Phytochemical Characterisation and Assessment of the Antifungal and Antioxidant Potential of the Hexane Extract of *Symphytum officinale*

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ABSTRACT

Symphytum officinale L. has been historically used to treat inflammatory and orthopaedic conditions. Despite its antifungal and antioxidant properties, the nonpolar extract of the plant has received little attention. The current study investigates hexane extract of *S. officinale* for its phytochemical composition and its antioxidant and antifungal properties. A Soxhlet system was utilised to obtain the hexane extract and its secondary metabolite profile was analysed by LC-MS. Among the 16 secondary metabolites identified, selected metabolites exhibited antifungal and antioxidant properties, including pyrrolizidine alkaloids, flavonoids, phenolic acids, glycosides and fatty acid derivatives. The extract's antifungal activity against *Fusarium solani* and *Aspergillus fumigatus* was assessed in the presence of physiologically relevant fat-soluble antioxidants using the agar well diffusion method. At 40 mg/mL, the extract exhibited a clear dose-dependent effect, with zones of inhibition of 13.15 ± 0.03 mm against *F. solani* and 14.12 ± 0.04 mm against *A. fumigatus*. According to the DPPH-based radical-scavenging assay, the hexane extracts exhibited substantial antioxidant capacity, with scavenging activity increasing gradually from 26.01% at 200 µg/mL to 54.21% at 400 µg/mL. These results confirm the presence of physiologically relevant fat-soluble antioxidants, although their activity is lower than that of ascorbic acid. The present study demonstrates the biochemical diversity of the nonpolar fraction of *S. officinale* and indicates that the hexane extract possesses antifungal and antioxidant properties.

***Keywords**:* Antifungal, Antioxidant, Hexane extract, Phytochemical, *Symphytum officinale*

INTRODUCTION

Symphytum officinale L., popularly known as Comfrey, is a vital medicinal plant that belongs to Boraginaceae family. It is mostly prevalent in Europe and Asia. It has been used to treat inflammation, gastrointestinal disorders and respiratory ailments. Research over the past decade has substantially supported the use of comfrey and its derived phyto-constituents in treating musculoskeletal diseases, including strains, blunt trauma, fractures, sprains, acute myalgia, and inflammatory and degenerative rheumatic illnesses (Smith *et al.,* 2011). It encompasses a wide range of

phytochemicals, including allantoin, phenolic acids, pyrrolizidine alkaloids, polysaccharides, triterpenes, fatty acids and sterols, which contribute to its therapeutic value. It is well known that lipophilic compounds, especially fatty acids, terpenoids, and phytosterols, have significant antioxidant and antibacterial properties (Trifan *et al.,* 2024). The increasing number of fungal infections, particularly those caused by *Aspergillus*, *Fusarium*, and *Candida* species, is a global health concern. The disadvantages of many conventional antifungal drugs include toxicity, decreased efficacy, and growing resistance. This has led to a rise in the search for plant-based antifungal medications that are more effective, safer, and more environmentally friendly. Although numerous studies have investigated phytochemical composition and pharmacological properties of *Symphytum officinale*, most research has focused on polar and semi-polar extracts, which are known to contain phenolic acids, flavonoids, and allantoin associated with antioxidant, anti-inflammatory, and wound-healing activities (Nastic *et al.,* 2020). In contrast, the biological potential of plant's non-polar fraction remains largely unexplored. Limited information is available on antifungal activity of lipophilic metabolites extracted with non-polar solvents, despite such extracts often being enriched in fatty acids, phytosterols, terpenoids, and other hydrophobic compounds with documented antimicrobial and antioxidant properties (Syed *et al.,* 2025). Furthermore, comprehensive characterization of non-polar constituents using advanced analytical techniques such as LC–MS has rarely been reported for *S. officinale*.

Therefore, the present study was designed to investigate the phytochemical profile, antifungal activity and antioxidant potential of the hexane extract of *S. officinale*. By specifically targeting the plant's non-polar metabolite fraction and characterising its constituents using LC–MS, this study provides novel insights into the bioactive compounds responsible for the observed biological activities. The findings contribute to a better understanding of the therapeutic potential of lipophilic metabolites from S. officinale and support their possible application in the development of natural antifungal and antioxidant agents.

MATERIAL AND METHODS

Collection of plant material

Symphytum officinale plant samples were collected from the Botanical Garden, University of Kashmir, Srinagar, J&K. The leaves were collected in sufficient quantity and washed thoroughly with running tap

water. They were further surface-sterilised with 70% ethanol for 2 min, then rinsed with distilled water. The leaves were shade-dried for two to three weeks and subsequently ground into a fine powder using an electric grinder. The powdered material was stored in an airtight container for future use (Trifan *et al.,* 2025; Luca *et al*., 2024).

Preparation of Hexane extract

Soxhlet Extraction

Approximately 50 g of dried leaf powder from the experimental plant was Soxhlet-extracted with 500 mL of hexane for 10-12 hours. The resulting extract was filtered through Whatman filter paper and concentrated using a rotary evaporator. A portion of the concentrated extract was sent to the Sophisticated Analytical Instrumentation Facility (SAIF), Chandigarh, India, for LC–MS analysis, while the remaining extract was stored in airtight glass vials at 4°C for future use (Mahire *et al.,* 2020; Hidayati *et al*.,2023).

LC-MS Analysis

LC–MS analysis was performed at the SAIF, India, using a DBA064 mass spectrometry system coupled with a Waters Acquity autosampler. Metabolite profiling was conducted through direct infusion mass spectrometry (MS and MS/MS) operated in positive electrospray ionisation (ESI+) mode. Data acquisition was carried out over a mass-to-charge (m/z) range of 50-3200, ensuring high analytical accuracy with a mass precision of <1 ppm. Spectral data were collected at a scanning rate of one spectrum per second, enabling tentative detection and characterisation of constituents present in the extract (Parekh *et al*., 2007).

Antifungal activity

Hexane extract of *S. officinale* was dissolved in 2% DMSO to prepare a stock solution of 100 mg/mL. Working solutions of 20, 30 and 40 mg/mL were prepared by diluting the stock solution with 2% DMSO. Antifungal activity was evaluated against *Aspergillus fumigatus* and *Fusarium solani*. The fungal strains were maintained and subcultured on Potato Dextrose Agar (PDA) and inoculum was prepared from actively growing cultures. The agar well diffusion method was employed for antifungal testing. PDA plates were uniformly inoculated with 100 µL of conidial suspension, and 6 mm wells were loaded with 50 µL of the extract solutions (20, 30, and 40 mg/mL). Nystatin (NS50) served as the positive control while 2% DMSO was used as negative control. Plates were incubated at 25-28°C for 48 h, after which the zones of inhibition were measured. All assays were performed in triplicate (Abrahim *et al.,* 2025).

Antioxidant activity

The DPPH radical scavenging assay was used to evaluate the antioxidant activity of the plant extract and the standard antioxidant.

Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine significant differences among treatment groups. When significant differences were observed, Tukey's honestly significant difference (HSD) post hoc test was applied for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. Statistical analyses and graphical representations were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

LC-MS-based phytochemical profiling

Based on the peak area and retention time, the chromatogram provides both semi-quantitative and qualitative information. Fig.1 shows the presence of various phytoconstituents, as evidenced by the LC–MS chromatogram of the hexane extract of *Symphytum officinale*, which exhibited several clearly defined peaks.

Metabolite identification was performed by comprehensively comparing the obtained mass spectra with the NIST spectral database and the literature. High-

incubation, the absorbance was measured at 517nm using a UV–Visible spectrophotometer, with methanol as the blank (Syed *et al*., 2026). 3.5 mL of freshly prepared methanolic served as the negative control. To prevent rapid photo-decomposition of DPPH, the samples (0.5 mL) at concentrations ranging from 200 to 400 µg/mL were mixed with the reaction mixtures, which were incubated in the dark at 37°C for 30 minutes. After DPPH solution (0.1 mM). Ascorbic acid was used as the positive control, while a mixture of methanol and DPPH solution. Confidence assignments were established by applying a stringent similarity threshold, whereby only compounds with matching scores exceeding 89% were retained. Based on representative MS spectra are provided in the Supplementary Data (Supplementary Fig. S1–S28). The chemical structures of these compounds were generated using ChemSketch software (Fig. 2).

These criteria indicate that the principal metabolites detected in the *S. officinale* extract belong to fatty acids, terpenoids, and sterols. Ten different compounds were identified tentatively (Table 1) and their

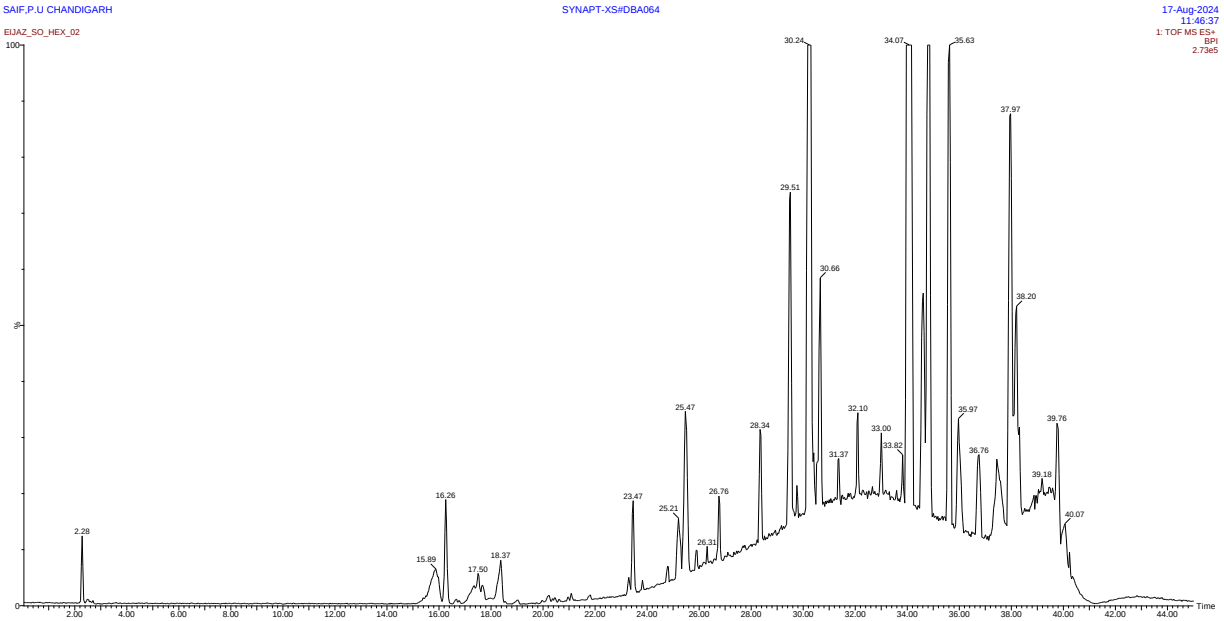

Fig. 1: LC-MS chromatogram of *Symphytum officinale* hexane extract. Retention times are utilised to detect key phytochemical components, and distinct peaks indicate the presence of different secondary metabolites.

Table 1: Tabulated data of LC-MS analysis of the hexane extract of *Symphytum officinale*

Compound Nature	Compound Name	Molecular Formula	m/z	RT	Adduct	Peak Area %	Match Score
Fatty acid	Stearic acid	$C_{18}H_{36}O_2$	285.28	17.70	M+H	2.9	98
	Palmitic acid	$C_{16}H_{32}O_2$	305	16.68	M+K	3.11	95
	γ-Linolenic acid	$C_{18}H_{30}O_2$	278.43	37.49	M+Na	2.88	89
	Lignoceric acid	$C_{24}H_{48}O_2$	391	39.72	M+Na	1.95	93
	Arachidic acid	$C_{20}H_{40}O_2$	335	34.61	M+Na	2.59	95
	Oleic acid	$C_{18}H_{34}O_2$	315.09	24.78	M+Na	3.96	87
Phytosterol	β-Sitosterol	$C_{29}H_{50}$	415	15.89	M+H	1.69	94
Triterpene	Squalene	$C_{30}H_{50}$	411	35.62	M+K	1.89	95
	α-Amyrin	$C_{30}H_{50}O$	427	28.34	M+H	2.74	92
	Isobauerenol	$C_{30}H_{50}O$	426.72	17.50	M+H	3.33	96

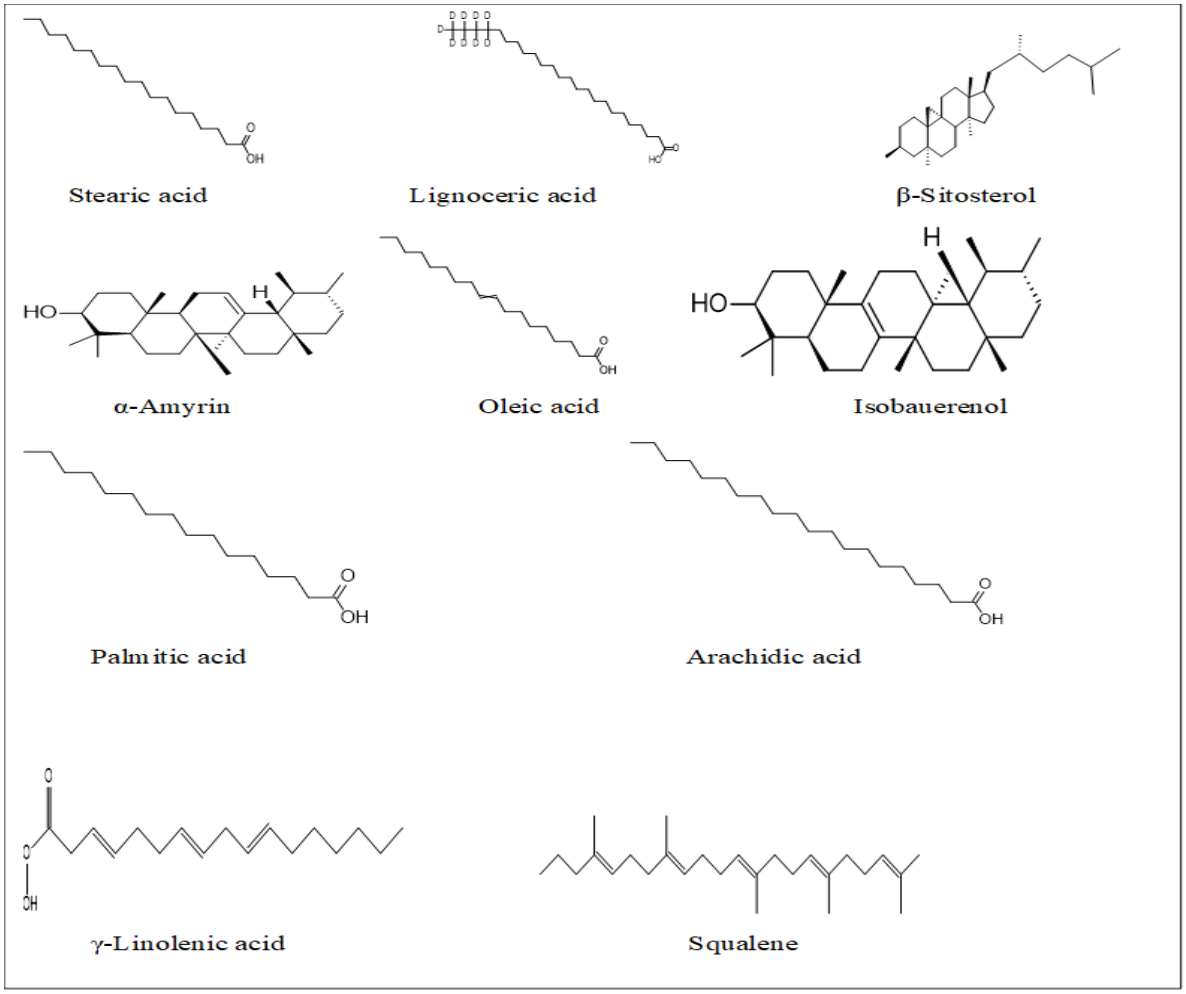

Fig. 2: Chemical structures of tentatively identified compounds.

Antifungal Activity

The hexane extract exhibited significant concentration-dependent antifungal activity against both *A. fumigatus* and *F. solani*. As shown in Table 2, the diameter of the inhibition zones increased progressively with increasing extract concentration (C1–C3), indicating enhanced suppression of fungal growth at higher doses. Against *F. solani*, the extract produced inhibition zones of 9.32±0.11 mm, 11.24±0.10 mm, and 13.15±0.03 mm at concentrations C1, C2, and C3, respectively. The gradual increase in inhibitory activity with concentration suggests a dose-dependent antifungal effect and indicates the presence of bioactive lipophilic constituents in the hexane extract that are effective against *F. solani*.

Similarly, the extract demonstrated appreciable antifungal activity against *A. fumigatus*, producing inhibition zones of 10.16±0.04 mm, 12.21±0.04 mm, and 14.12±0.04 mm at C1, C2, and C3, respectively. Notably, *A. fumigatus* exhibited greater susceptibility to the extract than *F. solani*, as evidenced by consistently larger

inhibition zones across all tested concentrations. The positive control produced the largest inhibition zones, with a maximum zone of 23.43 mm recorded against *A. fumigatus*, confirming its superior antifungal efficacy. Nevertheless, the observed inhibitory effects of the hexane extract demonstrate its potential as a source of natural antifungal compounds. These findings are further illustrated in Fig. 3 and 4.

Table 2: Antifungal activity of the hexane extract at different concentrations against fungal pathogens. Data are represented as the mean ± SD (standard deviation).

Fungal strains	Hexane extract (mg/mL)	Mean ± SD
Fusarium solani	C1	9.32 ± 0.11^{c}
	C2	11.24 ± 0.10^{b}
	C3	13.15 ± 0.03^{a}
Aspergillus fumigatus	C1	10.16 ± 0.04^{c}
	C2	12.21 ± 0.04^{b}
	C3	14.12 ± 0.04^{a}

Note: Values are expressed as mean ±SD. Means followed by different superscript letters within the same fungal strain are significantly different ($p<0.05$) according to Tukey's multiple comparison test following one-way ANOVA, where C1 = 20mg/mL, C2 = 30mg/mL, C3= 40 mg/mL.

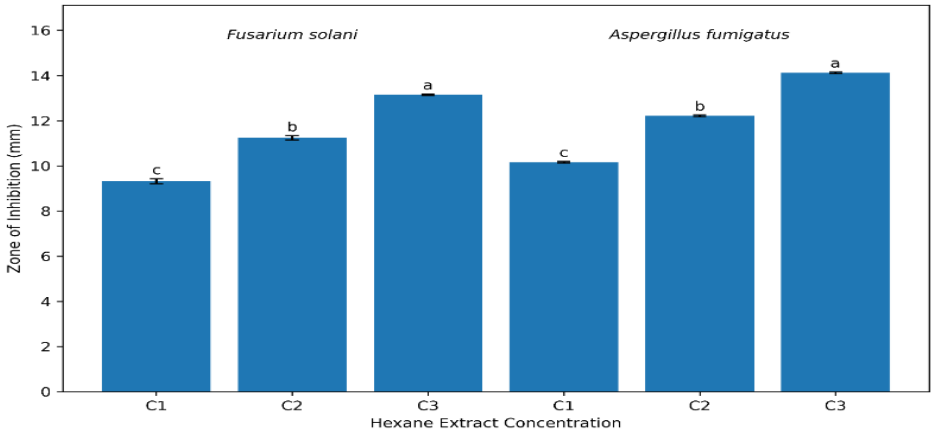

Fig. 3. Dose-dependent inhibition of different concentrations (C1-20mg/ml, C2-30mg/ml, C3-40mg/ml) of Hexane extract against selected fungal pathogens. PC (Positive control-50µg) and NC (negative control-2%DMSO).

Antioxidant Activity

The antioxidant potential of the *Symphytum officinale* hexane extract was evaluated using the DPPH radical-scavenging assay and compared with that of ascorbic acid, which served as the reference standard. The

results presented in Table 3 and illustrated in Fig. 5 demonstrate a clear concentration-dependent increase in antioxidant activity for both the standard and the plant extract. Ascorbic acid exhibited strong radical-scavenging activity, with percentage inhibition increasing from 53.41% at 1 µg/mL to 87.96% at 9 µg/mL, confirming its potent antioxidant capacity. Similarly, the hexane extract showed a gradual increase in DPPH radical inhibition with increasing concentration, ranging from 26.01% at 200 µg/mL to 54.21% at 400 µg/mL. This dose-dependent response indicates the presence of antioxidant constituents capable of neutralising free radicals.

The progressive increase in percentage inhibition observed for both samples confirms the reliability and reproducibility of the assay. Although the antioxidant activity of the hexane extract was lower than that of ascorbic acid, it demonstrated appreciable free-radical-scavenging potential. The concentration-dependent enhancement of antioxidant activity suggests that the extract contains bioactive lipophilic compounds that contribute to its antioxidant properties. Overall, these findings highlight the potential of the *S. officinale* hexane extract as a natural source of antioxidant agents.

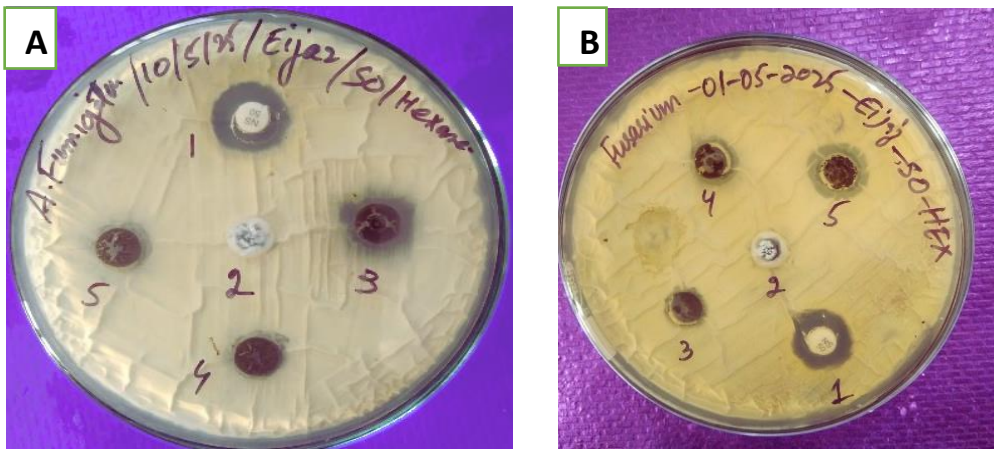

Fig.4: Antifungal activity of Hexane extract against (A) *Aspergillus fumigatus* and (B)*Fusarium solani.* 1(Positive control),2(Negative control),3(C1),4(C2), and 5(C3).

Table 3: Concentration-Dependent DPPH Scavenging Activity of Hexane Extract

Concentration (µg/ml)		**Mean +SD**	
Ascorbic acid	**Plant extract**	**Ascorbic acid**	**Plant extract**
1	200	53.41±0.09^{e}	26.01±0.09^{e}
3	250	66.78±0.09^{d}	35.20±0.15^{d}
5	300	70.45±0.30^{c}	45.91±0.35^{c}

7	350	74.92±0.11[b]	50.38±0.10[b]
9	400	87.96±0.21[a]	54.21±0.11[a]

Note: Data are presented as mean ± SD. Means followed by different letters within a column are significantly different at p 0.05 according to Tukey's multiple comparison test following one-way ANOVA.

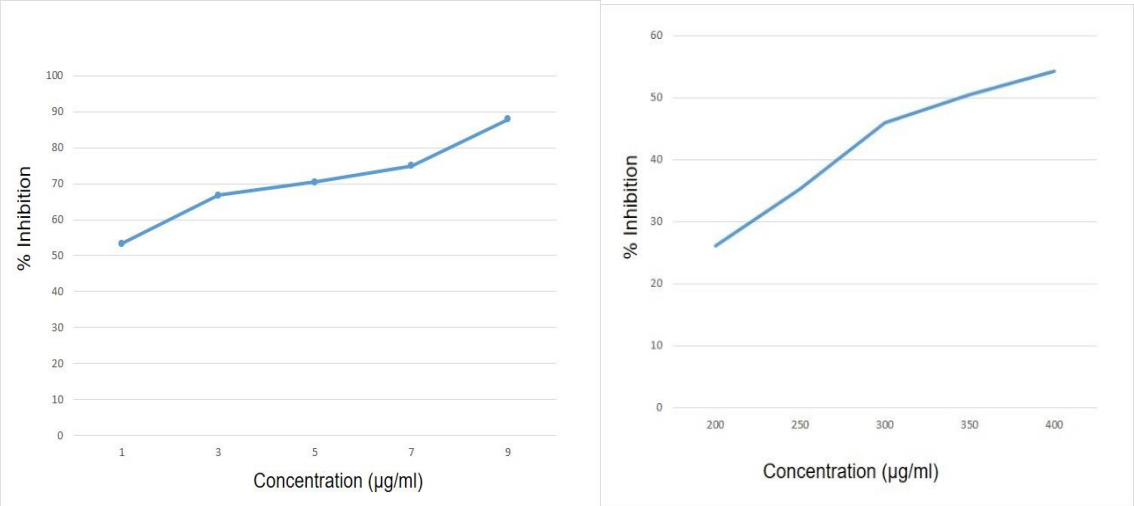

Fig.5: DPPH percentage scavenging activity of ascorbic acid (Reference standard) at different concentrations and DPPH percentage scavenging activity of plant extract (Hexane) at different concentrations.

DISCUSSION

With particular emphasis on the non-polar bioactive components of Symphytum officinale, the present research investigated the phytochemical composition and the antifungal and antioxidant properties of the hexane extract. Despite *S. officinale's* pharmacological properties having been thoroughly studied, most studies have focused on polar components; as a result, little is known about the activity of its lipophilic components (Nastic *et al.,* 2020; Trifan *et al.,* 2024). By demonstrating that the hexane extract contains a variety of secondary metabolites and exhibits measurable antifungal and antioxidant activity, the findings support the therapeutic potential of non-polar phytochemicals. 10 significant secondary metabolites were found by LC-MS investigation, most of which were classified as terpenoids, sterols, and fatty acids. The identification of substances such as gamma-linolenic acid, stigmasterol, Stearic acid, and oleic acid is consistent with previous research reporting these metabolites in different solvent extracts of *S. officinale* (Trifan *et al.,* 2024; Zengin *et al.,* 2021). Furthermore, several lipophilic constituents were characterised in the hexane extract, including γ-linolenic acid,

squalene, lignoceric acid, oleic acid and β-sitosterol. The predominance of these metabolites is consistent with the non-polar nature of hexane, which selectively extracts hydrophobic compounds such as fatty acids, terpenoids, sterols and long-chain hydrocarbons. Similar observations have been reported in previous phytochemical investigations of *S. officinale*, in which non-polar extraction systems preferentially recovered fatty acids and other lipid-derived metabolites (Nastić *et al.,* 2020). The identification of γ-linolenic acid, squalene, lignoceric acid, β-sitosterol, etc., is especially noteworthy because these compounds have been associated with diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial and membrane-protective effects. Thus, the enrichment of the hexane fraction with these bioactive lipophilic constituents may play an important part in the biological properties exhibited by *S. officinale* and highlights the potential of the non-polar extract as a valuable source of pharmacologically relevant natural products. The presence of nonpolar metabolites may be responsible for the reported antifungal and antioxidant activities.

A. fumigatus showed slightly greater susceptibility to the extract than *F. solani* at all tested concentrations. This difference in sensitivity may be related to variations in cell wall composition, membrane structure, or physiological characteristics between the two fungal species. Similar trends have been reported by Nastic *et al.* (2020), who observed higher susceptibility of *Aspergillus* species than *Fusarium* species to non-polar plant extracts. Nevertheless, further studies involving compound isolation and mechanistic investigations are required to identify the bioactive constituents and clarify their modes of action. The DPPH assay demonstrated that the hexane extract possesses moderate, concentration-dependent antioxidant activity, with radical-scavenging activity increasing from 26.01% to 54.21% across the tested concentrations. Although the antioxidant potential was lower than that of the reference standard, ascorbic acid, the results indicate the presence of bioactive constituents capable of neutralising free radicals. The observed antioxidant activity may be attributable to the collective action of the metabolites detected in the extract; however, no direct relationship between specific compounds and antioxidant activity can be established from the current data. The comparatively lower activity relative to polar extracts reported in previous studies may reflect the predominance of lipophilic constituents in the hexane fraction, as many potent antioxidant compounds are preferentially

extracted using more polar solvents (Fratianni *et al*., 2021). Additionally, lipophilic compounds, such as triterpenes and fatty acid derivatives, may contribute to antioxidant activity by preventing lipid peroxidation and quenching hydrophobic radicals, although they are less efficient radical scavengers than phenolics.

CONCLUSION

The findings from the experiment show that the hexane extract from *S. officinale* is a chemically important source of non-polar secondary metabolites, including fatty acid derivatives, terpenoids, and phytosterols. LC–MS-based phytochemical profiling of the hydrophilic fraction identified 10 distinct metabolites, highlighting the plant's rich biochemical diversity. Additionally, the extract demonstrated mild but considerable antioxidant capabilities, suggesting the significance of lipophilic fatty acids and sterols in its radical-scavenging activity. These findings highlight the effectiveness of the non-polar solvent hexane in selectively extracting lipophilic bioactive constituents that may not be efficiently recovered using more polar solvents, thereby providing a complementary approach for phytochemical profiling and bioactivity assessment. Therefore, based on

the findings of the present study, the hexane extract of *S. officinale* demonstrated significant bioactive potential and may serve as a promising source of natural antifungal and antioxidant agents. The pronounced biological activities observed are likely attributable to its diverse phytochemical composition, highlighting its potential application in the development of plant-based therapeutics and natural antioxidant formulations.

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