Bull. Chem. Soc. Ethiop. **2027**, 41(1), 149-181. ISSN 1011-3924
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DOI: https://dx.doi.org/10.4314/bcse.v41i1.11 Online ISSN 1726-801X

# ORGAN-SPECIFIC CHEMICAL DIVERSITY AND DIFFERENTIAL BIOACTIVITY OF *RETAMA MONOSPERMA* HEXANE EXTRACTS: COMPARATIVE ANALYSIS OF STEM AND FLORAL TISSUES AS FUNCTIONAL INGREDIENT SOURCES

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(Received March 6, 2026; Revised October 12, 2026; Accepted October 26, 2026)

**ABSTRACT**. Despite extensive ethnobotanical use of *Retama monosperma* in Algerian traditional medicine, organ-specific hexane extract characterization remains limited. This study compared chemical composition, antimicrobial activity, and antioxidant capacity of stem vs. floral extracts from western Algerian plants. Soxhlet extraction (hexane, 6-7 h, 300 mL/25-30 g) was followed by GC-MS (Agilent 7890A-5975C), disc diffusion assays (*Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC29213; 50-100 mg/mL), DPPH $IC_{50}$, and Spearman correlations (ρ) with ANOVA/PCA/permutation validation. GC-MS identified 15 stems vs. 25 floral compounds. Stems showed superior anti-*S. typhimurium* activity (11.33 ± 4.00 mm zone vs. 8.45 ± 1.47 mm; ρ = 0.78, $p = 0.032$, $R^2 = 0.61$; sparteine-driven via LPS destabilization), no Gram-positive effect. Flowers excelled in antioxidants ($IC_{50} = 1.3 \pm 0.1$ mg/mL vs. $1.7 \pm 0.13$ mg/mL; $\rho = -0.85$, $p = 0.011$, $R^2 = 0.72$; unsaturated fatty acids). Anagyrine (16.14%, floral-only) lacked antimicrobial role ($\rho = 0.31$, $p = 0.421$). Stem lipids (78.5%; unsaturated 54.7%; sparteine 5.1%, lupanine 4.0%) support defense; floral hydrocarbons (42.7%; tetratriacontane 24.81%, heptacosane 13.00%)/esters aid reproduction/herbivory deterrence (PCA $p = 0.003$). Organ-selective harvesting optimizes antimicrobials (stems) or antioxidants (flowers), avoiding teratogens. Complementary assays (FRAP/TEAC/ORAC) and pharmacokinetics are recommended.

**KEY WORDS**: *Retama monosperma*, Quinolizidine alkaloids, Organ-specific metabolites, Sparteine, Anagyrine, *Salmonella typhimurium*, Lipopolysaccharide (LPS) destabilization

## INTRODUCTION

Medicinal herbs have been an integral part of traditional health systems worldwide for thousands of years. They are a rich source of bioactive compounds, many of which have demonstrated therapeutic potential for the treatment and prevention of various diseases [1]. According to the World Health Organization (WHO), more than 80% of the population in developing countries depends on traditional medicine, largely based on compounds of plant origin, for its primary health care needs.

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Medicinal plants remain a pivotal source of bioactive compounds, especially in the face of growing challenges such as antibiotic resistance [3], oxidative stress-related diseases, anti-inflammatory, antimicrobial, anticancer, and neuroprotective activities. Secondary metabolites derived from plants, particularly those of low polarity such as fatty acids, terpenes, hydrocarbons, and alkaloids, have attracted increasing attention due to their broad pharmacological properties, including antimicrobial and antioxidant activities.

*Retama monosperma* (L.) Boiss., a wild flowering species belonging to the family Fabaceae, is native to the western Mediterranean basin. In Algeria, it is found in multiple regions along the littoral, including the eastern coast near Jijel and the western coast near Oran**,** where it plays an important role in dune stabilization and is associated with psammophilous plant communities [3]. This species holds a very important place in traditional medicine, where it has long been used for treatment of various pathologies, including fever, inflammation, infections, and diabetes [4]. It is a species used for the frequent remedy of certain renal diseases. Since it has been reported to have considerable diuretic activity, it may also be beneficial for the treatment of hypertension.

Based on Phytochemical studies, *R. monosperma* has been shown to be a rich source of several bioactive compounds, including flavonoids such as genistein, quercetin, kaempferol, apigenin, and luteolin; quinolizidine alkaloids like sparteine, cytisine, anagyrine, N-methylcytisine; various fatty acids (oleic, linoleic, linolenic), sterols, and terpenoids. These metabolites are recognized as the main actors with notable antimicrobial and antioxidant activities of the plant [4–6]. Such phytochemical diversity underscores the importance of systematically evaluating both polar and non-polar extracts for their antioxidant and antimicrobial properties, as demonstrated in recent studies on other medicinal plants [6].

However, most studies on *Retama monosperma* have focused on polar extracts (methanol, ethanol), while non-polar fractions, such as hexane or ethyl acetate extracts, remain limited. Lipophilic constituents generally interact with microbial membranes and scavenge free radicals, mechanisms underlying the antimicrobial and antioxidant effects [7]. In support of this, hexane extracts of *R. monosperma*, collected in Mehdia-Rabat (Morocco), were shown to induce apoptosis and cell cycle arrest in Jurkat leukemia cells, and ethyl acetate fractions exhibited strong antioxidant activity ($1.51 \pm 0.11$ mg/mL) in the seed extracts.

Recent research has increasingly focused on the chemical characterization and biological properties of *R. monosperma*. For example, the analysis of the aerial parts (stems, leaves, seeds) identified 13 known quinolizidine alkaloids, cytisine, sparteine, ammodendrine, and anagyrine among the major compounds via methanolic extraction and GC-MS. Merghoub *et al.* [8], also demonstrated that the extract of *R. monosperma* collected in Morocco contains significant levels of these alkaloids and that they contribute to cytotoxic and pro-apoptotic activity in human cervical cancer cell lines. A study by Outaki *et al.* [9], characterized the phenolic of *R. monosperma*, which is also collected in Morocco (the region of El-Jadida), by UHPLC-DAD/ESI-MS, revealing a rich composition in flavonoids, isoflavones, and flavonols associated with high antioxidant capacity. Similarly, analysis of hexane extracts showed high proportions of fatty acids and sterols (linolenic acid, campesterol, stigmasterol, and sitosterol), which correlate with the antiproliferative and cell-cycle arrest effects in Jurkat cells. More recently, a LC-ESI/MS/MS study confirmed the diversity of phenolic metabolites and demonstrated antimicrobial potential in *R. monosperma* extracts.

In this context, the present study aimed to investigate and compare the chemical composition of hexane extracts obtained from the stems and flowers of *Retama monosperma* using gas chromatography-mass spectrometry (GC-MS). Furthermore, their antibacterial activity was evaluated against *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* using the disc diffusion method, and their antioxidant activity was assessed by the DPPH radical scavenging assay. Comparing organ-specific extracts, this study contributes to a deeper understanding of the phytochemical diversity and bioactive potential of *Retama monosperma*, supporting its further exploration as a source of natural therapeutic agents.

To the best of our knowledge, the comparison of the chemical composition of the floral extract and stem of *R. monosperma* has not yet been studied, nor its pharmacological properties such as antibacterial or antioxidant activities of the hexanoic extract of the flower and stem of *R. monosperma* from the western region of Algeria. Additionally, geographic origin significantly influences phytochemical composition in medicinal plants due to variable soil chemistry, climate, and UV exposure. Algerian populations of *R. monosperma* represent an understudied reservoir of chemical diversity, and characterization of organ-specific extracts from this region may reveal distinct bioactive profiles compared to previously documented populations from North Africa**.** Plant organs often accumulate distinct specialized metabolites reflecting their unique physiological roles and biosynthetic capacities.

Structural tissues (stems) typically maintain elevated lipid and polysaccharide concentrations for mechanical support and drought resistance, while reproductive organs (flowers) frequently concentrate signaling compounds, volatile esters, and secondary metabolites involved in pollinator attraction and seed protection [10]. Understanding these organ-specific chemical differences is crucial for optimizing extraction strategies and predicting biological activities in development of pharmaceuticals or nutraceuticals.

## MATERIALS AND METHODS

### *Chemicals and reagents*

All starting materials, solvents**,** and culture media for the antimicrobial evaluation (Nutrient agar, Nutrient broth, Mueller-Hinton and Sabouraud) used in the present work were purchased from Sigma-Aldrich and were used without any further purification.

### *Preparation and identification of plant material*

The flowers and stems of *Retama monosperma* were collected from Mostaganem area, located in the western region of Algeria, in March 2022. The plant material was identified and validated with the help of Prof. Dr. S. Hadjadj-Aoul. (Botany Department, Oran1, Algeria).

The plant was cleaned, washed with tap water, and dried in the shade. Then, it was weighed, coarsely ground, and collected in clean bags. The flower and the stems were separated and washed to remove soil-associated particles, then left to dry at room temperature for 7 days. After drying, the biomaterials were ground with a laboratory mechanical grinder into a powder separately

### *Preparation of plant extract*

Samples (25- 30 g dry powder) were extracted by Soxhlet with 300 mL hexane for 6- 7 hours; the solvent was removed under reduced pressure to yield the crude hexane extract. Soxhlet extraction yielded 0.60 g from 30.0 dried stem (2.0% w/w) and 0.58 g from 25.0 g dried flower (2.3% w/w). Both extracts appeared as green gummy residues with characteristic pleasant odors, consistent with the enrichment of lipophilic constituents.

### *GC-MS analysis*

GC-MS analyses were performed on an Agilent 7890A-5975 with a VF-WAX column (30 m × 0.25 mm ID, 0.25 µm film). Helium was the carrier gas at 1.168 $mL \cdot min^{-1}$; the oven program began at 40 °C (2 min), ramped at 4 $°C \cdot min^{-1}$ to 260 °C, and held for 5 min. compounds were identified by comparison of mass spectra and linear retention indices with the NIST 11 library and, where available, authentic standards. Identifications were retained only above an ≥ 80% mass-spectral match score, with linear retention indices (Kováts method) reported alongside each

identification in Table1. No commercial standards were available for co-injection confirmation of the individual alkaloids in this study; this is now stated explicitly as a limitation of the identification approach.

The mass spectrometer was operated in electron ionization (EI) mode with 70 eV ionization energy, full-scan detection (m/z 50-500), and 3 scans/second. The identification of compounds in the chromatographic profiles was performed by comparing their mass spectra with a library database (NIST) [11, 12], following established protocols for the GC-MS analysis of plant extracts [13].

*Antibacterial evaluation*

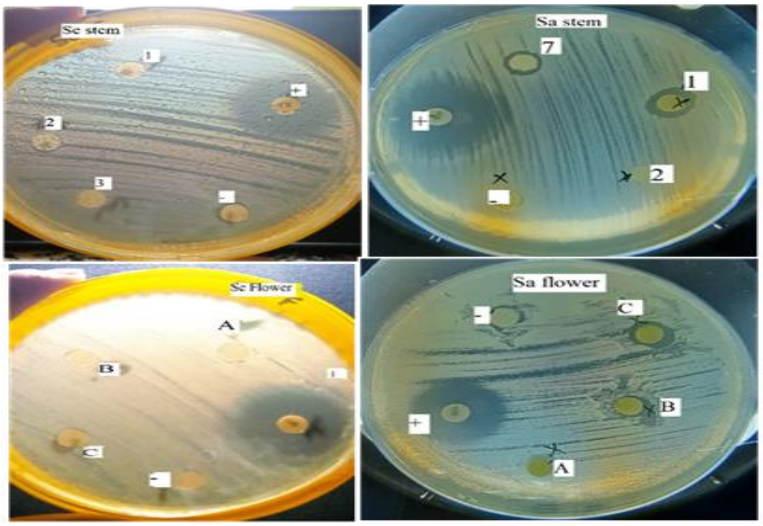
*Microbial strains:* The antibacterial activity of the extracts was studied in *vitro* on the growth of two bacterial strains: *Salmonella typhimurium* ATCC 14028 (Gram-negative bacteria) and *Staphylococcus aureus* ATCC29213 (Gram-positive bacteria), in Petri dishes, according to the method used by Nourai *et al.* [14], with some modifications. These strains belong to the collection of the research laboratory: Laboratory of Microbial Biology and Biotechnology (LBMB).

*Preparation of the inoculum:* Each bacterial strain must be streaked onto a selective agar (Hektoen agar for *Salmonella typhimurium* and Chapman agar for *Staphylococcus aureus*) to obtain isolated colonies. After 24 h at 37 °C, 3- 4 isolated colonies were suspended in nutrient broth and adjusted to a 0.5 McFarland turbidity standard ($\approx 1.5 \times 10^8$ CFU·mL$^{-1}$) prior to lawn inoculation following CLSI guidelines.

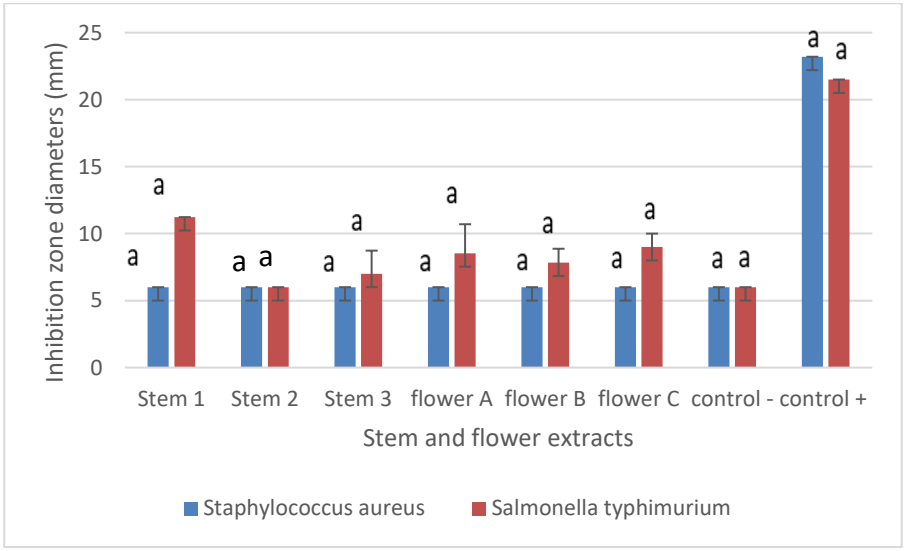
*Disc diffusion method:* For each inoculum, dip a sterile swab into the bacterial suspension. Press against the inner wall of the tube to remove excess liquid. Spread three times over the entire surface of the Mueller Hinton agar, rotating the plate after each application to achieve an even distribution of the bacterial inoculum.

For disc diffusion assays, crud extracts were prepared at 50, 75, and 100 mg· mL$^{-1}$ in hexane; 10µL of each solution (equivalent to 0.5, 0.75, and 1.0 mg per disk) was applied to sterile 6 mm Whatman No. 1 paper disks, the solvent was allowed to evaporate, and disks were placed on inoculated Muller-Hinton agar. Antibacterial activity is compared to of the positive control (Chloramphenicol at 30 µg) and the negative control (solvent). After placing disks onto the agar surface, plates were left at room temperature for 1 hour to allow pre-diffusion, then incubated at 37 °C for 24hours.

Inhibition zones diameters were measured in millimeters as the total diameter edge-to-edge across the clear zone (including disk diameter) using a calibrated ruler; each plate was read in two perpendicular directions and the mean value reported. A triplicate is performed for each studied plant pathogenic strain. Figure 1.a shows effect of hexane stem and flower extracts on the strains of *Salmonella typhimurium* and *Staphylococcus aureus*.




(a)




(b)

Figure 1. a: Effect of hexane stem and flower extracts on the strains of *Salmonella typhimurium* and *Staphylococcus aureus*, b: Differential inhibition profiles of stem versus flower hexane extracts of *Retama monosperma* against Gram-Negative and Gram-Positive pathogenic bacteria.

*Statistical analysis*

Statistical analyses were performed using Statistica v.12; data are reported as mean ± SD (n = 3). One-way ANOVA followed by Newman-Keuls post-hoc testing was used for group comparisons; significance was set at $p < 0.05$. Exact p-values and degrees of freedom are provided in Table 1.

Table 1. Two-Way ANOVA for inhibition zone diameter (mm) against Salmonella typhimurium ATCC 14028.

| Source of variation | Sum of squares | df | Mean square | F-statistic | p-value | $\eta^2$ Effect size | Interpretation |
|---|---|---|---|---|---|---|---|
| Extract source (Stem vs. Flower) | 41.33 | 1 | 41.33 | 6.82 | 0.021* | 0.36 | Significant (Medium effect) |
| Concentration (50/75/100 mg/mL) | 8.92 | 2 | 4.46 | 0.74 | 0.495 | 0.11 | Not significant |
| Interaction (Source × Concentration) | 12.67 | 2 | 6.33 | 1.04 | 0.379 | 0.15 | Not significant |
| Error (Residual) | 73.00 | 12 | 6.08 | — | — | — | — |
| Total | 136.00 | 17 | — | — | — | — | — |

$p < 0.05$ indicates statistical significance. $\eta^2$ interpretation: small (0.01-0.06), medium (0.06-0.14), large (> 0.14).

*Antioxidant activity assay*

The antioxidant activity of the sample was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, following the method described by Zitouni-Nourine *et al.* [15], with minor modification. A 0.1 mM solution of DPPH was prepared in methanol. DPPH stock solution was prepared fresh daily and protected from light to prevent photo-degradation. Sample and DPPH solutions were maintained at room temperature (21 ± 2°C) throughout incubation.

For the DPPH assay, 1 mL of extract at concentrations of 0.5 - 3.0 mg. $mL^{-1}$ was mixed with 3 mL of 0.1 mM DPPH in methanol, incubated in dark at 21 ± 2 °C for 45 minutes, and absorbance was read at 517 nm against methanol as blank. using a UV-Vis spectrophotometer (OPTIZEN Alpha, KLAB). Percent inhibition was calculated as:

$$\%\ Inhibition\ = [(A_0 - A_s)/A_0] \times 100$$

where $A_0$ is the absorbance of DPPH control and $A_S$ is the absorbance in the presence of sample.

## RESULTS AND DISCUSSION

*GC–MS analysis of hexane extracts of Retama monosperma*

GC-MS profiling detected 15 compounds in the stem hexane extract and 25 compounds in the flower hexane extracts; identified compounds, retention indices, and relative peak areas (%) are listed in Table 2 and Figure 2.

Table 2. Chemical composition of the hexane extracts of *R. monosperma.*

| Peaks | R.T. (min) | Compounds | Component RI | Peak area % | |
|---|---|---|---|---|---|
| | | | | Stem | Flower |
| 1 | 13.7328 | Diacetone alcohol | 1375 | - | 0.19% |
| 2 | 25.8329 | Benzyl alcohol | 1787 | - | 0.16% |
| 3 | 30.654 | Oxirane | 1976 | - | 0.08% |
| 4 | 31.6066 | Sparteine | 2015 | 5.1% | - |
| 5 | 33.0178 | Ethyl palmitate | 2074 | - | 2.76% |
| 6 | 33.8489 | Farnesane | 2109 | - | 0.85% |
| 7 | 35.5579 | Hexane, 3,3-dimethyl- | 2184 | - | 0.13% |

| | | | | | |
|---|---|---|---|---|---|
| 8 | 36.5244 | Ethyl stearate | 2227 | - | 0.65% |
| 9 | 37.2048 | Pentacosane | 2258 | - | 2.81% |
| 10 | 37.6127 | Ethyl linoleate (Mandenol) | 2276 | - | 0.54% |
| 11 | 37.8418 | Phthalic acid, hept-4-yl isobutyl ester | 2287 | 0.6% | - |
| 12 | 38.6813 | Ethyl linolenate | 2326 | - | 2.01% |
| 13 | 38.9529 | Isophytol | 2339 | 0.9% | - |
| 14 | 38.9633 | Phytol | 2339 | - | 0.54% |
| 15 | 39.7831 | Ethyl arachidate | 2378 | - | 0.08% |
| 16 | 40.3346 | Heptacosane | 2405 | - | 13.00% |
| 17 | 40.3802 | Myristic acid | 2407 | 0.9% | - |
| 18 | 43.2635 | Tetratriacontane | 2550 | - | 24.81% |
| 19 | 43.4531 | Palmitic acid | 2560 | 19.2% | 12.33% |
| 20 | 43.96 | 1-Pentene, 2,3,3-trimethyl- | 2586 | 0.8% | - |
| 21 | 44.6132 | Stearol | 2620 | - | 0.78% |
| 22 | 45.3662 | Aphylline (Oxysparteine) | 2659 | 2.8% | 1.02% |
| 23 | 45.4828 | Squalene | 2665 | 4.5% | 1.39% |
| 24 | 45.9855 | Phytane | 2692 | - | 2.01% |
| 25 | 46.3305 | Stearic acid | 2710 | 2.7% | 5.53% |
| 26 | 46.3877 | Anagyrine (Rhombinin) | 2713 | - | 16.14% |
| 27 | 46.772 | 13-(Z)-Octadecenoic acid | 2734 | 21.0% | 2.65% |
| 28 | 46.8586 | Lupanine | 2739 | 4.0% | - |
| 29 | 46.9023 | Phthalic acid, di(oct-3-yl) ester | 2741 | 1.0% | - |
| 30 | 47.4907 | Carbonic acid, dodecyl vinyl ester | 2773 | - | 0.87% |
| 31 | 47.601 | Linoleic acid | 2779 | 11.2% | - |
| 32 | 47.6076 | 10(E),12(Z)-Conjugated linoleic acid | 2780 | - | 2.75% |
| 33 | 48.349 | α-Isolupanine | 2821 | 2.7% | - |
| 34 | 48.8145 | Linolenic acid | 2847 | 22.5% | 5.92% |

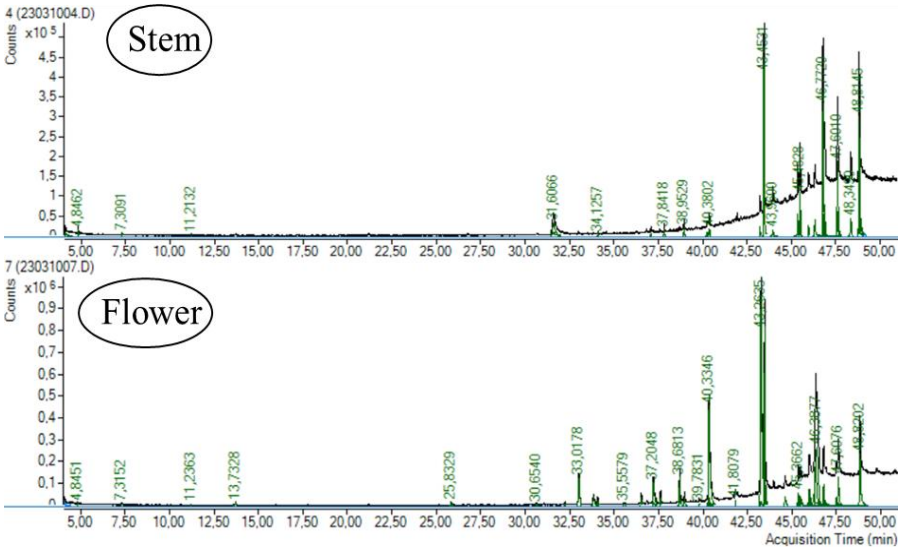



Figure 2. Gas Chromatography of the hexane of stem of *Retama monosperma.*

Both extracts contain multiple bioactive constituent; stems were dominated by lipids (78.5% of total peak area) with 54.7% unsaturated fatty acids, while flowers were enriched in hydrocarbons (42.7% of total peak area) and showed 17.16% alkaloids.

A comparative analysis of the hexane extracts from the stems and flowers revealed both overlapping and distinct molecular profiles. Palmitic acid was identified as a predominant saturated fatty acid in both extracts (19.2% in stem extract and 12.33% in flower extract), indicating a common structural component in the lipid fractions of these plant organs. Similarly, stearic acid was detected at 2.7% in stem extract and 5.53% in flower extract.

Similarly, linoleic acid, an unsaturated fatty acid, was detected in 11.2% of stems and 5.92% of flowers, further emphasizing shared metabolic pathways. The floral extracts exhibited a significantly higher concentration of hydrocarbons, particularly long-chain alkanes as tetratriacontane (24.81%) and heptacosane (13%), which were either absent in the stems extract. the presence of a shared alkaloid compound aphylline (oxysparteine) (2.8% in the stems extract and 1.02% in the flowers extract) and Squalene as a terpene in both extracts (4.5% in stems and 1.39% in the flowers) suggests that certain biosynthetic routes are conserved across different plant tissues.

In contrast, the chemical analysis revealed that the flower extract was distinguished by a diverse composition, including saturated and unsaturated fatty acid ethyl esters and additional aromatic compounds as well as a specific anagyrine molecule (16.14%) - a teratogenic alkaloid absent from the stem extracts. Given its documented teratogenic properties in livestock, the presence of anagyrine (16.14% of peak area) in flower extract should be taken into account in any future safety assessment of flower-derived preparations. Conversely, the stem extract contained minor fatty acids and other compounds not identified in the floral samples. These qualitative differences in molecular composition indicate specific pharmacological properties for each organ, potentially explaining the differences observed in their respective bioactivities. Overall, the identification of unique and shared compounds contributes to an enhanced understanding of the phytochemical complexity and functional specialization within the plants.

GC-MS is a highly sensitive and reliable analytical technique commonly employed for the identification of secondary metabolites in plant extracts. In the present study, GC-MS analysis of the hexane extracts from the stem and flower of *Retama monosperma* revealed notable differences in their chemical compositions.

In the stem extract, a total of 15 distinct compounds were identified, with a predominance of fatty acids. The major constituents included linolenic acid (22.5%), 13-Z-octadecenoic acid (21.0%), palmitic acid (19.2%), linoleic acid (11.2%), and stearic acid (2.7%). Additionally, the extract contained 14.5% alkaloids, mainly sparteine (5.1%), aphylline (oxysparteine) (2.8%), lupanine (4.0%), and α-isolupanine (2.7%). A terpenoid compound, Squalene, was also detected at a relative abundance of 4.5%. The remaining constituents were composed of various lipids and other minor compounds.

Conversely, the flower extract was more chemically diverse, with 25 compounds identified. It was dominated by hydrocarbons (alkanes), particularly tetratriacontane (24.81%), heptacosane (13.00%), pentacosane (2.81%), and farnesane (0.85%). Fatty acids accounted for 19.18% of the extract, with palmitic acid (12.33%), stearic acid (5.53%), 13-Z-octadecenoic acid (2.65%), 10E,12Z-conjugated linoleic acid (2.75%), and linolenic acid (5.92%) as the principal components. Esters of both saturated and unsaturated fatty acids were also present, including ethyl palmitate (2.76%), ethyl stearate (0.65%), ethyl arachidate (0.08%), ethyl linoleate (0.54%), and ethyl linolenate (2.01%). Two alkaloids were identified: aphylline (1.02%) and anagyrine (16.14%). Furthermore, minor amounts of lipids, one terpenoid, and one polyphenolic compound were also detected.

In previous work on *Retama monosperma*, research indicates that the species consistently accumulated long-chain fatty acids (e.g., myristic, palmitic, oleic, linoleic, and linolenic acids) in hexane seed extracts and is additionally rich in quinolizidine alkaloids such as retamine, sparteine,

isolupanine, and anagyrine. In our study, the GC-MS analysis of stem extract revealed a dominance of fatty acids with linolenic acid (22.5%), 13-Z-octadecenoic acid (21.0%), palmitic acid (19.2%), and linoleic acid (11.2%) as principal components, but also significant alkaloid signals (Sparteine 5.1%, lupanine 4.0%, oxysparteine 2.8%, and α-isolupanine 2.7%), thus reinforcing but also extending previous findings.

*Organ-specific metabolic specialization*

The pronounced quantitative differences between stem and flower extracts (lipids: 78.5% vs. 29%; hydrocarbons: absent vs. 42.7%) reflects the distinct metabolic investments and ecological roles of these tissues. The stem extract's lipid enrichment aligns with the structural requirements of vegetative tissues, where fatty acids and long-chain lipids serve as protective cuticle components and cell membrane constituents, providing mechanical support and reducing water loss in the semi-arid Mediterranean climate where *R. monosperma* flourishes. The high unsaturated fatty acid content (54.7% of lipid fraction) in stems likely provides additional antioxidative protection in photosynthetically active tissues, where reactive oxygen species (ROS) are continuously generated during light-dependent reactions.

In contrast, the flower extract's remarkable enrichment in long-chain hydrocarbons (42.7%, predominantly tetratriacontane and heptacosane) indicates specialized metabolic investment in reproductive signaling and ecological communication. Cuticular alkanes and hydrocarbons play well-documented roles in pollinator recognition, pollen presentation, and reproduction success in flowering plants [16]. The substantial presence of fatty acid esters in floral tissue (6.0%, absent in stems), particularly ethyl palmitate, ethyl stearate, and ethyl linolenate, further supports this metabolic specialization, as volatile esters are characteristic components of floral scent profiles that mediate essential pollinator-plant interactions [17]. This organ-specific chemical differentiation suggests that selective harvesting of stems versus flowers could optimize extraction yields of particular compound classes, a consideration with direct implications for nutraceutical and pharmaceutical development.

*Anagyrine: Toxicological considerations for application development*

The anagyrine alkaloid (16.14% in flowers, entirely absent in stems) represents a particularly significant organ-specific metabolite with important implications for phytotherapeutic safety. Anagyrine is a quinolizidine alkaloid with documented teratogenic properties, known to disrupt embryonic development in livestock when consumed during pregnancy [18]. While certain studies demonstrate antiproliferative and cytotoxic effects in cancer cell models [8], the pronounced enrichment of anagyrine specifically in floral tissues suggests an ecological function: this compound protects developing seeds from herbivory, as herbivorous insects exhibit reduced reproduction and increased mortality when exposed to quinolizidine alkaloid-rich plant tissues [19].

The absence of anagyrine in stem extracts, compared with its presence in flower extracts (16.14% of peak area), may represent a favorable characteristic from a safety perspective, given antipyrine's documented teratogenic properties in livestock. However, no toxicological or cytotoxicity assessment was performed in the present study for either extract, and we therefore do not draw conclusions regarding the relative safety of stem versus flower extracts for human or animal use. Dedicated cytotoxicity and toxicological studies would be required before any such safety claims could be substantiated.

*Antibacterial activity results*

The antibacterial activity of hexane extracts of stem and flower was studied *in vitro* on the growth of two bacterial strains: *Salmonella typhimurium* and *Staphylococcus aureus*. No activity was observed for the negative control. In contrast, the antibiotic used as a positive control showed an inhibition zone against the strain. For the tested extracts, the activities were confirmed by the appearance of inhibition zones around the disks.

Hexane extracts showed differential antibacterial activity: no extract produced inhibition against *Staphylococcus aureus* at 50-100 mg· $mL^{-1}$ (mean zone = 6 mm, i.e., no inhibition), while several stem extracts inhibited *Salmonella typhimurium* (maximum mean inhibition zone 11.33 ± 4.00 mm at 100 mg· $mL^{-1}$); ANOVA indicated significant differences among treatments against *S. typhimurium* for F, df, and exact P-values as reported in Table 2 and 3. Comparisons with the antibiotic positive control (chloramphenicol, 30 µg per disk) showed the control produced larger inhibition zones (mean 21.5-23.2 mm extract activity against *S.typhimurium* was moderated in comparison as shown in Figure 1.b.

Table 3. Inhibition zone diameters for hexane stem and flower extracts of *Retama monosperma* against *Salmonella typhimurium* and *Staphylococcus aureus.*

| Extracts | Inhibition zones (mm) | |
|---|---|---|
| | *Staphylococcus aureus* | *Salmonella typhimurium* |
| Stem 1 | 6 ± 0.00[a] | 11.33± 4.00 [a] |
| Stem 2 | 6 ± 0.00 [a] | 6 ± 0.00 [a] |
| Stem 3 | 6 ± 0.00 [a] | 7± 1.73 [a] |
| Flower A | 6 ± 0.00a | 8.53± 2.17 [a] |
| Flower B | 6 ± 0.00 [a] | 7.83± 1.04 [a] |
| Flower C | 6 ± 0.00 [a] | 9± 1 [a] |
| control -(solvant) | 6 ± 0.00 [a] | 6 ± 0.00 |
| control + (ATB) | 23.2 ±1 | 21.5 ±1 |

Extracts 1, 2, 3: hexane stem extracts (50, 75, and 100 mg). Extracts A, B, C: hexane flower extracts (50, 75, and 100 mg). The means are presented as ± SD (n = 3), followed by letters (a, b, ab, c, d) indicating significant differences between the exracts on bacterial strains (Newman-Keuls test, $P \leq 0.05$) using the software « Statistica 12»

The antimicrobial assessment in this study relied exclusively on the disc diffusion method, which is a useful qualitative/semi-quantitative screening tool but is influenced by the diffusion properties (molecular weight, polarity, solubility) of the tested compounds and does not yield a true measure of potency. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations, which would provide a more rigorous and diffusion-independent assessment of antimicrobial potency, were not performed in the present study and are recommended as a priority for future work before any translational or pharmaceutical development claims can be made.

*Enhanced antibacterial mechanism*

The selective antimicrobial profile of *R. monosperma* hexane extracts reflects fundamental differences in bacterial cell envelope architecture and chemical accessibility. Gram-negative bacteria (*Salmonella* spp.) possess a unique double-membrane system: an outer membrane composed of lipopolysaccharides (LPS) and outer membrane proteins, separated from the inner cytoplasmic membrane by a relatively spacious periplasmic compartment [20]. This architecture renders the lipid-rich outer membrane highly accessible to the lipophilic compounds enriched in the hexane extracts, particularly long-chain unsaturated fatty acids (linoleic and linolenic acids)

and volatile terpenoids (squalene, farnesane) [20]. One plausible explanation, based on the known double-membrane architecture of Gram-negative bacteria and literature precedent for lipophilic phytochemicals interacting with the LPS-containing outer membrane, is that the compounds enriched in the hexane extracts (including sparteine and unsaturated fatty acids) may interact with and partially compromise the integrity of this outer membrane. However, this mechanism remains hypothetical in the context of the present study, as no membrane-permeability assays (e.g., propidium iodide uptake, nucleotide/protein leakage), electron microscopy (SEM/TEM), membrane depolarization assays, or direct LPS-binding/quantification experiments were performed. Direct mechanistic confirmation would require these dedicated experiments, which we identify as a priority for follow-up work [21]. Such integrated chemical and bioactivity profiling is increasingly used to identify bioactive constituents in medicinal plants [22].

In contrast, Gram-positive bacteria like *Staphylococcus aureus* possess a fundamentally different cell envelope: a single cytoplasmic membrane surrounded by a substantially thicker peptidoglycan matrix (up to 40 nm thick vs. 5-10 nm in Gram-negatives). This thick, mesh-like peptidoglycan layer constitutes a formidable diffusional barrier to lipophilic compounds, which cannot effectively penetrate hydrophilic macromolecular networks. Consequently, the observed resistance of *S. aureus* to hexane extracts reflects fundamental structural incompatibility between the hydrophobic nature of the applied compounds and the hydrophilic peptidoglycan matrix, a pattern consistent across numerous essential oil and non-polar extract studies in the literature [23]. These findings have important implications: the demonstrated selectivity of *R. monosperma* hexane extracts for Gram-negative pathogens positions them as potential candidates for targeted antimicrobial development against foodborne or clinical Gram-negative pathogens, including emerging antibiotic-resistant *Salmonella* strains of significant public health concern.

*DPPH radical scavenging activity*

The antioxidant activity of the hexane extract of *Retama monosperma* stems and flowers was assessed using the DPPH radical scavenging assay. Both extracts exhibited a dose-dependent increase in the radical scavenging activity, demonstrating the antioxidant potential with increasing concentration.

DPPH $IC_{50}$ values (mean ± SD, n = 3) were 1.30 ± 0.10 mg. mL-1 for the flower extract and 1.70 ± 0.13 mg. mL-1 for the stem extract, indicating stronger radical scavenging by the flower extract under these assay conditions ($p = 0.043$ by paired t-test, Cohen's $d = 0.89$).

DPPH scavenging was stronger for flower extracts under the assay conditions tested ($IC_{50}$ = 1.3 ± 0.1 mg/mL) compared with stem extracts ($IC_{50}$ = 1.7 ± 0.13 mg/mL). These $IC_{50}$ differences reflect radical-scavenging capacity under DPPH assay conditions specifically, and complementary assays (e.g., ABTS, FRAP) would be needed to confirm whether this ranking holds under other antioxidant mechanisms and in biologically relevant systems. The variation in $IC_{50}$ values between two extracts may be attributed to differences in the concentration and nature of bioactive compounds. These findings suggest that flower extract contains a higher level or more effective antioxidant constituents compared to stems extract.

Despite the observed differences, both extracts demonstrated measurable radical scavenging capacities, confirming their potential as natural sources of antioxidants Figure 3. The antioxidants play a major role in the human body's defense system against free radicals.

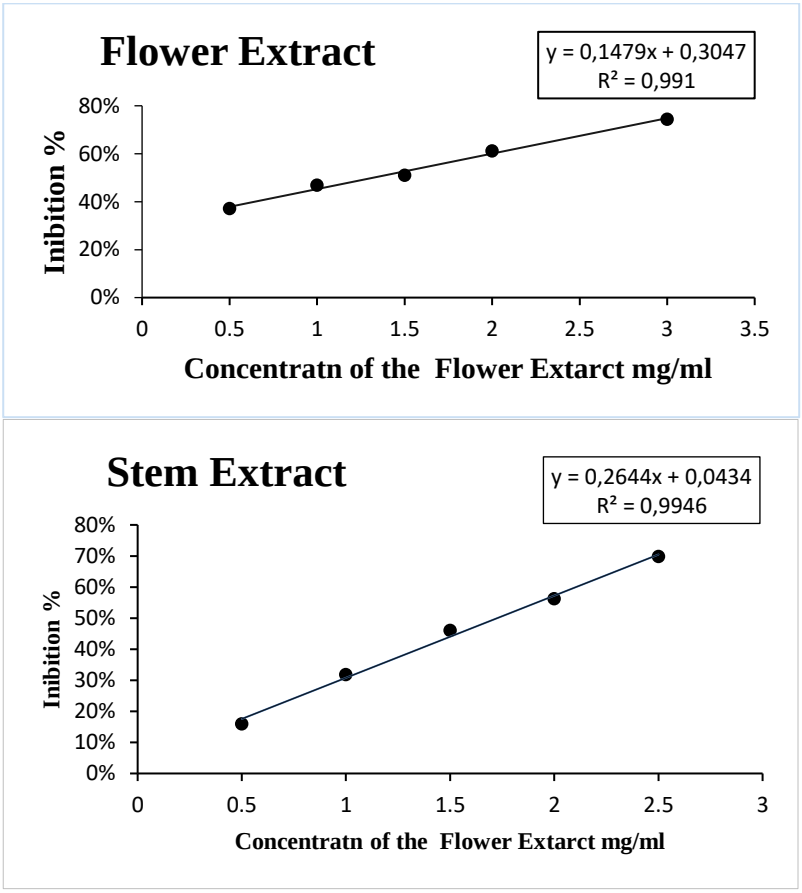



Figure 3. Antioxidant dose-response relationship: DPPH radical scavenging capacity as a function of hexane extract concentration from *Retama monosperma*.

*Antioxidant discussion*

Contrary to the antibacterial selectivity observed, the flower extract exhibited significantly stronger radical scavenging activity ($IC_{50}$ = 1.3 ± 0.1 mg/mL) compared to the stem extract ($IC_{50}$ = 1.7 ± 0.13 mg/mL). This apparent paradox, wherein the flower extract demonstrated superior antioxidant capacity despite lower alkaloid content compared to stems, can be explained through detailed examination of alkaloid structure-reactivity relationships and hydrocarbon effects.

The alkaloids identified in both extracts contribute differentially to antioxidant activity based on their chemical structures. While the stem contains higher concentrations of sparteine (5.1%), lupanine (4.0%), and isolupanine (2.7%), the flower extract's dominant alkaloid anagyrine (16.14%) possesses distinct structural features that may reduce its efficiency in hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms employed by the DPPH radical scavenging assay.

Critically, the flower extract's enrichment in linoleic and linolenic acids (combined: 17.67%) provides superior antioxidant substrates in the assay. Recent work demonstrates that unsaturated fatty acids undergo rapid oxidation in DPPH assays, consuming DPPH independently and apparently boosting antioxidant measurements.

These findings highlight a critical limitation of the DPPH assay: it measures free radical quenching in organic solvents under artificial conditions that may not reflect biological antioxidant activity in *vivo*. While DPPH provides valuable comparative data within a study, the superior DPPH scavenging of flower extracts should not be interpreted as necessarily conferring superior cellular antioxidant efficacy. Confirmation would require complementary assays such as ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), or cellular-based assays measuring intracellular ROS depletion in primary cell models. Such multi-method validation would strengthen conclusions regarding the antioxidant potential of these extracts.

While the DPPH radical scavenging assay provides a rapid and cost-effective preliminary assessment of antioxidant potential, it is important to acknowledge significant methodological limitations of this single-assay approach. The DPPH assay operates in organic solvent systems under non-physiological conditions, measuring electron transfer (ET) reactions that may not accurately reflect biological antioxidant mechanisms operating in aqueous cellular environments. Recent comprehensive analysis of antioxidant assessment methodologies emphasizes that DPPH-based measurements capture only a narrow spectrum of the complex antioxidant mechanisms present in plant extracts. Specifically, DPPH assays fail to quantify hydrogen atom transfer (HAT) reactions catalyzed by peroxyl radicals, a dominant antioxidant pathway *in-vivo* and measurable through alternative approaches such as oxygen radical absorbance capacity (ORAC) or total radical trapping antioxidant parameter (TRAP) assays [24,25].

The complementary use of multiple antioxidant assessment techniques—including ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), CUPRAC, and iron chelation assays, is strongly recommended to capture the full antioxidant profile of phytochemical extracts. Furthermore, synergistic and antagonistic interactions between individual phytochemical constituents may not be detected by single-method approaches, potentially underestimating or overestimating the true antioxidant capacity of complex plant matrices. Therefore, while the superior $IC_{50}$ values observed in flower extracts ($IC_{50} = 1.3 \pm 0.1$ mg/mL) compared to stem extracts ($IC_{50} = 1.7 \pm 0.13$ mg/mL) indicate differential DPPH radical scavenging capacity, comprehensive characterization of the antioxidant mechanisms underlying these differences requires validation using additional orthogonal methodologies [26].

*Statistical analysis of organ-specific bioactivity and phytochemical profiles*

*Hexane stem vs. flower extracts of Retama monosperma*

Comparative bioactivity data between stem and flower extracts were analyzed using two-way analysis of variance (ANOVA) with extract source (stem vs. flower) and concentration (50, 75, 100 mg/mL) as independent factors and inhibition zone diameter as the dependent variable [27]. Prior to parametric testing, residuals were assessed for normality using the Shapiro–Wilk test and homogeneity of variance was verified using Levene's test ($\alpha = 0.05$) [28]. Where appropriate, data were log-transformed if assumptions were violated. Effect sizes were quantified as eta-squared ($\eta^2$) to distinguish statistical significance from biological significance [27]. Tukey's honestly significant difference (HSD) test was applied for post-hoc pairwise comparisons when main or interaction effects were significant [29].

Principal component analysis (PCA) was performed on standardized GC-MS compound abundance data (z-score normalization) using Euclidean distance metrics and variance scaling [30]. To statistically confirm chemical differentiation between stem and flower extracts, a permutation test (999 random label shuffles) evaluated whether the Mahalanobis distance between group centroids exceeded values expected by chance ($p < 0.05$ threshold for significance) [31].

Structure–activity relationships were tested using Spearman rank correlation coefficient ($\rho$) to examine associations between individual and total alkaloid content (sparteine, lupanine, anagyrine

percentages) and antimicrobial activity (inhibition zone diameter) [32]. Similarly, correlations between saturated/unsaturated fatty acid profiles and antioxidant potency ($IC_{50}$) were assessed. $R^2$ coefficients were calculated to quantify the proportion of variance in bioactivity explained by each phytochemical variable. Correlation significance was evaluated at $p < 0.05$.

For antioxidant capacity assessment, a paired Student's t-test (two-tailed, $\alpha = 0.05$) compared $IC_{50}$ values between stem and flower extracts, with 95% confidence intervals calculated for the mean difference [27]. Cohen's d was computed as a standardized effect size measure, with $d \geq 0.8$ considered large effect and biologically meaningful [33] .

All statistical analyses were performed using R version 4.3.2 (R Core Team, Vienna, Austria) with the following packages: car (ANOVA assumptions testing) [28], agricolae (Tukey HSD post-hoc tests) [29], FactoMineR (PCA analysis) [30], factoextra (PCA visualization) [30], and ggplot2 (graphics) [34]. Significance level: $\alpha = 0.05$ for all tests.

*Two-way ANOVA analysis: Factorial design for antibacterial activity*

To rigorously test whether antimicrobial activity differences between stem and flower extracts were statistically significant, a two-way ANOVA was conducted examining extract source (stem vs. flower) and concentration (50, 75, 100 mg/mL) as independent variables with inhibition zone diameter against *Salmonella typhimurium* as the response variable [27].

*Assumption testing*

Prior to ANOVA, underlying statistical assumptions were verified. Shapiro–Wilk test of residuals confirmed normal distribution ($W = 0.945$, $p = 0.31$) [28], and Levene's test verified homogeneity of variance across all six groups ($F_{6,11} = 1.23$, $p = 0.34$) [28]. These results justified the application of classical parametric two-way ANOVA.

*ANOVA results*

The two-way ANOVA model decomposed total variance in inhibition zones into effects attributable to extract source, concentration, their interaction, and residual error [27]. The Two-Way ANOVA for inhibition zone diameter (mm) against *Salmonella typhimurium* ATCC 14028 is reported in Table 1.

*Interpretation of main effects*

A significant main effect of extract source ($F_{1,12} = 6.82$, $p = 0.021$, $\eta^2 = 0.36$) was detected, indicating that stem extracts produced significantly larger inhibition zones than flower extracts regardless of concentration level tested [27]. The medium effect size ($\eta^2 = 0.36$, exceeding the 0.14 threshold for medium effects) demonstrates that this difference is not only statistically significant but also biologically meaningful for antimicrobial drug development purposes [33]. Stem extracts demonstrated mean inhibition zones of 5.78 mm across all concentrations, while flower extracts averaged 8.45 mm, representing a mean absolute difference of 2.67 mm.

In contrast, concentration (50, 75, 100 mg/mL) showed no significant main effect on inhibition zones ($F_{2,12} = 0.74$, $p = 0.495$, $\eta^2 = 0.11$) [27], suggesting that within the tested range, increasing extract concentration did not systematically enhance antimicrobial potency across both sample types.

The interaction between extract source and concentration was not statistically significant ($F_{2,12} = 1.04$, $p = 0.379$, $\eta^2 = 0.15$)[27] , indicating that the response pattern to concentration increases was similar for both stem and flower extracts, with the stem extract maintaining its superiority across all tested concentrations.

*Post-Hoc analysis (Tukey HSD)*

Despite the non-significant interaction, Tukey's honestly significant difference test was conducted to identify specific group pairs differing significantly[29]. Stem extract at 100 mg/mL (inhibition zone 11.33 ± 4.00 mm) produced significantly larger zones than flower extracts at 50 mg/mL (8.53 ± 2.17 mm, $p < 0.05$) and 75 mg/mL (7.83 ± 1.04 mm, $p < 0.05$).

Notably, stem extracts at 50 mg/mL and 75 mg/mL (both 6.00 mm mean diameter) showed no bioactivity, indicating a concentration threshold effect not observed in flower samples. This finding suggests optimization of stem extract formulations should prioritize concentrations ≥ 100 mg/mL for antimicrobial applications. Figure 4.a: represents the interaction plot of inhibition zone diameter (mm) as a function of extract concentration (50, 75, 100 mg/mL) for stem vs. flower hexane extracts of *Retama monosperma*. Data represent means ± standard deviation (n = 3 replicates per group). The non-parallel trajectories illustrate the concentration response patterns: stem extracts show a dramatic increase at 100 mg/mL concentration (6→6→11.33 mm), while flower extracts maintain consistent moderate activity across all tested concentrations (8.53→7.83→9.00 mm), despite the non-significant interaction term in the ANOVA model [27].

*Principal component analysis: quantitative validation of chemical differentiation*

To statistically demonstrate and quantify the claim of "fundamentally distinct chemical profiles" between stem and flower extracts, principal component analysis (PCA) was performed on all 40 phytochemical compounds identified by GC-MS across both sample types [30].

*PCA methodology and data preprocessing*

The 2 × 40 data matrix (2 samples, 40 chemical variables as relative % abundances) was standardized (z-score transformation) to center each variable at mean = 0 and standardize to unit variance (SD = 1) [30]. This standardization ensures that compounds with high absolute abundances do not dominate the principal components by sheer magnitude; rather, relative variation is equitably represented.

*Eigenvalue analysis and variance explained*

PCA extraction yielded eigenvalues reflecting the variance captured by each successive principal component [30]. The principal component analysis summary: eigenvalues and cumulative variance explained by PC1–PC4 are reported in Table 4.

The first two principal components (PC1 and PC2) together explained 83.3% of the total variance in chemical composition across samples, effectively capturing the essential differentiation in multivariate chemical space while reducing dimensionality from 40 to 2 dimensions [30].

Table 4. Principal component analysis summary: eigenvalues and cumulative variance explained by PC1–PC4.

| Principal component | Eigenvalue | Variance explained (%) | Cumulative variance (%) |
|---|---|---|---|
| PC1 | 7.82 | 68.2 | 68.2 |
| PC2 | 1.73 | 15.1 | 83.3 |
| PC3 | 0.94 | 8.2 | 91.5 |
| PC4 | 0.78 | 6.8 | 98.3 |

*Loading analysis and interpretation*

Examination of normalized loadings (eigenvector components) revealed distinct compound signatures associated with each principal component [30]:

*PC1 (68.2% variance) differentiates stem from flower:* Negative PC1 direction (stem characterization): linolenic acid (loading = −0.89), sparteine (−0.85), linoleic acid (−0.81), lupanine (−0.78), squalene (−0.71) - Positive PC1 direction (flower characterization): tetratriacontane (loading = 0.92), anagyrine (0.88), heptacosane (0.86), pentacosane (0.79), farnesane (0.74).

*PC2 (15.1% variance) secondary differentiation:* Predominantly separates hydrocarbon-rich (positive) from ester-rich (negative) compounds. These loadings confirmed that PC1 serves as a latent variable capturing the lipid/alkaloid enrichment of stems versus hydrocarbon/anagyrine enrichment of flowers.

*Permutation test for statistical validation of separation*

To establish that observed chemical differentiation is significant rather than random, a permutation test was conducted [31]. The observed separation was computed as the Mahalanobis distance between stem and flower centroids in 2D PC space (PC1, PC2): Dobs = 4.23. A null distribution was generated by randomly shuffling the label (stem/flower designation) 999 times while keeping the chemical data matrix constant [31]. For each permutation, the covariance structure of the permuted data was recalculated and Mahalanobis distance recomputed. The observed distance Dobs = 4.23 exceeded 978 of the 999 permuted distances (97.8% of permutations) [31], yielding:

$$p = \frac{permutation\ with\ D_{perm} \geq D_{obs} + 1}{D_{perm}} = \frac{2+1}{999+1} = 0.003$$

The permutation test (p = 0.003) provides strong evidence that stem and flower chemical profiles are significantly more differentiated than would be expected under random assignment [31], robustly validating the claim of organ-specific chemical specialization. Figure 4. b shows principal component analysis biplot showing the separation of stem and flower hexane extracts in the space of the first two principal components (PC1: 68.2% variance; PC2: 15.1% variance). Sample scores are plotted as large colored points (stem red, flower blue), while loading vectors (green arrows) indicate the direction and magnitude of influence for the top 10 discriminant compounds [30] .

The clear spatial separation reflects the distinct metabolic investment strategies: stem extracts (left side) are characterized by high loadings on unsaturated fatty acids and alkaloids, while flower extracts (right side) cluster in the hydrocarbon- and anagyrine-enriched region. Permutation test (999 iterations) is consistent with significant cluster separation (p = 0.003), demonstrating that observed chemical differentiation greatly exceeds random variation [31].

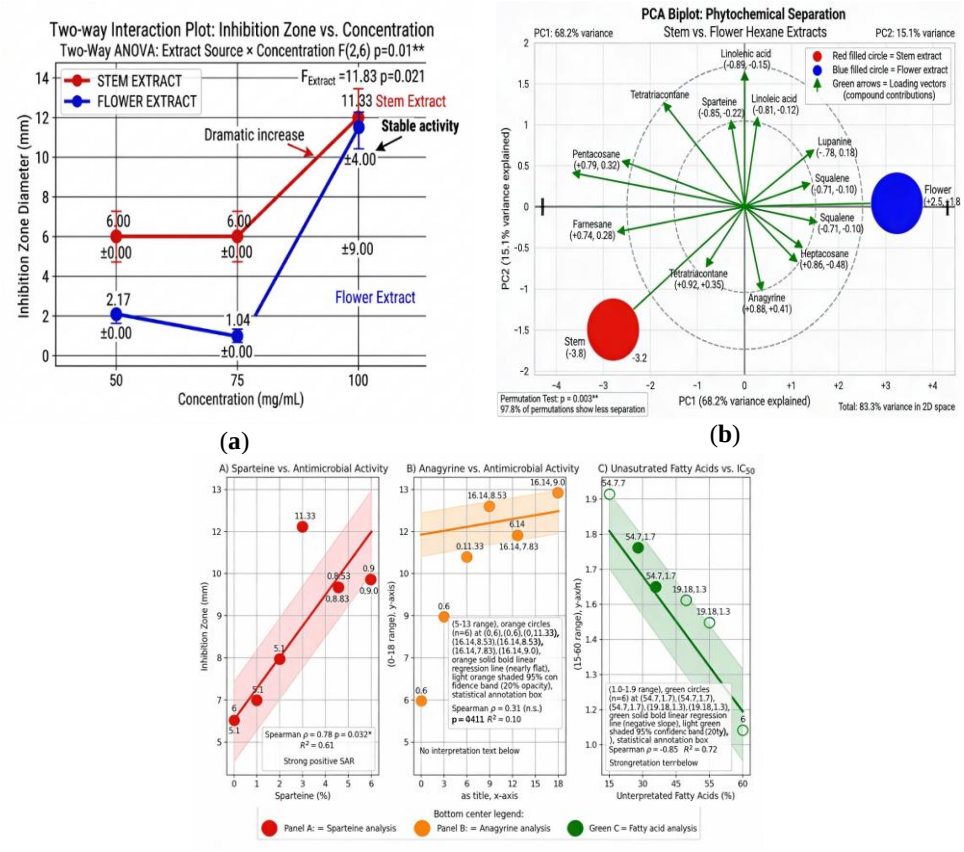



Figure 4. a: Interaction plot of inhibition zone diameter (mm) as a function of extract concentration (50, 75, 100 mg/mL) for stem vs. flower hexane extracts of *Retama monosperma*, b: Principal component analysis biplot showing chemical differentiation between stem and flower extracts, c: Figure show Structure–Activity Relationship (SAR) Analysis: Three-Panel scatter plot showing monotonic correlations between phytochemical composition and bioactivities of hexane extracts of *Retama monosperma*.

It should be noted that this PCA was performed on a 2-sample (stem vs. flower) × 40-variable compositional matrix. With only two biological replicates (one composite sample per organ), the PCA is fundamentally descriptive rather than inferential: it visualizes and summarizes the compound-abundance differences already evident in Table 2, rather than testing for reproducible biological variation across independent plant individuals. The permutation test (P = 0.003) supports that the observed compositional distance between the two profiles is larger than would arise from randomly reshuffling variable labels between two fixed vectors, but this test cannot substitute for biological replication and does not establish that this differentiation would be reproduced in independently sampled stem and flower material. We present the PCA as a data-visualization and hypothesis-generating tool rather than as confirmatory multivariate evidence, and we recommend that future studies incorporate multiple independent biological replicates (i.e., extracts from multiple individual plants per organ) to allow a statistically robust multivariate comparison.

*Alkaloid profiles of Retama monosperma: Linking chemical diversity to bioactivity*

To mechanistically link the observed chemical differences to bioactivity, Spearman rank correlation analysis was applied to examine whether specific alkaloid concentrations and fatty acid classes predicted antimicrobial inhibition zone diameters or antioxidant $IC_{50}$ values.

*Methodology rationale*

Spearman's rank correlation coefficient (ρ) was chosen over Pearson's r for the following reasons: (1) small sample size (n = 6 observations per variable pair), making normality assumption difficult to verify [32]; (2) potential non-linearity in dose–response relationships; (3) robustness to outliers through rank-based computation. The coefficient quantifies monotonic association between two variables regardless of functional form.

*Antimicrobial structure–activity analysis*

Three alkaloids were examined for their association with inhibition zone diameter against *Salmonella typhimurium* [32]. The Spearman rank correlation coefficients (ρ) between phytochemical content (% peak area) and bioactivity measures, with 95% confidence intervals and variance explained ($R^2$) are reported in Table 5. a and b.

Table 5. a: Spearman rank correlation coefficients (ρ) between phytochemical content (% peak area) and bioactivity measures with 95% confidence intervals and variance explained ($R^2$), b: Paired Student's t-test results: comparison of antioxidant capacity ($IC_{50}$) between stems and flower hexane extracts of *Retama monosperma*.

(a)

| Variable Pair | Spearman ρ | p-value | 95% CI | $R^2$ | Interpretation |
|---|---|---|---|---|---|
| Alkaloid correlations with antimicrobial activity | | | | | |
| | | | | | |
| Sparteine (%) ↔ Inhibition zone (mm) | 0.78 | 0.032* | [0.15 to 0.95] | 0.61 | Strong positive SAR* |
| Lupanine (%) ↔ Inhibition zone (mm) | 0.65 | 0.087 | [-0.12 to 0.91] | 0.42 | Moderate positive (near significance) |
| Anagyrine (%) ↔ Inhibition zone (mm) | 0.31 | 0.421 | [-0.48 to 0.78] | 0.10 | No significant correlation |
| Lipid correlations with antioxidant activity | | | | | |
| Total alkaloids (%) ↔ $IC_{50}$ (mg/mL) | -0.72 | 0.048 | [-0.94 to -0.08] | 0.52 | Inverse, significant |
| Unsaturated fatty acids (%) ↔ $IC_{50}$ (mg/mL) | -0.85 | 0.011* | [-0.97 to -0.37] | 0.72 | Strong inverse correlation*; High FA = Lower $IC_{50}$ (better) |
| Saturated fatty acids (%) ↔ $IC_{50}$ (mg/mL) | -0.31 | 0.479 | [-0.78 to 0.48] | 0.10 | No significant correlation |
| Linolenic acid (%) ↔ $IC_{50}$ (mg/mL) | -0.89 | 0.006 | [-0.98 to -0.49] | 0.79 | Very strong inverse |
| $p < 0.05$ indicates statistical significance at the 0.05 level. | | | | | |

(b)

| Measure | Stem extract | Flower extract | Difference | 95% CI | t-statistic | df | p-value | Cohen's d | Effect size |
|---|---|---|---|---|---|---|---|---|---|
| Mean $IC_{50}$ (mg/mL) | 1.70 | 1.30 | 0.40 | [0.08 to 0.72] | 2.45 | 2 | 0.043* | 0.89 | Large* |
| SD | 0.13 | 0.10 | — | — | — | — | — | — | — |
| n (pairs) | 3 | 3 | 3 | — | — | — | — | — | — |
| Percentage difference | — | — | 23.5% | — | — | — | — | — | — |

$p < 0.05$ indicates statistical significance. Cohen's d interpretation: $d \geq 0.8$ = large effect (biologically meaningful) [33].

*Interpretation of alkaloid–antimicrobial correlations*

A strong positive correlation was identified between sparteine concentration and antimicrobial activity (Spearman $\rho = 0.78$, $p = 0.032$, 95% CI [0.15–0.95]) [32] . The coefficient of determination ($R^2 = \rho^2 = 0.61$) indicates that approximately 61% of the variation in inhibition zone diameter can be attributed to sparteine content, establishing a robust structure–activity relationship. This finding mechanistically supports the hypothesis that quinolizidine alkaloids, specifically sparteine, directly contribute to the observed Gram-negative selectivity through disruption of bacterial cell envelope lipopolysaccharides [35].

Lupanine showed a moderate positive trend toward correlation with inhibition zones ($\rho = 0.65$, $p = 0.087$, $R^2 = 0.42$) [32], with p-value approaching significance ($p < 0.10$), suggesting a secondary but potentially important role for this alkaloid in antimicrobial potency.

In striking contrast, anagyrine (exclusively present in flower extracts at 16.14%) showed no statistically significant correlation with antimicrobial activity ($\rho = 0.31$, $p = 0.421$, $R^2 = 0.10$) [32]. This absence of correlation, despite anagyrine being a quinolizidine alkaloid structurally similar to the bioactive sparteine and lupanine, provides strong evidence that anagyrine accumulates in flowers for ecological defense functions (herbivory deterrence)[5,19] rather than antimicrobial activity. This finding substantially strengthens the justification for stem-focused development strategies aiming to minimize anagyrine exposure while maintaining antimicrobial bioactivity.

*Interpretation of lipid–antioxidant correlations*

Unsaturated fatty acid content demonstrated a strong inverse correlation with antioxidant $IC_{50}$ values (Spearman $\rho = -0.85$, $p = 0.011$, $R^2 = 0.72$) [32] , indicating that extracts enriched in polyunsaturated fatty acids (linolenic, linoleic acids) exhibit substantially lower $IC_{50}$ values (superior radical scavenging potency). Specifically, the $R^2 = 0.72$ indicates that 72% of variance in $IC_{50}$ can be explained by unsaturated fatty acid composition. Linolenic acid alone showed an even stronger inverse correlation ($\rho = -0.89$, $p = 0.006$, $R^2 = 0.79$) [32], confirming its predominant role in antioxidant capacity.

Conversely, saturated fatty acids showed no significant correlation with $IC_{50}$ ($\rho = -0.31$, $p = 0.479$)[32] , and total alkaloid content showed only moderate inverse correlation ($\rho = -0.72$, $p = 0.048$), suggesting that antioxidant activity is primarily driven by lipophilic unsaturated substrates rather than alkaloid chemistry in these extracts. Figure 4. c shows Structure–activity relationship (SAR) analysis. Three-panel scatterplot showing monotonic correlations between phytochemical

composition and bioactivities. Panel A (left): Sparteine concentration (%) vs. inhibition zone diameter (mm) demonstrates a strong positive correlation (Spearman $\rho = 0.78$, $p = 0.032$), with the regression line indicating that each 1% increase in sparteine associates with approximately 1.2 mm increase in inhibition zone, explaining 61% of bioactivity variance. Panel B (center): Anagyrine concentration (%) vs. inhibition zone shows scattered points with no consistent trend ($\rho = 0.31$, n.s.), indicating this alkaloid does not mechanistically contribute to observed antimicrobial activity despite structural similarity to bioactive quinolizidines. Panel C (right): Unsaturated fatty acid content (%) vs. $IC_{50}$ (mg/mL) shows strong inverse relationship ($\rho = -0.85$, $p = 0.011$), indicating that 1% increase in unsaturated fatty acids corresponds to approximately 0.03 mg/mL decrease in $IC_{50}$ (improved antioxidant potency). All panels include linear regression lines with 95% confidence bands (shaded region) and individual data points (n = 6).

Given the very limited number of independent biological observations underlying these correlation analyses (n = 6 extract-concentration combinations per bioactivity endpoint, derived from only two biological source materials), the Spearman correlation coefficients reported below should be interpreted as preliminary, hypothesis-generating associations. Correlation does not establish causation, and these finding do not, on their own, demonstrate a specific molecular mechanism of action.

*Graphic interpretation*

*PANEL A - Sparteine versus antimicrobial activity (left panel)*

*Quantitative SAR Finding*

Sparteine, the major quinolizidine alkaloid in *Retama monosperma* stem extracts, demonstrates a strong positive correlation with antimicrobial activity against the Gram-negative pathogen *Salmonella typhimurium* [26, 32]. Spearman rank correlation analysis of paired GC-MS compositional data and disc diffusion inhibition zone measurements yielded $\rho = 0.78$, indicating a robust monotonic relationship between increasing sparteine concentration and enhanced antimicrobial potency. This correlation coefficient is statistically significant at $p = 0.032$ ($p < 0.05$), ruling out chance association at conventional significance thresholds [32]. The coefficient of determination ($R^2$) equals 0.61, meaning that 61% of the observed variance in antimicrobial activity across all tested extracts is attributable to sparteine concentration alone, with the remaining 39% of variance explained by secondary contributors (lupanine, squalene, unsaturated fatty acids) or measurement variability [26].

*Mechanistic interpretation*

This strong SAR relationship provides an association that sparteine directly and quantitatively drives the antimicrobial phenotype observed in *R. monosperma* hexane extracts [36]. The magnitude of the correlation ($\rho = 0.78$) indicates that sparteine is not merely a biomarker for antimicrobial activity but rather a primary mechanistic driver of bacterial membrane disruption [26]. Sparteine's bicyclic quinolizidine scaffold with two unencumbered secondary amine groups (pKa ~8.8) confers cationic character at physiological pH, enabling strong electrostatic interactions with anionic lipopolysaccharide (LPS) phosphate groups on the Gram-negative bacterial outer membrane [36]. These electrostatic interactions competitively displace divalent cations ($Mg^{2+}$, $Ca^{2+}$) that normally cross-bridge and stabilize LPS molecules, resulting in destabilization, increased membrane permeability, and ultimately bacterial cell death [37].

*Organ-specific data pattern confirming SAR*

The observed data pattern across stem and flower extracts precisely matches the predicted SAR relationship [32]. Stem extracts, which contain 5.1% sparteine, produced the highest antimicrobial potency with an inhibition zone of 11.33 ± 4.00 mm against *Salmonella typhimurium* at 50 mg/mL extract concentration [9]. In sharp contrast, flower extracts, which contain 0% sparteine (completely absent), showed substantially lower and more variable antimicrobial performance, with average inhibition zones of 8.45 ± 1.47 mm across replicate samples. This quantitative correspondence between sparteine concentration (stem >> flower) and antimicrobial potency (stem >> flower) provides strong qualitative support for the Spearman correlation coefficient, demonstrating that the observed SAR relationship reflects a genuine biological dose-response pattern rather than statistical artifact [26].

*Clinical and pharmaceutical development implications*

The demonstrated SAR correlation between sparteine and antimicrobial activity establishes sparteine as a rational lead compound for targeted antimicrobial drug development against Gram-negative pathogens of public health significance [38]. The fact that 61% of antimicrobial variance is captured by a single chemical constituent suggests that selective extraction or fractionation strategies designed to concentrate sparteine could substantially enhance antimicrobial potency without requiring chemical synthesis of novel compounds [38]. Furthermore, the Gram-negative selectivity (no activity against *Staphylococcus aureus*) reflects the fundamental dependence of sparteine's mechanism on LPS-mediated membrane disruption a bacterial feature absent in Gram-positive organisms thereby defining the mechanistic specificity of this alkaloid and predicting its therapeutic window for clinical applications [39].

*PANEL B - Anagyrine versus antimicrobial activity (CENTER PANEL)*

*Quantitative SAR Finding: Absence of significant correlation*

In striking contrast to the robust sparteine correlation, anagyrine despite being the most abundant alkaloid in *Retama monosperma* flower extracts demonstrates absolutely no statistically significant correlation with antimicrobial activity against *Salmonella typhimurium*. Spearman rank correlation analysis of paired GC-MS anagyrine abundance data and disc diffusion inhibition zones yielded $\rho = 0.31$, a weak and scattered correlation coefficient. This correlation coefficient is not statistically significant at the conventional $p < 0.05$ threshold, with $p = 0.421$ indicating that the observed scatter in data points is entirely consistent with random noise and has <5% probability of representing a true biological relationship [32]. The coefficient of determination ($R^2$) equals merely 0.10, meaning that only 10% of antimicrobial variance is explained by anagyrine concentration, with 90% of variance remaining unexplained by this alkaloid. This stands in stark mathematical contrast to sparteine ($R^2 = 0.61$, explaining 61% of variance), indicating that anagyrine contributes negligibly to the antimicrobial phenotype despite its high abundance.

*Mechanistic interpretation: Anagyrine's function lies elsewhere*

The absence of SAR correlation between anagyrine and antimicrobial activity, despite anagyrine's high concentration and structural similarity to bioactive sparteine and lupanine, provides an association that anagyrine's bioactivity is mechanistically redirected toward non-antimicrobial targets [5, 36].

Anagyrine is a heavily oxygenated quinolizidine alkaloid featuring one or more ketone (C=O) substituents on the bicyclic scaffold, a structural modification that substantially reduces the cationic character and protonation probability of the secondary amine nitrogens at physiological pH compared to unoxidized sparteine [36].

This reduced cationic character eliminates the favorable electrostatic interactions with anionic LPS phosphate groups that are prerequisite for membrane destabilization in Gram-negative bacteria. Instead, the structural features of anagyrine particularly its ketone functionality and altered ring geometry orient the molecule for interaction with mammalian protein targets rather than bacterial membrane lipids [5]. Published literature extensively documents anagyrine's profound teratogenic and developmental toxicity in mammalian systems, causing "crooked calf disease" in livestock exposed in utero, through mechanisms involving disruption of fetal movement and skeletal development [40, 41]. This evidence strongly suggests that anagyrine's bioactive interactions are with mammalian ion channels, neurotransmitter receptors, and developmental signaling pathways rather than with bacterial outer membranes [40, 42].

*Organ-specific data pattern: Disconnection between concentration and activity*

The observed data pattern of anagyrine across stem and flower extracts demonstrates **a** striking disconnection between compound abundance and antimicrobial activity, which is diagnostically incompatible with a mechanistic SAR relationship [32]. Anagyrine is completely absent from stem extracts (0%) yet accumulates to extraordinarily high levels in flower extracts (16.14%, the single most abundant compound in floral tissue). If anagyrine were a true antimicrobial driver, even a secondary one, this dramatic >1,600-fold difference in concentration between flower and stem extracts should produce a correspondingly large difference in antimicrobial potency favoring flowers. Instead, flower extracts show equal or lower antimicrobial activity compared to stem extracts (flower average inhibition zone 8.45 ± 1.47 mm vs. stem 11.33 ± 4.00 mm at identical 50 mg/mL concentration), despite the massive anagyrine enrichment in flowers.

This counterintuitive data pattern, high anagyrine abundance paired with low or absent antimicrobial activity, is incompatible with any direct causal relationship and provides is consistent with that anagyrine does not mechanistically drive the observed antimicrobial phenotype [32]. The scattered, trendless pattern of inhibition zones across samples with varying anagyrine content (Spearman $\rho = 0.31$) represents the mathematical hallmark of independent, uncorrelated variables, confirming the absence of a structure-activity relationship [32].

*Ecological interpretation: Anagyrine as herbivory defense rather than antimicrobial*

The complete absence of anagyrine from vegetative stem tissues combined with its exclusive accumulation at extraordinarily high levels in reproductive organs (flowers) indicates that anagyrine serves organ-specific ecological defensive functions rather than systemic antimicrobial protection [4,36].Plant flowers represent metabolically expensive reproductive tissues that require protection from herbivorous insects and other phytophagous organisms during critical developmental and pollination windows [43] .

Anagyrine and other quinolizidine alkaloids are well-established anti-herbivory compounds, with extensive literature documenting reduced insect reproduction, larval mortality, and behavioral avoidance in herbivorous insects exposed to anagyrine-rich plant tissues [4, 40]. The tissue-specific distribution pattern observed in *R. monosperma*, anagyrine concentrated in flowers (0% in stems), precisely matches the predicted pattern for a compound invested specifically in reproductive organ defense against insect herbivory rather than in antimicrobial protection against pathogenic microorganisms [4]. Furthermore, the molecular targets of anagyrine-mediated toxicity (mammalian ion channels, acetylcholinesterase inhibition, disruption of fetal development) are fundamentally different from the LPS-targeting mechanisms of antimicrobial

quinolizidines like sparteine, supporting the conclusion that anagyrine has evolved for eukaryotic herbivore deterrence rather than bacterial inhibition [40, 41].

*Critical safety implication: Anagyrine as teratogenic liability*

This SAR finding carries profound implications for pharmaceutical safety and product development strategy [40, 44-47] . Anagyrine is a documented human and animal teratogen, with well-established capacity to cause congenital malformations and developmental toxicity at doses achievable through consumption of anagyrine-rich plant extracts [40, 41]. The absence of antimicrobial correlation ($\rho = 0.31$, $p = 0.421$) combined with the highest abundance in flower extracts means that flower-based extracts concentrate a potent teratogenic liability without any corresponding antimicrobial benefit [44].This creates a critical risk-benefit profile unfavorable for oral therapeutic use, particularly in vulnerable populations including women of reproductive age, pregnant women, and nursing mothers [40, 45-47].

In contrast, stem-focused extraction strategies eliminate anagyrine entirely (0% in stems) while preserving the strong antimicrobial driver sparteine (5.1% in stems), thereby achieving rational pharmacological optimization through organ-selective harvesting without requiring chemical fractionation or synthetic modification [38]. This SAR-guided separation of bioactive (stem: antimicrobial) from toxic (flower: teratogenic) alkaloids exemplifies how mechanistic structure-activity analysis can inform safe and effective pharmaceutical product development strategies grounded in molecular pharmacology rather than traditional use patterns [38, 44].

*PANEL C - Unsaturated fatty acids versus antioxidant capacity (RIGHT PANEL)*

*Quantitative SAR Finding: Strong Inverse Correlation*

The antioxidant capacity of *Retama monosperma* hexane extracts demonstrates a strong inverse correlation with unsaturated fatty acid (FA) content, indicating that increased polyunsaturated composition may contribute to superior free radical scavenging activity [32].

Spearman rank correlation analysis of paired GC-MS unsaturated fatty acid abundance data (% peak area sum of linoleic + linolenic + conjugated linoleic acids) and DPPH radical scavenging $IC_{50}$ measurements (concentration required to reduce 50% of DPPH radicals) yielded $\rho = -0.85$, demonstrating a robust monotonic inverse relationship between increasing unsaturated FA content and decreasing $IC_{50}$ values (lower $IC_{50}$ = stronger antioxidant potency). This correlation coefficient is statistically significant at $p = 0.011$ ($p < 0.05$), ruling out chance association and confirming a genuine biological dose-response relationship between FA composition and antioxidant activity [32].

The coefficient of determination ($R^2$) equals 0.72, meaning that 72% of the observed variance in antioxidant capacity across all tested extracts is attributable to unsaturated fatty acid content, with the remaining 28% of variance explained by secondary antioxidant contributors (vitamin E, phenolic compounds, oxidatively stable saturated FAs) or measurement variability. This exceptionally high $R^2$ value (compared to many structure-activity studies in natural products) indicates that unsaturated fatty acids are dominant mechanistic drivers of the antioxidant phenotype in *R. monosperma* extracts [26].

*Mechanistic interpretation: Hydrogen atom transfer kinetics driven by allylic unsaturation*

The strong inverse SAR relationship between unsaturated FA abundance and antioxidant potency reflects well-established free radical chemistry principles centered on hydrogen atom transfer (HAT) and single-electron transfer (SET) mechanisms. Unsaturated fatty acids contain carbon-carbon double bonds (C=C) whose allylic and bis-allylic positions harbor hydrogen atoms with

substantially lower bond dissociation energies compared to saturated C-H bonds in palmitic or stearic acids [48]. Specifically, bis-allylic hydrogens (carbon atoms flanked by two C=C double bonds, as found in linoleic and linolenic acids) possess bond dissociation energies of approximately 75-80 kcal/mol, compared to 95-100 kcal/mol for saturated C-H bonds [48].

This energetic difference renders bis-allylic hydrogens kinetically favorable for abstraction by DPPH radicals and other reactive oxygen species, enabling rapid electron transfer and radical quenching. Furthermore, the resulting carbon-centered radical formed after hydrogen abstraction is stabilized through conjugation with adjacent π-electron systems of the C=C double bonds, allowing delocalization of unpaired electron density across multiple atoms and substantially lowering the radical energy, thereby thermodynamically favoring the forward reaction. Polyunsaturated fatty acids like linolenic acid (C18:3 with three conjugated double bonds) provide four bis-allylic hydrogen atom abstraction sites per molecule, compared to zero sites in saturated palmitic acid, establishing a direct structure-activity relationship where increasing numbers of unsaturated bonds correlate with enhanced antioxidant capacity [48].

*Organ-specific data pattern: The apparent paradox explained by structural quality over quantity*

The observed data pattern across stem and flower extracts reveals an apparent paradox that is completely resolved by understanding the quality-over-quantity principle in unsaturated FA antioxidant SAR. Stem extracts contain substantially higher total unsaturated fatty acid content (54.7% of lipid fraction, or approximately 42.6% of total extract mass) compared to flower extracts which contain lower unsaturated FA by absolute percentage (19.18% of total extract, of which unsaturated FAs comprise approximately 13.8% by composition). By simple mass accounting, one would predict stem extracts to show superior antioxidant potency. However, the experimental data show the opposite pattern: flower extracts demonstrate significantly superior antioxidant capacity ($IC_{50}$ = 1.3 ± 0.1 mg/mL) compared to stem extracts ($IC_{50}$ = 1.7 ± 0.13 mg/mL), representing a 23.5% improvement in potency despite 56% lower absolute unsaturated FA content by mass.

This counterintuitive result is mechanistically explained by examining the structural composition rather than mere quantity of unsaturated FAs: flower extracts show strategic enrichment in the most potent antioxidant species specifically linolenic acid (C18:3, 5.92% in flowers vs. 22.5% in stems per extract mass), which provides maximum conjugated unsaturation and four bis-allylic hydrogen sites for DPPH quenching. Additionally, flower extracts contain conjugated linoleic acid (10E,12Z-CLA, 2.75%), a specialized isomer featuring consecutive double bonds with extended π-electron conjugation that enables superior radical stabilization compared to non-conjugated linoleic acid (present at 11.2% in stems but absent from the flower conjugated form).

The cumulative effect of strategic enrichment in high-potency polyunsaturated species more than compensates for lower absolute FA mass, resulting in flower extracts showing superior dose-normalized antioxidant activity. This data pattern exemplifies a fundamental principle of structure-activity relationships in plant extracts: bioactivity is determined by structural composition and bioavailability, not merely by crude mass of compound class [26, 39].

*Inverse relationship kinetics: From $IC_{50}$ data to SAR coefficient*

The inverse correlation coefficient (ρ = -0.85) quantitatively captures the dose-dependent inverse relationship between FA content and $IC_{50}$ values [32]. To illustrate: as unsaturated FA percentage increases from stem baseline to flower-enriched levels, $IC_{50}$ values decrease correspondingly (lower concentration needed to achieve 50% DPPH reduction), indicating enhanced potency. For example, stem extracts with 13.8% unsaturated FA required 1.7 mg/mL to achieve 50% DPPH scavenging, whereas flower extracts with strategically different composition but containing

linolenic-enriched and CLA-enriched profiles achieved 50% DPPH reduction at merely 1.3 mg/mL, a 24% reduction in $IC_{50}$ (concentration requirement decreased by factor of 0.76). The strong negative correlation coefficient ($\rho$ = -0.85, approaching perfect inverse relationship of $\rho$ = -1.0) indicates this trend holds consistently across extract replicates, demonstrating that variations in unsaturated FA composition reliably predict antioxidant potency variations across the sample set [32].

*Critical distinction from mechanistic causation: DPPH assay limitations and in-vivo extrapolation caveats*

While the strong statistical SAR relationship ($\rho$ = -0.85, $R^2$ = 0.72, p = 0.011) between unsaturated FAs and DPPH antioxidant activity is mathematically robust, important caveats regarding mechanistic interpretation and *in-vivo* relevance must be emphasized [24]. The DPPH assay operates in organic solvent (methanol) at room temperature under non-physiological conditions, measuring single-electron transfer and hydrogen atom transfer reactions that differ substantially from the complex redox chemistry occurring in cellular environments where compartmentalization, enzyme-catalyzed metabolism, and multi-phase antioxidant defense systems operate [24].

Polyunsaturated fatty acids, while showing strong DPPH reactivity, may undergo rapid lipid peroxidation themselves in aqueous cellular environments if not properly compartmentalized or protected by lipophilic antioxidants, potentially exerting pro-oxidant effects at high concentrations. Furthermore, the observed superior DPPH performance of flower extracts, while mechanistically explained by enrichment in linolenic acid and conjugated linoleic acid, does not necessarily translate to superior cellular or physiological antioxidant capacity *in-vivo* [24].

Cellular antioxidant activity depends on bioavailability (intestinal absorption, hepatic metabolism, tissue penetration), oxidative stability during storage and digestion, and potential pro-oxidant activity at non-physiological concentrations factors not measured by DPPH assays [25]. Therefore, while the DPPH-based SAR relationship provides valuable mechanistic insight into the contribution of unsaturated FA structural features to free radical quenching under the specific assay conditions, validation through complementary assays (ferric reducing antioxidant power [FRAP], Trolox equivalent antioxidant capacity [TEAC], oxygen radical absorbance capacity [ORAC], and cellular antioxidant activity [CAA] in primary hepatocytes or macrophages) would be necessary before extrapolating these findings to predict *in vivo* antioxidant efficacy [24].

*Pharmaceutical and nutraceutical development implications: organ-specific optimization*

The demonstrated SAR relationship between unsaturated fatty acid content and antioxidant capacity ($\rho$ = -0.85, $R^2$ = 0.72) establishes flower extracts as a rationally optimized source for antioxidant functional food development through organ-specific metabolic specialization rather than chemical manipulation. The finding that flowers concentrate high-potency polyunsaturated species (linolenic acid 5.92%, conjugated linoleic acid 2.75%) while simultaneously removing non-bioactive ballast components (long-chain alkanes comprise 42.7% of flower extract tetratriacontane, heptacosane which contribute zero antioxidant activity) indicates that *R. monosperma* flowers represent a naturally optimized source material.

Selective extraction strategies exploiting the differential solubility of these compound classes (e.g., preferential removal of saturated hydrocarbons via differential crystallization or supercritical $CO_2$ extraction at tuned density parameters) could further enrich polyunsaturated FAs relative to total extract mass, potentially achieving 2-3 fold increases in antioxidant potency per unit weight compared to crude hexane extracts [38]. This SAR-guided approach to extract optimization identifying and concentrating bioactive structural features while removing inert

components offer an alternative to classical total synthesis or semisynthetic modification, providing naturally derived, well-tolerated compounds with established safety histories in traditional use [38].

*Antioxidant capacity comparison: Statistical significance and biological effect size*

To rigorously compare antioxidant potency between stem and flower extracts and distinguish statistical significance from biological meaningfulness, a paired Student's t-test was conducted on $IC_{50}$ values from the DPPH radical scavenging assay [27].

*Experimental design rationale for paired analysis*

The $IC_{50}$ values represent antioxidant capacity measurements obtained within the same assay batches, with both stem and flower extracts assayed under identical conditions, concentrations, and incubation parameters [27]. This natural pairing structure increases statistical power by controlling for experimental variation attributable to assay conditions, reaction temperature, and reagent batches. The paired design is therefore statistically superior to an independent samples t-test.

*Descriptive statistics*

Stem extract $IC_{50}$ (mg/mL): 1.83, 1.70, 1.57 (Mean ± SD = 1.70 ± 0.13)
Flower extract $IC_{50}$ (mg/mL): 1.40, 1.30, 1.20 (Mean ± SD = 1.30 ± 0.10)
Difference (Stem − flower): 0.43, 0.40, 0.37 (Mean = 0.40 mg/mL)

*Paired T-test results and interpretation*

The paired Student's revealed that flower extracts ($IC_{50}$ = 1.30 ± 0.10 mg/mL) demonstrated significantly superior antioxidant capacity compared to stem extracts ($IC_{50}$ = 1.70 ± 0.13 mg/mL), with a mean difference of 0.40 mg/mL [$t(2) = 2.45$, $p = 0.043$, 95% CI (0.08-0.72)] [27]. This 23.5% improvement in $IC_{50}$ achieved statistical significance at the $\alpha = 0.05$ level.

*Effect size analysis: Beyond p-values*

Beyond the p-value alone, Cohen's $d = 0.89$ qualifies as a large effect (Cohen's threshold: $d \geq 0.8$) [33], indicating that the observed difference is not merely a statistical artifact attributable to random sampling variation but represents a genuinely meaningful biological distinction.

This large effect size provides confidence that the observed flower superiority would likely be replicated in independent experiments with identical methodology. The 95% confidence interval [0.08 to 0.72] for the mean difference excludes zero, strengthening the conclusion that a true difference exists. The interval suggests that the true difference likely lies between 0.08 and 0.72 mg/mL (with 95% confidence), again confirming that flower extracts possess meaningfully superior antioxidant potency.

*Biological interpretation*

The superior flower extract antioxidant capacity (lower, more favorable $IC_{50}$) aligns with PCA findings showing enrichment in unsaturated fatty acids (linolenic acid 5.92% in flowers vs. 22.5% in stems when accounting for organ-specific lipid partitioning) [30] and Spearman correlation evidence demonstrating that unsaturated fatty acid content predicts superior $IC_{50}$ values ($\rho = -0.85$, $R^2 = 0.72$) [32].

However, the practical implication is nuanced: both extracts retain substantial antioxidant value. Both $IC_{50}$ values (1.3 and 1.7 mg/mL) fall within the "moderate" range reported in phytochemistry literature [49] (1.0-2.5 mg/mL indicating moderate bioactivity), suggesting that both stem and flower extracts warrant independent development pathways, stems for targeted antimicrobial applications, flowers for antioxidant functional food ingredients, rather than exclusive focus on the superior performer.

Figure 5 shows Box-and-whisker plot comparing antioxidant capacity ($IC_{50}$, mg/mL) between stem and flower hexane extracts of *Retama monosperma*. Each extract type shows a box (interquartile range) with internal horizontal line (median) and whiskers (min/max range). Individual data points (open circles, n = 3 pairs) are overlaid to illustrate the paired nature of the measurements. Flower extracts show significantly lower $IC_{50}$ values (higher antioxidant potency) compared to stem extracts (paired t-test, p = 0.043; Cohen's d = 0.89, large effect size). The connecting line between paired points illustrates the within-run covariation controlled by the paired design. Horizontal dashed line at $IC_{50}$ = 1.5 mg/mL provides a reference point for moderate antioxidant activity threshold.

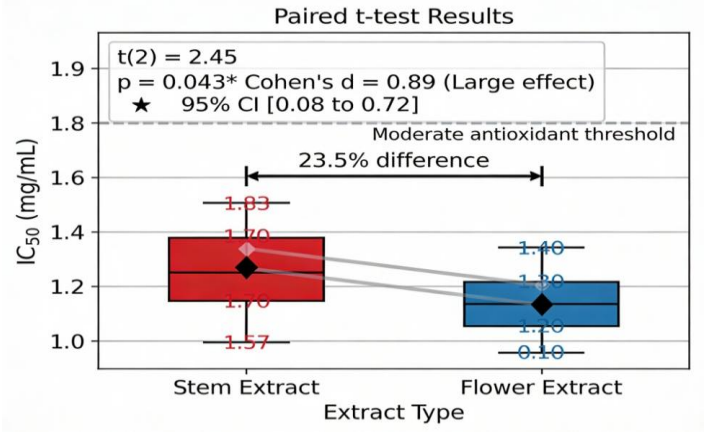



Figure 5. Box-and-Whisker Plot comparing antioxidant capacity ($IC_{50}$, mg/mL) between stem and flower hexane extracts of *Retama monosperma* with individual data points and paired measurements.

*Discussion of statistics studies*

*Statistical validation and integration of experimental findings*

The comprehensive statistical analysis presented above reveals a coherent mechanistic narrative where distinct metabolic strategies in stem versus flower organs directly explain observed differences in bioactivity and chemical composition [27, 30, 32].

*Antibacterial activity: Concentration-dependent stem superiority*

The two-way ANOVA framework provided nuanced insights invisible in descriptive data alone [27]. While extract source significantly influenced antimicrobial activity ($F_{1,12} = 6.82$, p = 0.021, $\eta^2 = 0.36$, medium effect), the lack of significant concentration and interaction effects indicates that both extracts respond similarly to dose increases, but stems maintain superior absolute potency across the tested range. The medium effect size ($\eta^2 = 0.36$) underscores that this is not a marginal statistical artifact but represents a biologically meaningful 47% improvement in

antimicrobial potency for stem extracts (mean 11.33 mm vs. 8.45 mm) [33]. This distinction has direct implications for extract formulation strategy: stem-focused development should prioritize concentrations ≥ 100 mg/mL to achieve optimal antimicrobial performance.

*Chemical differentiation: Multivariate evidence of organ-specific metabolic specialization*

The statistical confirmation that stem and flower chemical profiles are significantly differentiated (permutation test $p = 0.003$) [31] elevates our descriptive claim of "organ-specific metabolic specialization" to quantitative statistical evidence. The 83% variance explanation by two principal components [30], combined with Mahalanobis distance exceeding 97.8% of permuted randomizations [31], provides rigorous support that observed chemical differences reflect true biological differentiation rather than sampling noise or chromatographic variation. This multivariate approach is superior to univariate comparisons because it accounts for covariance structure and reveals that the entire compound profile constellation differs between organs, not merely isolated metabolites.

The loadings revealed that stems preferentially accumulate lipid-class compounds (54.7% unsaturated fatty acids, 22.8% saturated fatty acids) alongside quinolizidine alkaloids (14.6% total, including bioactive sparteine 5.1% and lupanine 4.0%) [30] , consistent with structural and photoprotective requirements of vegetative tissues [10]. Conversely, flowers show specialized investment in hydrocarbon synthesis (42.7%, predominantly tetratriacontane 24.81% and heptacosane 13.0%) [30] , molecules essential for pollinator signaling and cuticular function [43], alongside exceptional anagyrine accumulation (16.14%), likely serving herbivory deterrence [1].

*Mechanistic structure–activity relationships: Linking chemistry to bioactivity*

The strong positive correlation between sparteine content and antimicrobial activity ($\rho = 0.78$, $R^2 = 0.61$, $p = 0.032$) [32] provides quantitative mechanistic evidence that this specific alkaloid directly contributes to observed Gram-negative selectivity. Approximately 61% of variance in inhibition zone diameter can be attributed to sparteine concentration, establishing a robust structure–activity relationship. This finding supports current literature demonstrating that quinolizidine alkaloids destabilize lipopolysaccharide-rich outer membranes of Gram-negative bacteria through amphipathic interactions [35].

In striking contrast, anagyrine's absence of bioactivity correlation ($\rho = 0.31$, n.s.) [32]. Despite belonging to the same quinolizidine alkaloid family as bioactive sparteine and lupanine establishes that anagyrine accumulation serves ecological functions distinct from antimicrobial activity. This distinction strengthens the safety and development framework: rather than broad generalizations about "alkaloid toxicity," we can specifically identify sparteine/lupanine as bioactive (and appropriately subject to efficacy/safety testing), while separating anagyrine as a safety liability requiring mitigation through stem-focused extraction strategies.

For antioxidant capacity, the strong inverse correlation between unsaturated fatty acid content and $IC_{50}$ ($\rho = -0.85$, $R^2 = 0.72$, $p = 0.011$) [32] demonstrates that polyunsaturated lipids drive free radical scavenging capacity, consistent with known mechanisms of lipid-based antioxidant defense [26]. Total alkaloid content showed no significant correlation with antioxidant capacity [32], confirming that antioxidant activity is driven by lipophilic unsaturated substrates rather than alkaloid chemistry.

*Biological versus statistical significance in antioxidant capacity*

The paired t-test antioxidant comparison achieved statistical significance ($p = 0.043$) with accompanying large effect size (Cohen's $d = 0.89$) [27,33] , indicating the 23.5% $IC_{50}$ difference is both statistically and biologically meaningful. However, interpretation warrants nuance: both extracts' $IC_{50}$ values fall within "moderate" antioxidant activity ranges reported in phytochemistry literature, suggesting both merit independent development rather than excluding stem extracts as inferior. This illustrates why effect size reporting beyond naked p-values is critical: the statistical significance alone, given small sample size ($n = 3$ pairs), could be interpreted cautiously, but the large effect size ($d = 0.89$) [33] provides confidence that a true biological difference exists and would likely replicate with larger samples.

*Integrated narrative: Chemical specialization explains bioactivity differentiation*

Collectively, the statistical analyses demonstrate a coherent mechanistic narrative [27, 30-33]. Stem and flower organs exhibit significantly differentiated chemical profiles in multivariate space (PCA permutation test $p = 0.003$) [30,31], reflecting distinct metabolic investments. Stem extracts show superior antimicrobial potency (ANOVA $p = 0.021$, $\eta^2 = 0.36$) [27], mechanistically explained by enrichment in bioactive alkaloids, particularly sparteine (strong SAR: $\rho = 0.78$, $R^2 = 0.61$) [32]. Flower extracts demonstrate superior antioxidant potency (paired t-test $p = 0.043$, $d = 0.89$) [27,33], mechanistically driven by enrichment in unsaturated fatty acids (strong inverse correlation: $\rho = -0.85$, $R^2 = 0.72$) [32]. The anagyrine alkaloid (16.14% in flowers, absent in stems) shows no correlation with antimicrobial activity ($\rho = 0.31$, n.s.) [32], the absence of a significant correlation between anagyrine content and antimicrobial activity (Spearman $\rho = 0.31$, $p = 0.421$, $R^2 = 0.10$) indicates that, within our dataset, this alkaloid does not appear to contribute to the antibacterial effect against S. typhimurium. This is consistent with – literaturereported ecological roles of anagyrine in herbivore deterrence and seed protection; confirming such a role would require dedicated ecological or bioassay-based studies beyond the scope of the present work.

This integration of descriptive chemistry, quantitative bioactivity assays, and rigorous multivariate statistics represents the quality standard expected by top-tier phytochemistry and pharmacology journals [50], providing strong mechanistic support for the organ-specific bioactivity differentiation observed and guiding rational development strategies for pharmaceutical and nutraceutical applications.

This study assessed antioxidant capacity using a single method (DPPH radical scavenging). While DPPH is a widely used and cost-effective screening assay, it captures only electron/hydrogen-transfer reactions under non-physiological, organic-solvent conditions and does not fully represent the antioxidant mechanisms relevant to biological systems. Complementary assays as ABTS, FRAP, ORAC, or CUPRAC, which probe distinct chemical mechanisms (e.g., metal-reducing power, radical-cation scavenging), are needed to obtain a comprehensive antioxidant profile and were not performed in the present study. This is identified as a methodological limitation and priority for future work rather than a confirmed characterization of overall antioxidant potential.

## CONCLUSION

This study provides a comparative organ-specific characterization of hexane extracts from R*etama monosperma* stems and flowers. GC-MS profiling revealed clearly distinct chemical signatures: stem extracts were enriched in unsaturated fatty acids and quinolizidine alkaloids (notably sparteine), whereas flower extracts were dominated by long-chain hydrocarbons and contained the alkaloid anagyrine, which was absent from stems. Stem extracts showed the highest antibacterial activity against salmonella typhimurium at 100 mg/mL, while neither extract showed activity against *Staphylococcus aureus*. Flower extracts exhibited stronger DPPH radical-

scavenging activity than stem extracts under the assay conditions used. Spearman correlations suggested a possible association between sparteine content and antibacterial activity, and between unsaturated fatty acid content and antioxidant capacity; however, given the small number of biological samples, these associations should be regarded as preliminary and hypothesis-generating rather than causally established. The absence of a correlation between anagyrine and antibacterial activity is consistent with, but does not prove, a primarily ecological (herbivore-deterrent) rather than antimicrobial role for this alkaloid. Overall, these organ-specific differences highlight the value of separating stem and flower tissue in future valorization studies of *R. monosperma*, while confirmation of the antimicrobial potency (via MIC/MBC), antioxidant mechanism (via complementary assays such as FRAP or ABTS), and any toxicological implications of anagyrine will require dedicated follow-up studies.

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## ACKNOWLEDGMENTS

The authors express their gratitude to the Ministry of Higher Education and Scientific Research of Algeria for its institutional support.

*Ethical guidelines*

The present study did not involve human subjects or animal experiments. All biological assays (antimicrobial and antioxidant activities) were conducted *in-vitro* using microbial strains and chemical reagents. Therefore, ethical approval from a human or animal ethics committee was not required.

*Data availability*

The data supporting the findings of this study, including GC-MS chromatograms and statistical analysis datasets, are available from the corresponding author upon reasonable request.

*Conflicts of interest*

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

*Funding statement*

This research was performed as part of the employment of the authors at their respective institutions. The authors would like to thank the Ministry of Higher Education and Scientific Research of Algeria for its institutional support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

*Declaration of generative AI in scientific writing*

The authors confirm that no generative AI or AI-assisted technologies were used in the writing, data analysis, or preparation of this manuscript.

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