatography, coumarin, *Caryopteris* species, cyclooxygenase, DPPH

INTRODUCTION

The genus *Caryopteris* comprises small shrubs and perennial herbs, many of which are valued in traditional medicine for their diverse therapeutic properties [1,2]. The genus reportedly contains a wide array of bioactive phytochemicals [3], including flavonoids, phenolic acids, terpenoids, and essential oils, which contribute to their pharmacological potential. Several *Caryopteris* species have been used in folk remedies for treating fever, cough, inflammation, skin disorders, and microbial infections [4]. Despite their ethnomedicinal relevance, only a limited number of species within this genus have undergone comprehensive biochemical and pharmacological investigation, leaving many with uncharacterized therapeutic profiles [5,6].

Phytochemicals play an important role in plant defense systems and are increasingly recognized as vital sources of health-promoting compounds for humans [7,8]. Among these, phenolic compounds and flavonoids exhibit potent antioxidant activity, neutralizing reactive oxygen species (ROS) and reducing oxidative stress, which is implicated in the pathogenesis of chronic diseases such as cardiovascular disorders, cancer, diabetes, and neurodegenerative conditions [9]. Moreover, these secondary metabolites are linked to anti-inflammatory activity through modulation of

pro-inflammatory mediators and signaling pathways [2]. Evaluating the phytochemical composition and bioactivity of medicinal plants is, therefore, essential to identify potential candidates for natural antioxidant and anti-inflammatory therapeutics [10-12].

The antioxidant potential of plant metabolites is commonly assessed using both radical-scavenging and lipid-peroxidation inhibition assays, which together provide insight into their mode of action [9,13]. The DPPH assay evaluates the ability of compounds to donate electrons to neutralize free radicals, while the FTC method measures inhibition of lipid peroxidation, a critical process in oxidative damage to biological membranes [14]. These approaches, combined with anti-inflammatory evaluation, enable a comprehensive assessment of plant bio-efficacy. Despite the recognized medicinal relevance of *Caryopteris* species [15], systematic antioxidant and anti-inflammatory profiling using complementary assays remains limited, particularly for species from poorly explored regions.

Azad Jammu and Kashmir (AJK), located in the western Himalayas, is known for its biodiversity-rich regions due to its varied topography and climate [16]. The Nakyal mountainous zone of Kotli district represents a unique ecological spot where medicinal plant species are found under specific environmental pressures that may influence their phytochemical composition. *Caryopteris odorata* and *Caryopteris grata*, the focus of the present study, are naturally distributed in this region but have not been comprehensively evaluated for their antioxidant and anti-inflammatory properties. Given the increasing interest in region-specific bioresources, understanding the bioactive potential of these species from AJK can contribute to both conservation and sustainable utilization efforts.

The present study was designed to quantify and compare the radical scavenging abilities of *C. odorata* and *C. grata* extracts prepared using solvents of varying polarity, to evaluate their anti-inflammatory potential by the inhibition of key enzymatic mediators, and to study the relationship between solvent polarity and bioactivity. We hypothesized that the plant polar extracts would exhibit higher antioxidant and anti-inflammatory activities due to higher phenolic and flavonoid contents. *C. odorata* has been extensively studied for its strong antioxidant and anti-inflammatory potential. Previous research demonstrated that the ethyl acetate fraction of *C. odorata* exhibited exceptional free radical scavenging activity (IC_{50}=8.01±0.254 μg/mL) and the highest total antioxidant capacity among the tested fractions [3]. Singh et al. [6] further confirmed these properties, reporting significant DPPH radical scavenging activity in the leaf essential oils. Moreover, four new iridoid glucosides were isolated from *C. odorata* that showed notable antioxidant activity and inhibition of lipoxygenase, suggesting a possible anti-inflammatory mechanism [5]. In contrast, information on *C. grata* remains scarce. Although a study reported the chemical composition of its essential oils [10], it did not evaluate their antioxidant or anti-inflammatory activities. Therefore, despite substantial evidence supporting the bioactivity of *C. odorata*, comparative studies exploring both *C. odorata* and *C. grata* (particularly using solvent extracts of varying polarities and modern analytical profiling such as GC-MS) are lacking. This gap limits our understanding of the differences and the potential pharmacological relevance of *C. grata*. The obtained findings are expected to inform future bioprospecting, validate traditional medicinal claims [6,14,15], and highlight the potential of these species as sources of natural health-promoting agents.

MATERIALS AND METHODS

Ethics statement

The collection of plant material complies with relevant institutional, national, and international guidelines and legislation [17].

Sample collection and extractions

Leaves of *C. grata* (Wall. ex Walp.) Benth. & Hook. f. ex C. B. Clarke and *C. odorata* (D. Don) B. L. Rob. were collected from the Nakyal Mountains, District Kotli, Azad Jammu and Kashmir, Pakistan, during the spring season (March 2023). To ensure botanical accuracy, the plant was authenticated, and a reference sample (MUH No. 014221) was deposited in the Department of Botany's plant collection at Mirpur University of Science and Technology, Mirpur, Pakistan. Healthy, mature leaves were randomly collected from multiple plants to ensure representative sampling. The leaves were washed with distilled water to remove surface

debris, air-dried under shade at ambient temperature (25-28°C) for 10-15 days, and ground to a fine powder using a laboratory grinder. For extraction, 20 g of powdered leaves were macerated separately in 200 mL of each solvent (methanol, ethyl acetate, hexane, aqueous) for 72 h at room temperature with occasional shaking [15]. Extracts were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator (for organic solvents) and lyophilized (for aqueous extracts). Concentrated extracts were stored in airtight vials at 4°C until further phytochemical analysis [18-20].

Quantitative assessment of total flavonoids, phenolic, and tannin contents

The total phenolic contents were quantified using the Folin-Ciocalteu colorimetric assay, which relies on the reduction of the Folin-Ciocalteu reagent by phenolic compounds under alkaline conditions, producing a blue complex measurable at 765 nm. Results were expressed as gallic acid equivalents (GAE). Total flavonoids were estimated by the aluminum chloride ($AlCl_3$) colorimetric method**,** based on the formation of a stable flavonoid aluminum complex that produces a yellow color quantifiable at 415 nm. Values were expressed as quercetin equivalents (QE). For total tannin contents, the study was conducted via the Folin-Ciocalteu method after polyvinylpolypyrrolidone (PVPP) precipitation**,** where PVPP binds and removes tannins. The phenolic contents before and after PVPP treatment were compared, and the difference was used to represent the tannin content in units as tannic acid equivalents [21,22].

GC-MS analysis protocol

GC-MS analysis was performed on methanol leaf extracts of *C. grata* and *C. odorata* using an Agilent GC-MS system operated by MassHunter software. Dried methanol extracts were reconstituted in HPLC-grade methanol (1.0 mg/mL), vortexed, sonicated for 5 min, and filtered through 0.22 µm PTFE syringe filters before injection. A 1 µL aliquot of each sample was introduced in split-less mode (purge on at 1.0 min) onto an HP-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film) with helium as the carrier gas at a constant flow rate of 1.0 mL min_^{1}. The injector temperature was set to 250°C, and the oven temperature program was as follows: initial 60°C (2 min hold), ramp at 3°C min-^{1} to 150 °C, then 10 °C min–^{1} to 280°C with a final hold of 10 min. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV, scanning from m/z 40–550 with a solvent delay of 3.0 min; ion source, quadrupole, and transfer line temperatures were 230°C, 150°C, and 280°C, respectively. Compound identification was performed by comparing the acquired mass spectra with the NIST17 library (C:\MassHunter\LIBRARY\NIST17.L). For each detected peak (e.g., Peak 1 at 9.476 min, Area 4,708,803, Area % 2.16), the three best library matches were recorded, and the highest-quality match with consistent retention behavior was selected for the results table. Peaks with a match score ≥80% and not present in blanks above 10% intensity were reported. Relative abundances were expressed as normalized peak area percentages [8].

DPPH radical scavenging assay

The antioxidant activity of *C. odorata* and *C. grata* extracts was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [23,24]. Stock solutions (10 mg/mL) of methanol, ethyl acetate, hexane, and aqueous extracts were prepared, and serial dilutions (0.0156-1 mg/mL) were made in the respective solvents. A 0.1 mM DPPH solution in methanol was freshly prepared, and 1 mL was mixed with 1 mL of each extract dilution. Reaction mixtures were incubated in the dark for 30 min at room temperature, and absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Trolox was used as a positive control. Percentage inhibition was calculated as:

$$Percent\ Inhibition\ (\%) = \frac{A\ control - A\ sample}{A\ control} \times 100$$

where *A control* is the absorbance of the DPPH solution without extract, and *A sample* is the absorbance with extract.

FTC inhibition assay

Ferric thiocyanate (FTC) inhibition activity was determined following a modified method to evaluate the lipid peroxidation inhibitory potential of *C. grata* and *C. odorata* extracts [19]. Briefly, extract solutions were prepared in appropriate solvents and incubated with

a linoleic acid emulsion under controlled conditions. At predetermined time intervals (0, 24, 48, and 72 h), aliquots were withdrawn and reacted with ammonium thiocyanate and ferrous chloride under acidic conditions. The absorbance was measured at 500 nm using a spectrophotometer. Percentage inhibition of lipid peroxidation was calculated relative to the control according to the formula:

$$FTC\ \%\ Inhibition\ = \frac{A\ control\quad A\ sample}{A\ control} \times 100$$

Cyclooxygenase (COX) enzyme inhibition assay

The inhibitory activity of *C. grata* and *C. odorata* leaf extracts against cyclooxygenase enzymes was determined using a commercially available COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol with minor modifications [25]. Dried leaf extracts obtained using methanol, hexane, ethyl acetate, and aqueous solvents were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 10 mg/mL. Working concentrations were prepared by serial dilution with assay buffer. The assay quantifies the conversion of arachidonic acid to prostaglandin catalyzed by COX enzymes. In brief, 50 µL of COX enzyme solution was added to each well of a 96-well microplate, followed by 10 µL of test extract at various concentrations. After incubation at 37°C for 10 min, 10 µL of arachidonic acid substrate (1.0 mM) was added to initiate the reaction. The mixture was incubated for an additional 2 min. Prostaglandin production was detected by adding the chromogenic substrate, and absorbance was measured at 590 nm using a microplate reader (BioTek Epoch, USA). Indomethacin, a known COX inhibitor, was used as a positive control. Negative controls included reactions without plant extracts. All samples, controls, and blanks were assayed in triplicate. The percent inhibition of COX activity was calculated using the formula:

$$COX\ \%\ Inhibition\ = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Statistical analysis

All statistical analyses for the quantification of phytochemicals and data visualizations were performed using RStudio 2025.05.1 Build 513, "Mariposa Orchid" Release (Posit Software, PBC, 2025). A two-way ANOVA was conducted for each phytochemical to evaluate the effects of species (*C. odorata, C. grata*), extract type, and their interaction. Effect sizes were calculated as generalized eta-squared (ges) for ANOVA tables and partial eta squared (η^2_p) with 95% confidence intervals for each factor. Where significant main effects were detected, Tukey's HSD post-hoc tests were applied for pairwise comparisons. Regarding the DPPH assay, percent inhibition values for each concentration were calculated and plotted to generate dose-response curves for each extract. A four-parameter logistic regression model was used to fit the curves and estimate IC_{50} values (the concentration required to achieve 50% inhibition). All measurements were performed in triplicate, and data are presented as mean ± standard deviation (SD). Mean per cent inhibition values at selected concentrations were visualized using bar plots to compare antioxidant activity between species and extracts. The effects of species and extract type on antioxidant activity were evaluated using two-way ANOVA, with species (*C. odorata*, *C. grata*) and extract type as fixed factors. For the FTC assay statistics, a repeated-measures ANOVA test was conducted. Data from 24, 48, and 72 h were used for time-course statistical comparison. Additionally, the area under the inhibition–time curve (AUC) was calculated for each replicate to represent the cumulative inhibitory effect over the 72-h incubation period. Regarding the COX assay, the concentration–response data were fit by nonlinear regression using a four-parameter log-logistic model (4PL; bottom constrained to 0 when appropriate). IC_{50} estimates and 95% confidence intervals were obtained from the fitted models; fits were inspected for leverage points and lack-of-fit. To evaluate the group-wise effects, mean per cent inhibition values were analyzed using a two-way ANOVA with fixed factors species (2 levels: *C. odorata*, *C. grata*) and extract (4 levels: aqueous, hexane, ethyl acetate, methanol). Multiple comparisons were performed with Tukey's HSD, and compact letter displays were used on the bar plot to denote groups that differ at $\alpha = 0.05$. Analyses were performed in R (version to be stated) using drc or nls for 4 PL fits, rstatix/afex for ANOVA and effect sizes, and emmeans for Tukey contrasts. Figures (dose-response curves and grouped bar plots with the mean±SE and letters) were generated with ggplot2. Correlation analysis was conducted using the cor function in R, with significance levels

assessed by the cor.mtest function from the corrplot package. PCA was conducted using the PCA function from the FactoMineR package, and visualized with the factoextra and ggplot2 packages [17,26].

RESULTS

Quantification and assessment of the major bioactive polyphenol groups

Multivariate analysis of variance (MANOVA) was conducted to assess the combined effects of species (*C. grata* and *C. odorata*) and extraction solvents on leaf phenolic, flavonoid, and tannin contents (Supplementary Table S1). The MANOVA (Pillai's Trace) indicated a highly significant main effect of species (Pillai=0.9685, F (3,14) =143.35, P<0.001) and a significant main effect of extract (Pillai=0.9341, F (9,48)=2.41, P=0.024). The species × extract interaction was not significant (Pillai=0.6963, F (9,48) =1.61, P=0.139), indicating that extraction effects were broadly similar for both species.

Given the significant multivariate effects for MANOVA analysis, we conducted separate two-way ANOVAs for each phytochemical (Table 1). The species exerted a strong and highly significant influence on all measured compounds: phenolics ($F_{1,16}$ = 135.42, P<0.001), flavonoids ($F_{1,16}$ = 95.38, P<0.001), and tannins ($F_{1,16}$ = 110.62, P<0.001). The extraction method significantly influenced the phenolic content (F (3,16) =4.52, P=0.018) and flavonoids (F (3,16)=4.25, P=0.022), but not tannins (F (3,16)=0.79, P=0.517). The interaction terms were non-significant for all compounds (P>0.1), reinforcing the MANOVA finding that extraction effects were consistent across species. Species effects were exceptionally large for phenolics (η^2p=0.894), flavonoids (η^2p=0.856), and tannins (η^2p=0.874), indicating that species identity explained most of the variations in compound concentrations (Supplementary Table S2). Extraction effects were moderate for phenolics (η^2p=0.459) and flavonoids (η^2p=0.444), but small for tannins (η^2p=0.129). Interaction effects were small to moderate in magnitude ($\eta^2p\leq0.299$).

Table 1. Two-way ANOVA results for phenolics, flavonoids, and tannins in leaves of *C. grata* and *C. odorata*

Compound	Effect	DFn	DFd	F	p-value	Significance	ges
Phenolics	Species	1	16	135.42	<0.001	***	0.894
	Extract	3	16	4.524	0.018	*	0.459
	Species × Extract	3	16	2.280	0.118	ns	0.299
Flavonoids	Species	1	16	95.381	<0.001	***	0.856
	Extract	3	16	4.253	0.022	*	0.444
	Species × Extract	3	16	0.683	0.575	ns	0.114
Tannins	Species	1	16	110.62	<0.001	***	0.874
	Extract	3	16	0.789	0.517	ns	0.129
	Species × Extract	3	16	2.212	0.126	ns	0.293

ges – generalized eta-squared effect size measure from ANOVA; ***P<0.001, **P<0.01, *P<0.05, ns – not significant

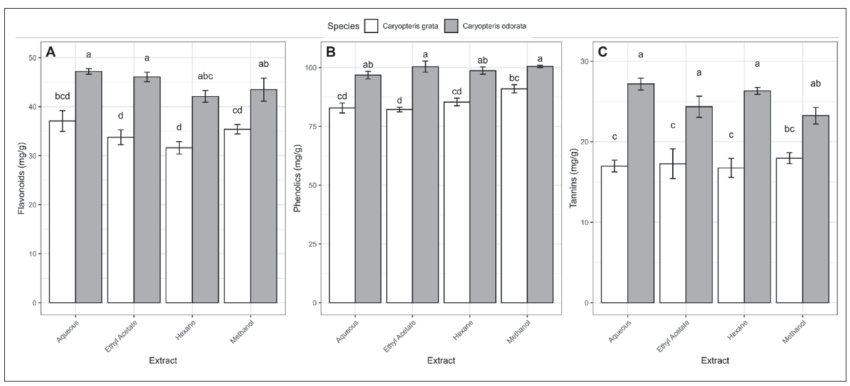

Fig. 1. Quantification and comparative analysis of the major bioactive polyphenol groups in Caryopteris species collected from the Nakyal mountainous region of Kotli, AJK, Pakistan. **A** – flavonoids; **B** – phenolics; **C** – tannins. Data are presented as mean±standard deviation (n=3). Bars sharing different letters within each compound group represent statistically significant differences (P<0.05) according to Tukey's HSD post-hoc test.

For flavonoids (Fig. 1A), *C. odorata* consistently demonstrated higher concentrations than *C. grata* for all extraction types, consistent with the large and significant species effect found in the ANOVA (η^2p = 0.856). In *C. odorata*, aqueous and ethyl acetate extractions yielded the highest flavonoid levels (both labeled "a" in Tukey's test), followed by methanol and hexane. In *C. grata*, the highest flavonoid content was recorded in the aqueous extracts, while the hexane extracts produced the lowest values. The significant extraction effect (P=0.022) reflects these variations

Table 2. GC-MS profile of *C. grata* leaf extract

Peak No.	RT (min)	Area %	CAS No.	IUPAC Name	Common Name
1	9.476	2.16	2033-30-9	5,6-dimethyl-1h-benzimidazol-2-one	—
2	9.754	2.15	54789-69-4	2-methyl-4h-3,1-benzoxazin-4-one	—
3	10.760	1.73	3368-21-6	3-(4-isopropylphenyl)prop-2-enoic acid	4-isopropylcinnamic acid
4	10.861	1.21	73573-81-6	1,2-dimethoxy-4-[(1e)-2-[(1r,2r)-2-(3,4-dimethoxyphenyl) cyclohex-3-en-1-yl]ethenyl]benzene	—
5	11.011	61.93	63785-74-0	methyl 7-methylcyclopenta[c]pyran-4-carboxylate	—
6	12.594	0.60	504-96-1	7,11,15-trimethyl-3-methylidenehexadec-1-ene	—
7	13.536	2.57	57-10-3	hexadecanoic acid	palmitic acid
8	14.440	1.73	150-86-7	(2e,7r,11r)-3,7,11,15-tetramethylhexadec-2-en-1-ol	phytol
9	14.691	2.38	506-44-5	(9z,12z,15z)-octadeca-9,12,15-trien-1-ol	α-linolenyl alcohol
10	16.461	10.21	451-79-6	(e)-n'-(2-fluorophenyl)methanimidoyl hydroxylamine	o-fluorobenzaldoxime
11	16.884	0.77	1000377-93-5	bis(2-propylpentyl) benzene-1,2-dicarboxylate	di(2-propylpentyl) phthalate
12	17.494	1.02	128535-01-3	n-phenyl-2-methylcyclopent-2-ene-1-carboxamide	—
13	17.579	1.04	35963-08-7	(13α,17aα)-1,2:14β,15β:21,23-triepoxy-7α-hydroxy-4,4,8-trimethyl-17-oxa-d-homo-24-nor-5α-chol-20,22-diene-3,16-dione	—
14	17.799	2.66	5682-82-6	2,5-dicyclopentylidenecyclopentan-1-one	—
15	18.638	2.74	1000435-69-4	4-chloro-2-(9-fluoro-[1,2,4]triazolo[1,5-c]quinazolin-2-yl) phenol	—
16	18.975	1.06	1000212-78-7	5-(4-fluorophenylsulfanyl)-6-methoxy-4-methyl-8-nitroquinoline	—
17	19.253	2.92	20390-12-9	6a,11a-dihydro-3,8,9-trimethoxy-6h-benzofuro[3,2-c][1] benzopyran	—
18	20.671	1.13	1139-17-9	isolongifol-5-ol	Isolongifolol

Table 3. GC-MS Profile of *C. odorata*

Peak No.	RT (min)	Area %	CAS No.	IUPAC Name	Common Name
1	7.572	6.42	496-16-2	2,3-dihydro-1-benzofuran	dihydrobenzofuran
3	9.722	30.59	91-64-5	2h-1-benzopyran-2-one	coumarin
5	13.525	7.02	57-10-3	hexadecanoic acid	palmitic acid
6	14.445	10.39	150-86-7	(2e,7r,11r)-3,7,11,15-tetramethylhexadec-2-en-1-ol	phytol
7	14.681	5.14	463-40-1	(9z,12z,15z)-octadeca-9,12,15-trienoic acid	α-linolenic acid
8	16.435	6.51	1000353-20-4	3-methoxythiophenol, s-trifluoro-	—
9	16.884	7.10	1000377-93-5	bis(2-propylpentyl) benzene-1,2-dicarboxylate	di(2-propylpentyl) phthalate
10	17.794	4.24	1000443-23-4	4-cyclohexyl-1-(furan-2-ylmethyl)piperazine	—
11	19.831	6.97	20535-83-5	6-methoxy-1h-purin-2-amine	—

among solvent types, though the absence of a significant interaction suggests similar extraction patterns in both species. For phenolics, *C. odorata* consistently exhibited higher total contents than *C. grata* across all extraction types (Fig. 1B). In both species, methanol extraction yielded the highest phenolic concentrations, as indicated by shared significance letters in Tukey's test. In *C. odorata*, phenolic levels from ethyl acetate and methanol extractions did not differ significantly, while in *C. grata*, aqueous and hexane extractions produced statistically similar phenolic contents. These patterns align with the significant species and extraction main effects detected in the ANOVA, with species differences being particularly pronounced.

For tannins (Fig. 1C), *C. odorata* again had consistently higher concentrations than *C. grata* across all extracts, in agreement with the large species effect ($\eta^2p=0.874$) and non-significant extraction main effect. In *C. odorata*, aqueous, ethyl acetate, and hexane extracts showed the highest tannin yields (all labeled "a"), whereas methanol extracts were slightly lower ("ab"). In *C. grata*, methanol extraction produced

the highest tannin content, with aqueous, ethyl acetate, and hexane extracts showing similarly lower levels (all "c"). The extraction significance (P=0.517) in the ANOVA suggests these visual differences are not statistically robust.

GC-MS analysis of *C. grata* and *C. odorata*

The GC-MS profile of *C. grata* revealed a distinct dominance of a single compound, 7-methylcyclopenta[c]pyran-4-carboxylic acid methyl ester (61.93% area), which was absent from *C. odorata* (Table 2). In contrast, *C. odorata* exhibited a more balanced chemical composition (Table 3), with coumarin (30.59%) as the principal constituent, followed by phytol (10.39%) and *n*-hexadecanoic acid (7.02%). Both species contained three compounds, i.e., n-hexadecanoic acid, phytol, and phthalic acid, di(2-propylpentyl) ester, with significant differences in their relative abundance. While *C. grata* contained these at 2.57%, 1.73%, and 0.77% respectively, *C. odorata* possessed markedly higher proportions of 7.02%, 10.39%, and 7.10%, indicating a greater contribution of these shared metabolites to its overall chemical profile.

Several compounds were unique to each species. *C. grata* has O-fluorobenzaldoxime (10.21%) along with multiple synthetic or halogenated compounds such as 4-chloro-2- {9-fluoro- [1,2,4] triazolo[1,5-c] quinazolin-2-yl} phenol and other complex aromatic heterocycles. *C. odorata* was characterized by naturally occurring phytochemicals such as 9,12,15-octadecatrienoic acid (5.14%), benzofuran derivatives, and nitrogen-containing heterocycles like alkyl piperazines and purine derivatives. Collectively, *C. grata* demonstrated a chemical profile dominated by a single ester and a suite of nitrogen/halogen-containing heterocycles, suggesting the presence of specialized metabolites or potential environmental contamination markers. In contrast, *C. odorata* presented a more typical phytochemical fingerprint, enriched with coumarins, fatty acids, and terpenoid alcohols, reflecting a broader spectrum of natural plant secondary metabolites.

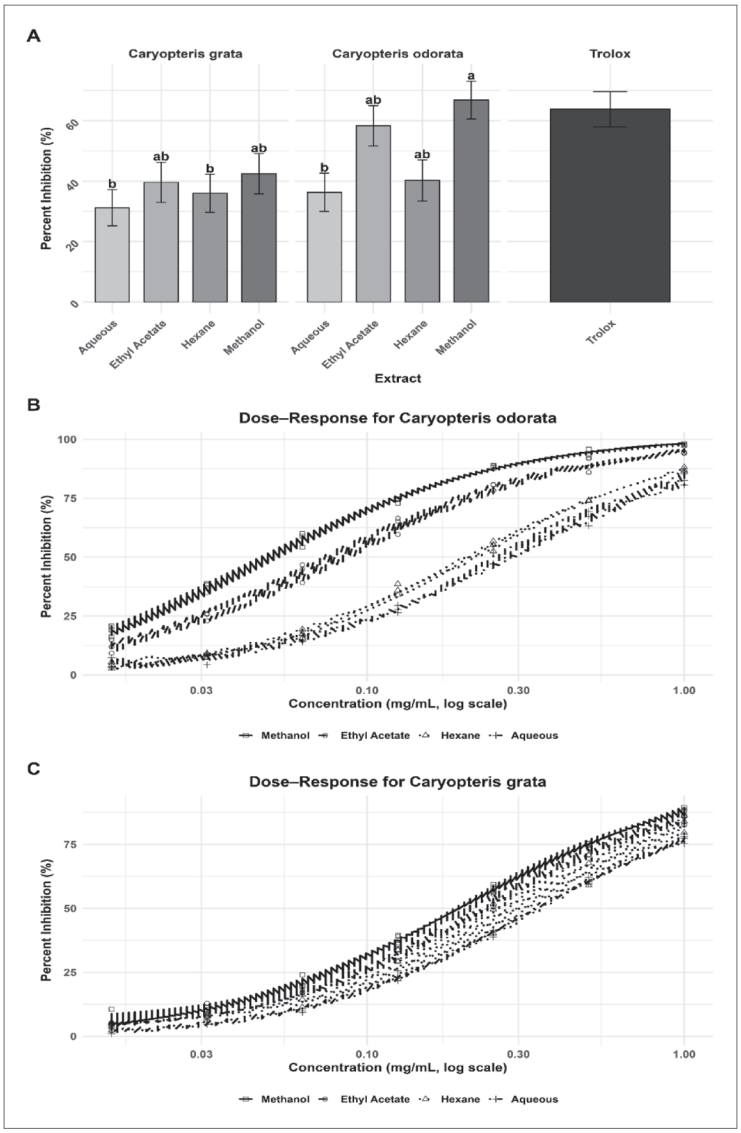

Fig 2. Quantitative and comparative evaluation of antioxidant activities in Caryopteris species. **A** – DPPH percentage inhibition of *C. grata* and *C. odorata* extracts. Data represent the mean±standard deviation (n=3). Bars with different letters within each compound group indicate statistically significant differences (P<0.05) according to Tukey's HSD post-hoc test; **B** – dose-response curves showing DPPH radical scavenging activity of extracts for C. grata; **C** – dose-response curves showing DPPH radical scavenging activity of extracts for *C. odorata*. Curves were fitted using a four-parameter logistic model (LL.4).

DPPH radical scavenging activity

The DPPH radical scavenging activity of *C. odorata* and *C. grata* indicated differences between species and extraction solvents (Fig. 2A). Among *C. odorata* extracts, methanol exhibited the highest inhibition (~67%), statistically comparable to the synthetic antioxidant

Trolox (~64%), followed by ethyl acetate (~58%), hexane (~40%), and aqueous (~36%) extracts. In contrast, *C. grata* demonstrated comparatively lower activity, with methanol (~42%) and ethyl acetate (~40%) extracts outperforming hexane (~36%) and aqueous (~31%) fractions. *C. odorata* polar extracts (methanol, ethyl acetate) showed significantly greater radical scavenging potential than corresponding *C. grata* extracts, suggesting a higher abundance of phenolic or other antioxidant constituents in *C. odorata*. The antioxidant activity of *C. odorata* and *C. grata*, evaluated through DPPH radical scavenging dose-response curves and IC_{50} values, revealed marked differences in potency between species and extracts. In *C. odorata*, methanol and ethyl acetate extracts displayed steep, left-shifted sigmoidal inhibition curves, achieving >90% scavenging at relatively low concentrations (Fig. 2B). These extracts exhibited the lowest IC_{50} values (methanol: 38.51-48.72 µg/mL; ethyl acetate: 62.31-89.85 µg/mL), indicating strong efficacy. Hexane and aqueous extracts showed weaker activity with flatter curves and much higher IC_{50} values (189.16-235.20 µg/mL and 224.00-354.63 µg/mL, respectively). In comparison, *C. grata* demonstrated generally right-shifted dose-response curves, reflecting the need for higher concentrations to achieve comparable inhibition (Fig. 2C). Its methanol (184.48-237.21 µg/mL) and ethyl acetate (195.64-230.66 µg/mL) extracts, while the most active for this species, remained substantially less potent than *C. odorata* equivalents. Hexane and aqueous extracts of *C. grata* were the least effective, with IC_{50} values exceeding 247 µg/mL. Across both species, Trolox, used as a positive control, displayed vastly superior activity (IC_{50}≈1.79-2.15 µg/mL). Overall, *C. odorata*, particularly its methanol and ethyl acetate extracts, exhibited markedly stronger radical scavenging capacity than *C. grata*, suggesting higher concentrations or greater efficiency of antioxidant phytochemicals. Trolox, used as a positive control, exhibited a markedly lower IC_{50} (1.79-2.15 µg/mL; Supplementary Table S3), with its full dose-response curve (Supplementary Fig. S1).

The two-way ANOVA for DPPH radical scavenging activity showed significant main effects of species and extract type. Across all extracts, *C. odorata* and *C. grata* differed significantly in antioxidant potential (F=8.207, P=0.00473), indicating that one species consistently exhibited higher activity than the other. Similarly, antioxidant activity varied significantly among the four extracts irrespective of species (F=4.419, P=0.00515), suggesting that solvent type strongly influenced the extraction of active compounds. The interaction between species and extract was not significant (F=1.203, P=0.31064), implying that the relative ranking of extract effectiveness was similar for both species, with no extract showing contrasting patterns between the two (Supplementary Table S4).

FTC inhibition assay

The repeated-measures ANOVA indicates that the species and extract type had highly significant main effects on per cent inhibition, as well as a significant Species × Extract interaction**,** meaning that the effect of the extract differs between species (Supplementary Table S5). Time also had a small but significant effect, suggesting that percentage inhibition changed over the 24-72 h period. However, none of the interactions involving Time (Species × Time, Extract × Time, Species × Extract × Time) were significant, indicating that the temporal change in inhibition was consistent across species and extracts. This suggests that while the type of extract and species strongly influence antioxidant activity, the effect of time is modest and does not depend on the species or extract type.

In the FTC assay, *C. grata* exhibited relatively low antioxidant potential across all extracts and time points (Supplementary Fig. S2). The bar plot visually reinforces the two-way ANOVA findings by showing clear separation between *C. odorata* and *C. grata* in COX inhibitory activity across all extract types. *C. odorata* consistently produced higher inhibition values, with its methanol extract approaching 65% inhibition and ethyl acetate extract exceeding 55%, both substantially outperforming the corresponding *C. grata* extracts. The positive control (indomethacin) showed ~85% inhibition, while the untreated control had negligible activity. Among the tested extracts, the methanol fraction consistently produced the highest inhibition of lipid peroxidation, with values reaching approximately 12% at 72 h. The ethyl acetate extract showed moderate activity, ranging from 7–9% over time, while the aqueous and hexane extracts demonstrated only weak inhibition, remaining below 5%. Statistical analysis indicated that the methanol extract differed significantly from the aqueous and hexane fractions, whereas ethyl acetate showed intermediate

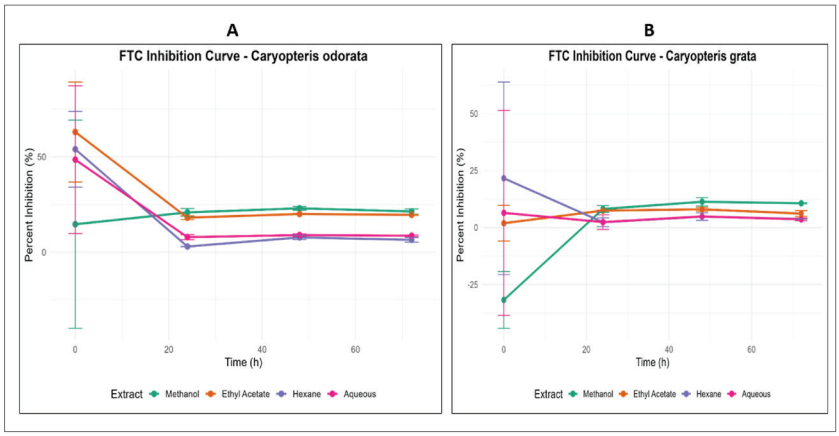

Fig. 3. Time-course curves and comparison of FTC inhibition across four tested extracts **A** – *C. odorata*; **B** – *C. grata*.

behavior. *C. grata* contains modest levels of antioxidant constituents, with methanol extract being the most effective solvent for their recovery. *C. odorata* showed considerably higher antioxidant activity in the FTC assay compared to C. grata. The methanol extract was the most potent, producing inhibition values between 21-24% throughout the assay period, followed closely by the ethyl acetate fraction (18-20%). Both extracts differed significantly from the aqueous and hexane fractions. The aqueous extract displayed moderate inhibition of 8–10%, whereas the hexane extract remained weak (3-7%). The sustained inhibition by methanol and ethyl acetate suggests that *C. odorata* possesses a substantial pool of phenolic and other polar antioxidant metabolites.

C. odorata demonstrated substantially higher FTC % inhibition than *C. grata*, highlighting a stronger antioxidant potential against lipid peroxidation (Fig. 3A). Methanol extracts were proven to be the most potent, peaking at 48 h (23.01±1.78%) before a modest decline at 72 h (21.35±2.47%). The ethyl acetate extracts maintained consistently high inhibition (~18-20%) across all time points, with minimal variability, reflecting stable and reproducible activity. Aqueous extracts showed moderate inhibition (~8-9%), whereas the hexane extracts displayed a weaker trend overall, and a marked increase from 2.97±0.25% at 24 h to 7.75±1.97% at 48 h before declining. Across the extracts, peak inhibition often occurred at 48 h, possibly due to optimal accumulation or release of active compounds before a gradual decline at 72 h. The smaller standard deviations in strong extracts confirm the reliability of these measurements. For *C. grata*, FTC % inhibition remained low across all extracts and time points, indicating limited lipid peroxidation inhibition capacity (Fig. 3B). Of the extracts, methanol consistently achieved the highest inhibition, peaking at 48 h (11.33±3.23%) before a slight decline at 72 h (10.67±0.45%). Ethyl acetate showed a moderate activity, maintaining values between ~6-8% across time, with a small peak at 48 h. Aqueous and hexane extracts demonstrated minimal inhibition (<5% in most cases), with slight increases observed at 48 h. Stronger extracts exhibited relatively low standard deviations, indicating good reproducibility, whereas weaker extracts (e.g., aqueous at 24 h) showed greater variability. Overall, the time trend suggests that antioxidant activity in *C. grata* peaks at mid-incubation (48 h) before stabilizing or declining slightly. The control group showed negligible FTC inhibition across all time points, with mean values fluctuating around zero and standard deviations reflecting baseline variability rather than any true inhibitory effect. No progressive inhibition trend was observed, confirming that changes in the test samples were attributable to plant extracts rather than assay drift or experimental noise (Supplementary Fig. S3).

Comparative COX inhibition profiles of *C. odorata* and *C. grata*

The comparative COX inhibition profiles (Supplementary Fig. S4) indicated that *C. odorata* possesses substantially stronger anti-inflammatory potential. Across all tested solvents, *C. odorata* extracts exhibited lower IC_{50} values and steeper inhibition curves, with methanol and ethyl acetate fractions showing particularly high potency, approaching the activity of the reference drug indomethacin. In contrast, *C. grata* demonstrated only moderate activity in its methanol and ethyl acetate extracts, while hexane and aqueous extracts were largely inactive within the tested concentration range. In the control group (Fig. 4A), the baseline response was flat, with no detectable COX inhibition at any tested

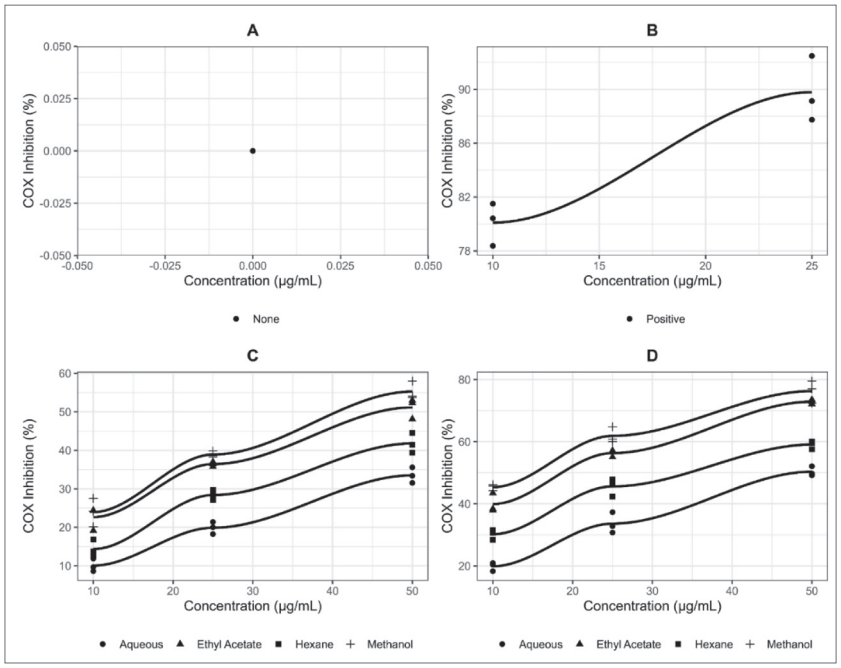

Fig. 4. Dose-response data for cyclooxygenase (COX) inhibition as depicted by various tested extracts **A** – Dose-response plot for the control group showing baseline COX inhibition in the absence of any treatment. Only the untreated point at 0 µg/mL is present, confirming no intrinsic inhibition and validating the use of this panel as a baseline reference; **B** – indomethacin (positive control), demonstrating high COX inhibition (~78–92%) across all tested concentrations; **C** – dose–response curves for *C. grata* extracts; **D** – dose-response curves for *C. odorata* extracts with methanol and ethyl acetate fractions showing high potency with IC_{50} values of ~14 µg/mL and ~19 µg/mL, respectively.

concentration. Only a single data point was present at 0 µg/mL, representing the untreated condition, which maintained 0% inhibition. The indomethacin panel (Fig. 4B) served as the positive control, showing strong COX inhibitory activity even at low concentrations. Inhibition values ranged from approximately 78% to 92% across the tested range, with near saturation observed from the lowest dose onwards, preventing a typical sigmoidal curve fit. For *C. grata*, methanol and ethyl acetate extracts displayed measurable COX inhibition with moderate potency, reaching IC_{50} values of approximately 41.95 µg/mL and 48 µg/mL, respectively (Fig. 4C). In contrast, hexane and aqueous extracts did not reach 50% inhibition within the tested range, suggesting negligible activity under the assay conditions. Overall, *C. grata* demonstrated weaker COX inhibitory potential, with methanol extracts being the most active among the tested solvents but still requiring comparatively higher concentrations to achieve inhibitory effects.

The *C. odorata* extracts (Fig. 4D) exhibited markedly higher potency across all solvents compared to *C. grata*. The methanol and ethyl acetate extracts were the most active, with IC_{50} values of ~14 µg/mL and ~19 µg/mL, respectively, indicating strong inhibitory effects at relatively low doses. The hexane extract showed moderate potency (IC_{50}≈33 µg/mL), while the aqueous extract was weaker but still capable of reaching 50% inhibition (IC_{50}≈49 µg/mL). These results suggest that *C. odorata*, particularly its methanol and ethyl acetate extracts, holds promising potential as a source of bioactive compounds for COX inhibition, surpassing the inhibitory efficacy observed in *C. grata*. Further details regarding IC_{50} (µg/mL) of COX inhibition by extract (mean of triplicates) and IC_{50} per replicate summary (mean ± SD) for COX inhibition have been updated in the supplementary data (Supplementary Tables S6 and S7). Two-way ANOVA analysis confirmed that both plant species and solvent extract type independently and significantly influenced COX inhibitory activity (Supplementary Table S8; Supplementary Fig. S5). The main effect of species was highly significant ($F(1,64) = 34.923$, $P<0.001$, ges=0.353), with *C. odorata* consistently showing higher inhibition than *C. grata* when averaged across all extracts. The large effect size indicates that species identity accounted for approximately 35% of the explainable variance, reinforcing the superior bioactivity of *C. odorata* observed in the dose–response curves.

The main effect of extract type was also significant ($F(3,64) = 11.109$, $P<0.001$, ges=0.342), reflecting the general trend that methanol and ethyl acetate fractions were more potent than aqueous or hexane extracts, regardless of species. In contrast, the interaction between species and extract type was not significant ($F(3,64) = 0.352$, $P=0.788$, ges=0.016), indicating that the magnitude of difference between the two species was consistent across all solvent extractions. This lack of interaction suggests a broad, species-driven difference in COX inhibition rather than a selective solvent-specific advantage.

Spearman correlation and PCA biplot

The correlation heatmap (Supplementary Fig. S6) demonstrated consistently strong positive associations among the measured phytochemicals and antioxidant activities for *C. grata* and *C. odorata* collected from the Nakyal mountains, Kotli, AJK, Pakistan. DPPH, FTC, and COX assays were almost perfectly correlated (r=0.94-0.99), indicating they capture overlapping antioxidant properties. Tannins showed a near-perfect correlation with flavonoids (r=0.98) and a strong association with phenolics (r=0.88), while flavonoids and phenolics were also closely related (r=0.88), suggesting co-occurrence of these compounds in the plant tissues. Moderate correlations (r=0.36-0.59) between some phytochemical groups and specific antioxidant assays indicate that while overall trends are similar, individual phytochemicals may contribute differently to each bioassay (Supplementary Table S9).

The PCA (Fig. 5) revealed that PC1 accounted for the largest proportion of variance, clearly separating the two species. *C. odorata* samples clustered on the positive PC1 side, associated with elevated tannin, flavonoid, and phenolic contents, and stronger antioxidant activities, whereas *C. grata* grouped on the negative PC1 side, reflecting lower levels of these traits. The 95% confidence ellipses showed no overlap, indicating distinct phytochemical-bioactivity profiles. These findings suggest that *C. odorata* from this region may be a comparatively richer source of phenolic antioxidants, with greater pharmacological potential.

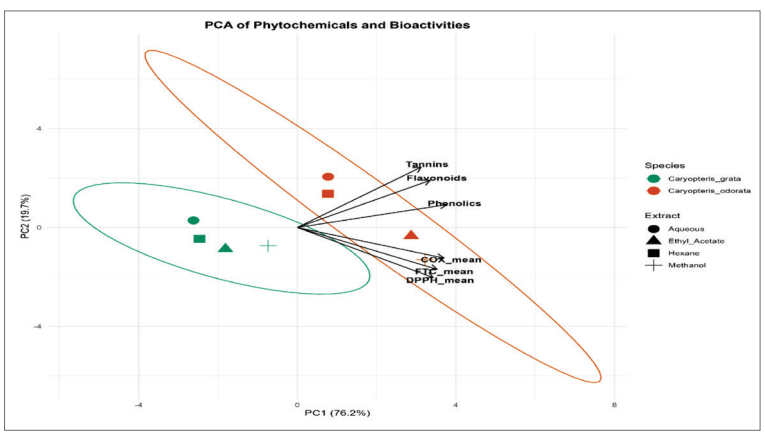

Fig. 5. Principal component analysis (PCA) of phytochemical traits and bioactivities in *C. grata and C. odorata*. The biplot shows species clustering and variable loadings, highlighting the major contributors to variation across extracts and assays.

DISCUSSION

The present investigation provides further information on the phytochemical composition, antioxidant capacity, and anti-inflammatory potential of *C. odorata* and *C. grata* from the Nakyal mountainous zone of Azad Jammu and Kashmir. The detection of phytochemicals aligns with earlier reports on Caryopteris species from other regions [27,28], which have demonstrated a rich phytochemical profile contributing to diverse pharmacological effects [29]. The significant variation in phytochemical content across extraction solvents confirms the solvent polarity dependency of secondary metabolite recovery. The polar and mid-polar solvents, methanol and ethyl acetate, yielded the highest phenolic and tannin contents in the plant species. This is consistent with the well-documented solubility of polyphenols in polar solvents [30].

The GC-MS profiling of *C. odorata* detected multiple bioactive constituents previously reported to possess antioxidant and anti-inflammatory properties [2,5,31]. These compounds may explain the biological effects observed in our assays. Ahmed et al. [2] reported on the pharmacological roles of coumarins. With literature reporting coumarin as a natural anti-inflammatory compound [32] with cultural and medicinal significance [3,5], the study investigated the anti-inflammatory role of the *Caryopteris* species. The detected compound, dihydrobenzofuran, along with its derivatives, has been reported to possess strong radical-scavenging properties [33], attributed to the electron-donating hydroxyl and methoxy groups on its aromatic structure. This is in agreement with the antioxidant activity measured in our study. By inhibiting pro-inflammatory enzymes, including COX and lipoxygenase (LOX), dihydrobenzofurans have been shown to decrease the production of inflammatory mediators [34]. These findings support our GC-MS and subsequent trials on COX inhibition, well supported by our dose-responsive curves.

Our results demonstrated the superior efficacy of *C. odorata* extracts in terms of antioxidant and anti-inflammatory assays. The *C. odorata* GC-MS profile was unique to coumarin, a naturally occurring benzopyrone exhibiting strong antioxidant activity through multiple mechanisms,

including direct free radical scavenging, singlet oxygen quenching, and metal ion chelation [35]. Literature reports that coumarin derivatives also downregulate inflammatory pathways such as NF-κB and suppress COX-2 expression, thereby reducing prostaglandin production [36]. The antioxidant and anti-inflammatory action aligning with the high DPPH scavenging and COX inhibitory activity recorded for *C. odorata* methanol and ethyl acetate extracts, is due to coumarin being a major component (30.59%).

Furthermore, *n*-hexadecanoic acid, commonly known as palmitic acid, exhibits moderate anti-inflammatory properties [37]. While excessive dietary palmitic acid is pro-inflammatory, in plant-derived extracts it may serve as a bioactive lipid with antimicrobial and mild anti-inflammatory effects, possibly via modulation of macrophage activity. It also exhibits some antioxidant potential by stabilizing cell membranes and reducing oxidative damage to lipids [38]. GC-MS also reported the presence of phytol, which has antioxidant capacity through ROS scavenging and inhibition of lipid peroxidation. It exhibits anti-inflammatory effects by suppressing nitric oxide production and downregulating pro-inflammatory cytokines in activated macrophages. These properties likely contribute to the sustained FTC inhibition observed in *C. odorata* extracts [39,40]. α-Linolenic acid is recognized for its potent anti-inflammatory effects through competitive inhibition of arachidonic acid metabolism, leading to reduced synthesis of pro-inflammatory eicosanoids [41]. Its antioxidant role is partly indirect, stemming from membrane stabilization and modulation of oxidative signaling pathways. The presence of α-linolenic acid in *C. odorata* may therefore enhance both radical scavenging and COX inhibitory effects. Presumably, the presence of dihydrobenzofuran, coumarin, phytol, and α-linolenic acid provides a strong chemical basis for the superior performance of *C. odorata* in both DPPH radical scavenging and COX inhibition assays. The crosstalk between these metabolites, particularly the combination of polyphenolic and fatty acid-derived compounds, may enhance overall bio-efficacy through complementary mechanisms of ROS neutralization, membrane protection, and suppression of inflammatory mediator production.

The GC-MS profile of *C. grata* was characterized by 4-isopropylcinnamic acid, palmitic acid, phytol, α-linolenyl alcohol, O-fluorobenzaldoxime, di(2-propylpentyl) phthalate, and isolongifolol. Several of these phytochemicals, including phytol [40,42], palmitic acid [43], and α-linolenyl alcohol [44], have documented antioxidant and mild anti-inflammatory effects through free radical neutralization, lipid peroxidation inhibition, and modulation of eicosanoid biosynthesis. 4-Isopropylcinnamic acid possesses moderate radical scavenging and antimicrobial properties, while isolongifolol [45,46] is reported to have anti-inflammatory and cytoprotective effects. However, some constituents, such as O-fluorobenzaldoxime and di(2-propylpentyl) phthalate, have limited evidence for strong bioactivity in these contexts and may not contribute significantly to pharmacological efficacy. While *C. grata* exhibits a bioactive profile capable of moderate antioxidant and anti-inflammatory activity, the broader and phenolic-enriched metabolite composition of *C. odorata* confers both potency and a broader spectrum of action. The GC-MS analysis revealed species-specific differences in dominant compounds. *C. odorata* contained coumarin, phytol, and *n*-hexadecanoic acid, all of which have documented antioxidant, anti-inflammatory, and antimicrobial properties [47]. In contrast, the predominance of 7-methylcyclopenta[c]pyran-4-carboxylic acid methyl ester in *C. grata* suggests a narrower spectrum of bioactive contribution. This might have marked superior efficacy of *C. odorata* compared to *C. grata.*

The marked superiority of *C. odorata* over *C. grata* in phenolic and tannin contents was mirrored in its stronger antioxidant performance in both DPPH and FTC assays. Phenolics and tannins are known to effectively donate hydrogen atoms or electrons to neutralize free radicals, as well as chelate pro-oxidant metal ions [48]. The high activity of *C. odorata* methanol and ethyl acetate extracts in DPPH radical scavenging confirms their efficiency in targeting stable free radicals, while sustained FTC inhibition indicates effective suppression of lipid peroxidation, a critical pathway in oxidative stress-related cellular damage. The strong positive correlations observed between phytochemical levels and antioxidant assays reinforce the central role of polyphenols in these bioactivities [49].

Caryopteris species, such as *C. incana* extracts have [8] anti-inflammatory activity. There is little data on *C. odorata* from the AJK region. The comparative

underperformance of *C. grata* may reflect either a lower abundance of active polyphenolic compounds or differences in the composition of its bioactive metabolites, as indicated by the GC-MS profiles. The high-altitude ecological origin of these plants in Nakyal may also contribute to their phytochemical richness [50]. Plants growing under environmental stresses such as high UV exposure, temperature fluctuations, and low nutrient availability often accumulate higher levels of secondary metabolites as adaptive mechanisms [51]. This environmental influence might partly explain the antioxidant and anti-inflammatory activities observed in *Caryopteris sp*.

This study addresses a key research gap by being the first to systematically compare *C. odorata* and *C. grata* from this region through complementary antioxidant and anti-inflammatory assays, alongside phytochemical quantification and GC-MS profiling. The findings suggest that *C. odorata*, particularly its methanol and ethyl acetate extracts, hold promise as a natural source of therapeutic agents targeting oxidative stress and inflammation. Further research should focus on bioassay-guided isolation of active compounds, *in vivo* validation, and mechanistic studies, to fully realize the pharmacological potential of these underexplored species. Additionally, *in vitro* findings should be complemented with *in vivo* models to assess pharmacokinetics, bioavailability, and safety profiles. This study underscores the pharmacological potential of *C. odorata* as a natural antioxidant and anti-inflammatory source, and contributes to the phytochemical and bioactivity data available for the *Caryopteris* genus. The study reinforces the significance of AJK's high-altitude flora as a reservoir of bioactive compounds, potentially shaped by unique environmental and ecological factors

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Conflict of interest disclosure: The authors have no competing interests to declare.

Data availability: The data supporting this article are available in the online dataset: https://www.serbiosoc.org.rs/NewUploads/Uploads/Shabbir%20et%20al_Dataset.zip

REFERENCES

1. Joshi A, Pant AK, Prakash O, Kumar R, Stocki M, Isidorov VA. Chemical composition, antimicrobial, and antioxidant activities of the essential oils from stem, leaves, and seeds of *Caryopteris foetida* (D. don) Thell. Indian J Nat Prod Resour. 2021;12(2):214-224. https://doi.org/10.56042/ijnpr.v12i2.38172
2. Ahmed MG, Mehmood MH, Mehdi S, Farrukh M. *Caryopteris odorata* and its metabolite coumarin attenuate characteristic features of cardiometabolic syndrome in high-refined carbohydrate-high fat-cholesterol-fed diet rats. Front Pharmacol. 2023;14:1097407. https://doi.org/10.3389/fphar.2023.1097407
3. Shahzadi T, Abbasi MA, Ur-Rehman A, Siddiqui SZ, Ajaib M. *Caryopteris odorata*: a rich source of antioxidants for protection against chronic diseases and food products. J Chil Chem Soc. 2011;56(2):678-81. http://dx.doi.org/10.4067/S0717-97072011000200012
4. Shao YZ, Hou QZ, Xie ZY, Yang YY, He CY, Zhou F, Zhang J, Liang JY. Bioactivities and chemical constituents of essential oils extracted from Caryopteris mongholica bunge against two stored-product insects. J Essent Oil Bear Plants. 2021;24(1):22-30. https://doi.org/10.1080/0972060X.2021.1875888
5. Shahzadi T, Abbasi MA, Ur-Rehman A, Riaz T, Khan KM, Ashraf M, Afzal I, Akhtar MN, Ajaib M. Antioxidant and lipoxygenase inhibiting new iridoid glucosides from *Caryopteris odorata*. Nat Prod Res. 2013;27(4-5):302-13. https://doi.org/10.1080/14786419.2012.668692
6. Singh D, Mathela CS, Panwar A, Pande V. Sesquiterpene hydrocarbon rich essential oils of Caryopteris odorata (D. Don) Robin: chemical composition, antioxidant and antimicrobial activity. J Essent Oil Res. 2014;26(4):274-81. https://doi.org/10.1080/10412905.2014.922507
7. Khanum H, Ishtiaq M, Maqbool M, Hussain I, Bhatti KH, Mazhar MW, Gul A. Evaluating the conservation value and medicinal potential of wild herbaceous flora in the Sanghar Mountains of District Bhimber, AJK, Pakistan. Ethnobotany Res Appl. 2024;27:1-41. https://doi.org/10.32859/era.27.6.1-41
8. Mushtaq W, Ishtiaq M, Maqbool M, Ajaib M, Hussain T, Mazhar MW, Sardar T. Exploration of antidiabetic potential of traditional ethno-medicinal plant *Viscum cruciatum* Sieber ex Boiss.(Loranthaceae) from Rawalakot district, Poonch, Azad Jammu and Kashmir, Pakistan. Pak J Bot. 2024;56(6):2315-25. https://doi.org/10.30848/pjb2024-6(15)
9. Mazhar MW, Ishtiaq M, Maqbool M, Jafri FI, Siddiqui MH, Alamri S, Akhtar MS. Synergistic effects of selenium nanoparticles and LED light on enhancement of secondary

metabolites in sandalwood (Santalum album) plants through in-vitro callus culturing technique. PeerJ. 2024;12:e18106. https://doi.org/10.7717/peerj.18106
10. Upadhyaya K, Dixit VK, Padalia RC, Mathela CS. Chemical composition of the essential oil of Caryopteris grata Benth. J Essent Oil Res. 2009;21(1):69-70. https://doi.org/10.1080/10412905.2009.9700113
11. Sardar T, Ishtiaq M, Mazhar MW, Maqbool M, Moussa IM, Zaman W, Mahmoud EA. Methyl jasmonate and iron oxide nanoparticles act as elicitors to stimulate production of bioactive antioxidants and metabolites in the in vitro callus cultures of Bergenia ciliata (haw.) Sternb. S Afr J Bot. 2023;162:201-10. https://doi.org/10.1016/j.sajb.2023.09.016
12. Mazhar MW, Ishtiaq M, Maqbool M, Akram R. Efficacy of zinc oxide nanoparticles on bitter gourd growth, yield, and some phytochemicals through seed priming. Int J Veg Sci. 2023;29(2):145-55. https://doi.org/10.1080/19315260.2022.2142876
13. Maqbool M, Ishtiaq M, Mazhar MW, Casini R, Mahmoud EA, Elansary HO. Enhancing bioactive metabolite production in Aerva sanguinolenta callus cultures through silver nanoparticle and salicylic acid elicitation. Sustainability. 2023;15(13):10395. https://doi.org/10.3390/su151310395
14. Ajaib M, Kamran SH, Siddiqui MF, Qasim M, Azeem M, Abideen Z, Elnaggar A, El-Keblawy A. Exploring the phytochemical, antioxidant, antimicrobial and analgesic potentials of Solanum erianthum as an alternative biological feedstock for producing sustainable biochemicals. Biocatal Agric Biotechnol. 2024;58:103183. https://doi.org/10.1016/j.bcab.2024.103183
15. Ajaib M, Ishtiaq M, Bhatti KH, Ahmed KS, Maqbool M, Hussain T. Traditional ethnobotanical knowledge of wild plants of Tilla Jogian district Jhelum, Pakistan. Pak J Bot. 2021;53(4):1303-406. https://doi.org/10.30848/pjb2021-4(19)
16. Mustafa A, Ahmad KS, Mehmood A, Nawaz F, Ahmed H, Hamid A, Liyaqat I. Ethnobotany of Mountain Region of Himalaya, District Poonch, Azad Kashmir. In: Ethnobiology of Mountain Communities in Asia. Cham: Springer; 2021. p. 245-67. https://doi.org/10.1007/978-3-030-55494-1_13
17. Arooj ZE, Ishtiaq M, Mazhar MW, Maqbool M, Hussain T. Assessment of plant community composition and diversity across cemeteries: indicator species of burial sites in Tehsil Samahni, Azad Kashmir, Pakistan. Community Ecol. 2025;1-15. https://doi.org/10.1007/s42974-025-00278-x
18. Gorse MK, Bele P, Udaipure SK. Phytochemical screening and quantitative analysis of active phytocontents of Guizotia abyssinica seed to know their therapeutic values. Int J Health Sci. 2022;6(S2):2880-92. https://doi.org/10.53730/ijhs.v6ns2.5794
19. Evans WC. Trease and Evans' pharmacognosy. Gen Pharmacol. 1997;2(29):291. https://doi.org/10.1016/s0306-3623(97)89724-8
20. Blainski A, Lopes GC, De Mello JCP. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. Molecules. 2013;18(6):6852-65. https://doi.org/10.3390/molecules18066852
21. Aparna B, Hema BP. Preliminary screening and quantification of flavonoids in selected seeds of Apiaceae by UV-visible spectrophotometry with evaluation study on different aluminium chloride complexation reaction. Indian J Sci Technol. 2022;15(18):857-68. https://doi.org/10.17485/ijst/v15i18.131
22. Verza SG, Pavei C, Ortega GG. Study of the specificity of cross-povidone (PVPP) as binding agent in the quantification of polyphenolic compounds. J Braz Chem Soc. 2008;19:1627-33. https://doi.org/10.1590/s0103-50532008000800025
23. Gulcin I, Alwasel SH. DPPH radical scavenging assay. Processes. 2023;11(8):2248. https://doi.org/10.3390/pr11082248
24. Bandonienė D, Murkovic M, Pfannhauser W, Venskutonis P, Gruzdienė D. Detection and activity evaluation of radical scavenging compounds by using DPPH free radical and on-line HPLC-DPPH methods. Eur Food Res Technol. 2002;214(2):143-7. https://doi.org/10.1007/s00217-001-0430-9
25. Badieyan ZS, Moallem SA, Mehri S, Shahsavand S, Hadizadeh F. Virtual screening for finding novel COX-2 inhibitors as antitumor agents. Open Med Chem J. 2012;6:15. https://doi.org/10.2174/1874104501206010015
26. Lê S, Josse J, Husson F. FactoMineR: an R package for multivariate analysis. J Stat Softw. 2008;25:1-18. https://doi.org/10.18637/jss.v025.i01
27. Luczkiewicz M, Jesionek A, Kokotkiewicz A, Migas P, Mardarowicz M, Szreniawa-Sztajnert A, Zabiegala B, Bucinski A. Production of essential oils from in vitro cultures of Caryopteris species and comparison of their concentrations with in vivo plants. Acta Physiol Plant. 2015;37(3):58. https://doi.org/10.1007/s11738-015-1804-0
28. Wu M, Mao X, Wang J, Jin J, Shen L, Zhou J, Huang C, Hu X. Abietane diterpenoids from Caryopteris incana (Thunb.) Miq. and their HIF-2α inhibitory activities in vitro. Phytochemistry. 2024;224:114145. https://doi.org/10.1016/j.phytochem.2024.114145
29. Zhang X, Cao Y, Pan D, Yao X, Wang F, Zhang G, Luo Y. Antifibrotic pyridine-containing monoterpene alkaloids from Caryopteris glutinosa. Phytochemistry. 2022; 203:113378. https://doi.org/10.1016/j.phytochem.2022.113378
30. Montenegro I, González B, Domínguez Á, Gómez E. Solubility study of several polyphenolic compounds in pure and binary solvents. J Chem Thermodyn. 2025;203:107434. https://doi.org/10.1016/j.jct.2024.107434
31. Costa JP, Islam T, Santos PS, Ferreira PB, Oliveira LS, Alencar VOB, Paz FCJ, Ferreira LF, Feitosa M, Citó MGL, Sousa P. Evaluation of antioxidant activity of phytol using non- and pre-clinical models. Curr Pharm Biotechnol. 2016;17(14):1278-84. https://doi.org/10.2174/1389201017666161019155715
32. Saadati F, Chahardehi AM, Jamshidi N, Jamshidi N, Ghasemi D. Coumarin: A natural solution for alleviating inflammatory disorders. Curr Res Pharm Drug Discov. 2024;7:100202. https://doi.org/10.1016/j.crphar.2024.100202
33. Chand K, Hiremathad A, Singh M, Santos MA, Keri RS. A review on antioxidant potential of bioactive heterocycle ben-

zofuran: Natural and synthetic derivatives. Pharmacol Rep. 2017;69(2):281-95. https://doi.org/10.1016/j.pharep.2016.11.007
34. Ayoub AJ, El-Achkar GA, Ghayad SE, Hariss L, Haidar RH, Antar LM, Mallah ZI, Badran B, Grée R, Hachem A, Hamade E. Fluorinated benzofuran and dihydrobenzofuran as anti-inflammatory and potential anticancer agents. Int J Mol Sci. 2023;24(12):10399. https://doi.org/10.3390/ijms241210399
35. Todorov L, Saso L, Kostova I. Antioxidant activity of coumarins and their metal complexes. Pharmaceuticals. 2023;16(5):651. https://doi.org/10.3390/ph16050651
36. Revankar HM, Bukhari SNA, Kumar GB, Qin HL. Coumarins scaffolds as COX inhibitors. Bioorg Chem. 2017;71:146-59. https://doi.org/10.1016/j.bioorg.2017.02.001
37. Aparna, Vasudevan Kalarickal V Dileep, Pradeep K Mandal, Ponnuraj Karthe, Chittalakkottu Sadasivan, Madathilkovilakathu Haridas. Anti-inflammatory property of n-hexadecanoic acid: structural evidence and kinetic assessment. Chem Biol Drug Des. 2012;80(3):434-9. https://doi.org/10.1111/j.1747-0285.2012.01418.x
38. Ganesan T, Subban M, Christopher Leslee DB, Kuppannan SB, Seedevi P. Structural characterization of n-hexadecanoic acid from the leaves of Ipomoea eriocarpa and its antioxidant and antibacterial activities. Biomass Convers Biorefin. 2024;14(13):14547-58. https://doi.org/10.1007/s13399-022-03576-w
39. Islam MT, Ayatollahi SA, Zihad SNK, Sifat N, Khan MR, Paul A, Salehi B, Islam T, Mubarak MS, Martins N, Sharifi-Rad J. Phytol anti-inflammatory activity: Pre-clinical assessment and possible mechanism of action elucidation. Cell Mol Biol. 2020;66(4):264-9. https://doi.org/10.14715/cmb/2020.66.4.31
40. Naikwadi PH, Phatangare ND, Mane DV. Ethanopharmacological anti-inflammatory study of phytol in ethanolic extract of Woodfordia floribunda Salisb. Ann Phytomed. 2022;11(2):426-37. https://doi.org/10.54085/ap.2022.11.2.52
41. Yuan Q, Xie F, Huang W, Hu M, Yan Q, Chen Z, Zheng Y, Liu L. The review of alpha-linolenic acid: Sources, metabolism, and pharmacology. Phytother Res. 2022;36(1):164-88. https://doi.org/10.1002/ptr.7295
42. Ejiofor EU, Onoja SO, Agwamba EC, Louis H, Onyedikachi UB, Onwuasoanya WE, Aguwamba C, Kube MT, Nkume PI, Ekeleme MN, Onodugo AC. Computational, chemical profile, in vitro antioxidant, hypoglycaemic, and anti-inflammatory activity of hexane extract of some selected dark green vegetables. Proc Indian Natl Sci Acad. 2023;89(2):386-400. https://doi.org/10.1007/s43538-023-00169-7
43. Selenge T, Vieira SF, Gendaram O, Reis RL, Tsolmon S, Tsendeekhuu E, Ferreira H, Neves NM. Antioxidant and anti-inflammatory activities of Stellera chamaejasme L. roots and aerial parts extracts. Life. 2023;13(8):1654. https://doi.org/10.3390/life13081654
44. He WS, Zhao L, Sui J, Li X, Huang S, Ding H, Zhu H, Chen ZY. Enzymatic synthesis of a novel antioxidant octacosanol lipoate and its antioxidant potency in sunflower oil. J Agric Food Chem. 2024;72(39):21781-93. https://doi.org/10.1021/acs.jafc.4c07240
45. Li J, Li J, Fang H, Yang H, Wu T, Shi X, Pang C. Isolongifolene alleviates liver ischemia/reperfusion injury by regulating AMPK-PGC1α signaling pathway-mediated inflammation, apoptosis, and oxidative stress. Int Immunopharmacol. 2022;113:109185. https://doi.org/10.1016/j.intimp.2022.109185
46. Balakrishnan R, Vijayraja D, Mohankumar T, Manimaran D, Ganesan P, Choi DK, Elangovan N. Isolongifolene mitigates rotenone-induced dopamine depletion and motor deficits through anti-oxidative and anti-apoptotic effects in a rat model of Parkinson's disease. J Chem Neuroanat. 2021;112:101890. https://doi.org/10.1016/j.jchemneu.2020.101890
47. Behera B, Meher RK, Mir SA, Nayak B, Satapathy KB. Phytochemical profiling, in vitro analysis for anti-inflammatory, immunomodulatory activities, structural elucidation and in silico evaluation of potential selective COX-2 and TNF-α inhibitor from Hydrilla verticillata (Lf) Royle. J Biomol Struct Dyn. 2025;43(2):859-73. https://doi.org/10.1080/07391102.2023.2283871
48. Seidi F, Liu Y, Huang Y, Xiao H, Crespy D. Chemistry of lignin and condensed tannins as aromatic biopolymers. Chem Soc Rev. 2025;54(6):3140-232. https://doi.org/10.1039/D4CS00440J
49. Mitra S, Tareq AM, Das R, Emran TB, Nainu F, Chakraborty AJ, Ahmad I, Tallei TE, Idris AM, Simal-Gandara J. Polyphenols: A first evidence in the synergism and bioactivities. Food Rev Int. 2023;39(7):4419-41. https://doi.org/10.1080/87559129.2022.2026376
50. Mansour G, Ghanem C, Mercenaro L, Nassif N, Hassoun G, Del Caro A. Effects of altitude on the chemical composition of grapes and wine: A review. OENO One. 2022;56(1):227-39. https://doi.org/10.20870/oeno-one.2022.56.1.4895
51. Ashraf MV, Khan S, Misri S, Gaira KS, Rawat S, Rawat B, Khan MH, Shah AA, Asgher M, Ahmad S. High-altitude medicinal plants as promising source of phytochemical antioxidants to combat lifestyle-associated oxidative stress-induced disorders. Pharmaceuticals. 2024;17(8):975. https://doi.org/10.3390/ph17080975

SUPPLEMENTARY MATERIAL

Supplementary Table S1. Multivariate analysis of variance (MANOVA) results for the effects of species (*C. grata* and *C. odorata*) and extracts on combined leaf phenolic, flavonoid, and tannin contents

Effect	df	Pillai's Trace	Approx. F	num df	den df	P value	Significance
Species	1	0.9685	143.35	3	14	< 0.001	***
Extract	3	0.9341	2.41	9	48	0.024	*
Species × Extract	3	0.6963	1.61	9	48	0.139	ns
Residuals	16						

***P<0.001, **P<0.01, *P<0.05, ns=not significant. MANOVA conducted using Pillai's trace to account for correlated dependent variables

Supplementary Table S2. Partial eta-squared (η^2p) effect sizes for phenolics, flavonoids, and tannins with 95% confidence intervals

Compound	Effect	η^2_p	95% CI Low	95% CI High
Phenolics	Species	0.894	0.792	1.000
	Extract	0.459	0.075	1.000
	Species × Extract	0.299	0.000	1.000
Flavonoids	Species	0.856	0.720	1.000
	Extract	0.444	0.059	1.000
	Species × Extract	0.114	0.000	1.000
Tannins	Species	0.874	0.753	1.000
	Extract	0.129	0.000	1.000
	Species × Extract	0.293	0.000	1.000

Supplementary Table S3. IC_{50} values (μg/mL) of *C. odorata*, *C. grata*, and Trolox in the DPPH radical scavenging assay. Values represent individual replicates. Lower IC_{50} indicates higher antioxidant potency

Species	Extract	Replicate	IC50
Caryopteris odorata	Methanol	1	48.7218
Caryopteris odorata	Methanol	2	38.50549
Caryopteris odorata	Methanol	3	37.74657
Caryopteris odorata	Ethyl Acetate	1	70.51308
Caryopteris odorata	Ethyl Acetate	2	62.30806
Caryopteris odorata	Ethyl Acetate	3	89.84921
Caryopteris odorata	Hexane	1	189.1582
Caryopteris odorata	Hexane	2	196.7987
Caryopteris odorata	Hexane	3	235.1968
Caryopteris odorata	Aqueous	1	284.0669
Caryopteris odorata	Aqueous	2	224.0028
Caryopteris odorata	Aqueous	3	354.6311
Caryopteris grata	Methanol	1	184.4775
Caryopteris grata	Methanol	2	191.9407
Caryopteris grata	Methanol	3	237.2114
Caryopteris grata	Ethyl Acetate	1	230.6579
Caryopteris grata	Ethyl Acetate	2	215.461
Caryopteris grata	Ethyl Acetate	3	195.641
Caryopteris grata	Hexane	1	416.2569
Caryopteris grata	Hexane	2	247.7964
Caryopteris grata	Hexane	3	263.0368
Caryopteris grata	Aqueous	1	325.4794
Caryopteris grata	Aqueous	2	337.2168
Caryopteris grata	Aqueous	3	284.4847
Trolox	Trolox	1	2.151186
Trolox	Trolox	2	1.788839
Trolox	Trolox	3	1.906322

Supplementary Table S4. Results of two-way ANOVA for DPPH radical scavenging activity as affected by species and extract type

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Species	1	7215	7215	8.207	0.00473	**
Extract	3	11653	3884	4.419	0.00515	**
Species × Extract	3	3172	1057	1.203	0.31064	ns
Residuals	160	140646	879	—	—	—

df=degree of freedom; Pr(>F) = probability value from the F-test; **P<0.01; *P<0.05; ns=not significant (P≥0.05)

Supplementary Table S5. Repeated-measures ANOVA employed for FTC inhibition

Effect	df (num)	df (den)	F value	P value	Significance
Intercept	1	16	2152.72	< 2.2e-16	***
Species	1	16	319.73	5.34e-12	***
Extract	3	16	160.58	3.75e-12	***
Species × Extract	3	16	33.42	4.02e-07	***
Time	2	32	5.84	0.0069	**
Species × Time	2	32	0.096	0.909	ns
Extract × Time	6	32	0.378	0.888	ns
Species × Extract × Time	6	32	0.425	0.857	ns

***P<0.001, **P<0.01, *P<0.05

Supplementary Table S6. IC_{50} (µg/mL) of COX inhibition by extract (mean of triplicates)

Species	Extract	IC_{50} (µg/mL)
C. odorata	Methanol	14.22
C. odorata	Ethyl Acetate	19.21
C. odorata	Hexane	33.17
C. grata	Methanol	41.95
C. odorata	Aqueous	49.49
C. grata	Ethyl Acetate	48.04
C. grata	Hexane	>50 (not reached)
C. grata	Aqueous	>50 (not reached)

Supplementary Table S7. IC_{50} per replicate summary (mean ± SD) for COX inhibition

Species	Extract	n valid / 3	Mean IC_{50}	SD
C. grata	Aqueous	0/3	—	—
C. grata	Ethyl Acetate	2/3	45.82	0.68
C. grata	Hexane	0/3	—	—
C. grata	Methanol	3/3	42.18	2.05
C. odorata	Aqueous	1/3	46.48	—
C. odorata	Ethyl Acetate	3/3	19.06	1.74
C. odorata	Hexane	3/3	32.71	3.07
C. odorata	Methanol	3/3	14.29	1.20

Supplementary Table S8. Two-way ANOVA analysis confirming both plant species and solvent extract type independently and significantly influenced COX inhibitory activity

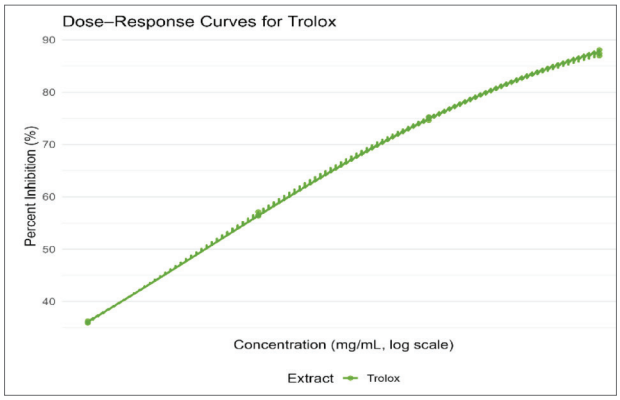
Effect	DFn	DFd	F	P value	Significance
Species	1	64	34.923	1.45×10★	***
Extract	3	64	11.109	5.81×10★	***
Species × Extract	3	64	0.352	0.788	ns

***P<0.001; ns: non-significant

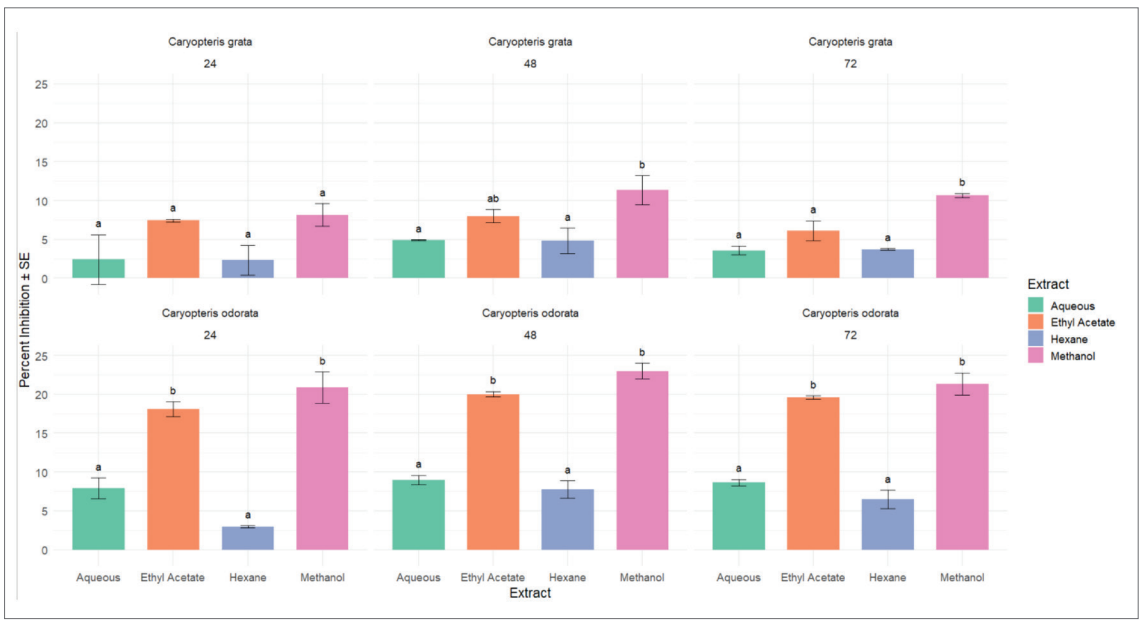
Supplementary Table S9. Pearson correlation coefficients between phytochemical contents and antioxidant activities in *C. grata* and *C. odorata*

	DPPH	FTC	COX	Phenolics	Flavonoids	Tannins
DPPH	1	0.969★★	0.944★★	0.690	0.479	0.362
FTC	0.969★★	1	0.908★★	0.708★	0.586	0.410
COX	0.944★★	0.908★★	1	0.816★	0.582	0.557
Phenolics	0.690	0.708★	0.816★	1	0.856★	0.880★
Flavonoids	0.479	0.586	0.582	0.856★	1	0.919★★
Tannins	0.362	0.410	0.557	0.880★	0.919★★	1

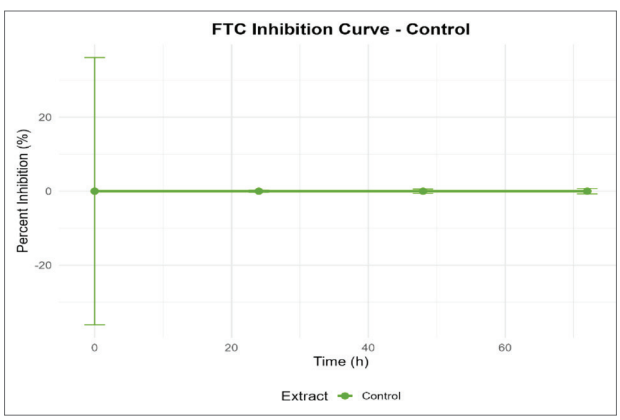
DPPH_mean – 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (mean value); FTC_mean – ferric thiocyanate assay (mean value); COX_mean – cyclooxygenase inhibitory activity (mean value); Phenolics – total phenolic content; Flavonoids – total flavonoid content; Tannins – total tannin content. Significant correlations are marked with stars: ★P<0.05, ★★P<0.01.



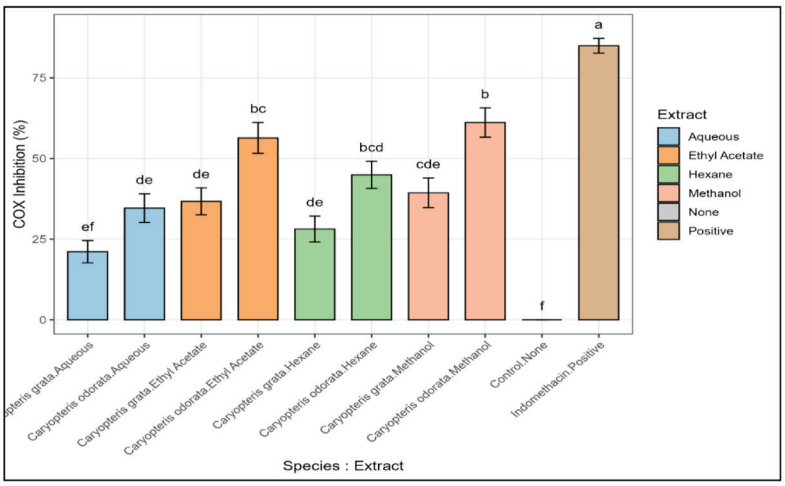
Supplementary Fig. S1. Dose–response curves for DPPH radical scavenging activity of the positive control Trolox (1.79-2.15 µg/mL IC_{50}).



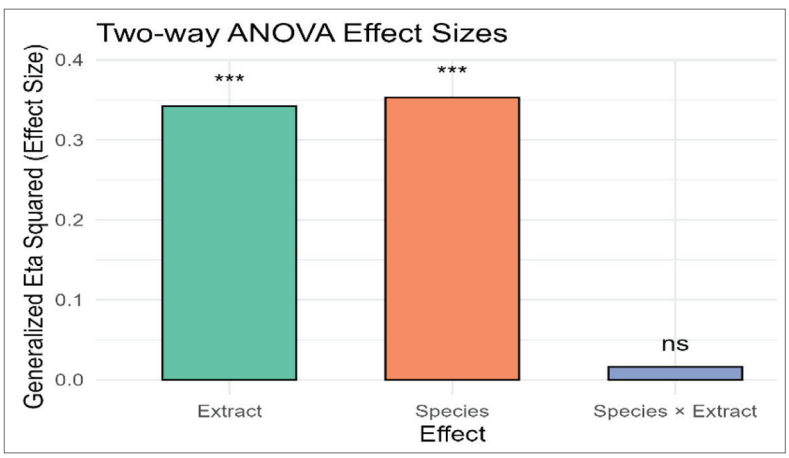
Supplementary Fig. S2. Mean±SE for FTC inhibition (%) over time (24, 48, 72 h) for all extracts of *C. grata* and *C. odorata*, including control.



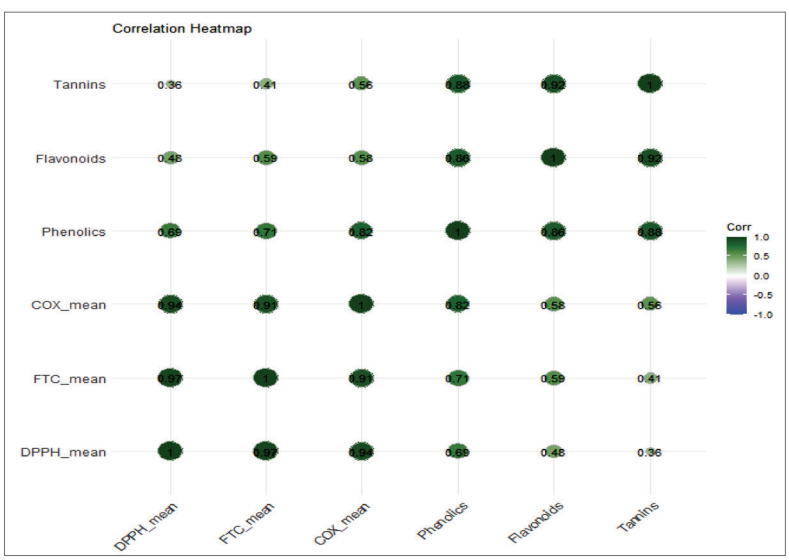
Supplementary Fig. S3. FTC inhibition profile of the control group showing baseline stability across time.



Supplementary Fig. S4. Bar plot showing mean COX inhibition (%)±standard error for *C. grata* and *C. odorata* across four solvent extracts (aqueous, ethyl acetate, hexane, methanol), alongside the untreated control (None) and positive control (Indomethacin). Different lowercase letters above the bars indicate statistically significant differences between groups (P<0.05, Tukey's HSD).



Supplementary Fig. S5. Main effects of species and extract type on COX inhibition, with effect sizes (generalized eta squared) indicated above each bar and significant differences marked with asterisks (P<0.05, P< 0.01, P< 0.001). Magnitude categories: ~0.02 small, ~0.13 medium, ~0.26 large (Cohen's benchmarks adapted for ges).



Supplementary Fig. S6. Correlation heatmap of phytochemical contents and bioactivities of *C. grata* and *C. odorata*. The heatmap illustrates pairwise Pearson correlation coefficients, with color intensity indicating the strength and direction of association between measured traits.