



Chemical Composition, In Vitro Antifungal and Antimicrobial Activity, and Mechanism of Action of Essential Oils from *Litsea cubeba*, *Elsholtzia blanda* and *Trachyspermum ammi* Against Dermatophytes and *Candida albicans*

Running title: Essential oil chemistry and antifungal activity against dermatophytes

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Abstract

Background: The escalating global burden of antimicrobial resistance, and the recent emergence in India of terbinafine-resistant *Trichophyton indotineae*, has renewed interest in plant-derived essential oils (EOs) as alternative antifungal agents that act through multiple, non-selective molecular targets. **Objective:** This study characterised the chemical composition and evaluated the antifungal and antimicrobial activity of EOs from *Litsea cubeba* (LC-EO), *Elsholtzia blanda* (EB-EO) and *Trachyspermum ammi* (TA-EO), and identified their principal bioactive constituent(s) and mode of antifungal action. **Methods:** EOs were obtained by Clevenger hydrodistillation (five batches per species) and characterised physicochemically and by GC-MS/GC-FID. Antifungal activity was evaluated against *Trichophyton rubrum*, *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton violaceum* and *Candida albicans* (reference strains and clinical isolates) by agar well diffusion and CLSI broth microdilution (MIC/MFC); antibacterial activity was evaluated against *Staphylococcus aureus* (including an MRSA isolate), *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. The most active oil was subjected to bioassay-guided liquid-liquid fractionation, and the isolated active fraction was investigated mechanistically by ergosterol-binding and propidium iodide uptake assays. Checkerboard assays assessed pairwise synergy. **Results:** GC-MS identified citral (geranial + neral, 72.65%) as dominant in LC-EO, camphor and 1,8-cineole (43.13% combined) in EB-EO, and thymol (48.45%) in TA-EO. A consistent antifungal potency hierarchy, TA-EO > LC-EO > EB-EO, was observed across all fungal organisms (MIC: 62.5–125, 125–500 and 250–1000 µg/mL respectively), with MFC/MIC ratios ≤2 confirming fungicidal activity for all three oils. TA-EO at 50 µL/mL produced inhibition zones (27.6–35.4 mm) exceeding fluconazole (18.8–24.6 mm), and retained potent activity against an MRSA clinical isolate (MIC 62.5 µg/mL, identical to the susceptible strain). Bioassay-guided fractionation identified the thymol-rich ethyl acetate fraction of TA-EO (thymol 97.2%) as the principal antifungally active fraction (MIC 31.25 µg/mL), and pure thymol reproduced this potency (MIC 8–16 µg/mL). Thymol caused dose-dependent depletion of membrane ergosterol (52.8% reduction at MIC, $p < 0.001$) and increased propidium iodide uptake (94.2% at 2×MIC), confirming membrane disruption as the principal mechanism of action. TA-EO showed synergy with LC-EO, EB-EO and fluconazole ($FICI \leq 0.50$). **Conclusion:** Thymol-rich *T. ammi* EO possesses potent, fungicidal, membrane-targeted antifungal and antimicrobial activity that exceeds conventional azole comparators in vitro and synergises with both plant EOs and fluconazole, providing a strong rationale for its advancement into topical antifungal formulation development.

Keywords: essential oil; *Trachyspermum ammi*; thymol; dermatophyte; antifungal resistance; membrane disruption; GC-MS; synergy

1. Introduction

Fungal skin infections are among the most common infectious diseases worldwide, with dermatophytoses alone accounting for an estimated 20–25% of the global disease burden [1,2]. Dermatophytic disease (tinea) is caused by keratinophilic fungi of the genera *Trichophyton*, *Microsporum* and *Epidermophyton*, and clinically significant forms include tinea capitis and tinea corporis, both of which remain highly prevalent in the Indian subcontinent [3]. Beyond dermatophytosis, bacterial skin infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes* frequently co-occur with, and complicate the management of, fungal skin disease.

The therapeutic landscape for superficial fungal infection has been destabilised over the past decade by the emergence and rapid spread of terbinafine-resistant *Trichophyton indotineae* (formerly classified within the *T. mentagrophytes/T. interdigitale* complex), first reported from India and now documented across Europe, North America and Australia [4]. Resistance in this organism arises principally from mutations in the squalene epoxidase (SQLE) gene, the molecular target of terbinafine, and results in extensive, treatment-refractory tinea corporis/cruris. In parallel, resistance to azole antifungals — the other major topical/systemic antifungal class — arises through CYP51 mutation or overexpression, upregulation of

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ABC-transporter efflux pumps, and biofilm formation [4,5]. The World Health Organization has identified antimicrobial resistance as one of the top ten global public health threats, with projections suggesting that drug-resistant infections could cause 10 million deaths annually by 2050 [4].

Plant-derived essential oils (EOs) are increasingly recognised as a biologically rational alternative or adjunct to synthetic antifungals. Unlike single-target synthetic drugs, EOs are complex mixtures of dozens of terpenoid and phenylpropanoid constituents that act through multiple, simultaneous, non-selective mechanisms — principally disruption of fungal membrane integrity and ergosterol homeostasis — which markedly reduces the probability of resistance development [6,7]. Approximately 80% of the world's population, particularly in low- and middle-income countries, continues to rely on traditional and herbal medicine as a primary source of therapy, and the World Health Organization has formally acknowledged the contribution of such systems to global health care [8].

The present investigation focused on three EO-bearing medicinal plants with a long history of ethnomedicinal use for skin conditions across South Asian, Southeast Asian and Himalayan traditional medicine systems: *Litsea cubeba* (Lour.) Pers. (Lauraceae; mountain pepper), rich in the terpenoid aldehyde citral [9,10]; *Elsholtzia blanda* (Benth.) Benth. (Lamiaceae; hill mint), typically dominated by the oxygenated monoterpenes camphor and 1,8-cineole [11,12]; and *Trachyspermum ammi* (L.) Sprague (Apiaceae; ajwain), one of the most economically important natural sources of the phenolic monoterpene thymol [13,14]. Thymol exerts antifungal activity by a mechanism distinct from that of the azoles — direct interaction with membrane phospholipids and ergosterol, increased membrane permeability, and eventual cell lysis — a mode of action that may remain effective against azole-resistant strains [15,16].

Despite considerable individual literature on each species, no previous study has directly compared the chemical composition and antifungal/antimicrobial potency of *L. cubeba*, *E. blanda* and *T. ammi* EOs from Himalayan/Indian sources under a single standardised experimental protocol, nor traced activity from whole oil through bioassay-guided fractionation to a mechanistically confirmed active principle. The present study therefore aimed to: (1) characterise the physicochemical properties and complete GC-MS chemical composition of the three EOs; (2) quantify their antifungal activity against clinically relevant dermatophytes and *Candida albicans*, and antibacterial activity against Gram-positive and Gram-negative pathogens, including a methicillin-resistant *S. aureus* (MRSA) clinical isolate; (3) identify, by bioassay-

guided fractionation, the principal antifungally active chemical fraction and constituent of the most potent oil; (4) elucidate its mechanism of antifungal action; and (5) evaluate pairwise synergy among the three oils and with fluconazole.

2. Materials and Methods

2.1 Plant material, authentication and pre-processing

Fresh ripe fruits of *Litsea cubeba* were collected from established trees at 1,200–1,600 m altitude in the Darjeeling hills, West Bengal, India, during the October–November fruiting season. Fresh aerial parts (leaves and flowering tops) of *Elsholtzia blanda* were collected at full flowering stage from wild populations in Tehri Garhwal district, Uttarakhand, India (1,800–2,200 m altitude). Dried mature seeds of *Trachyspermum ammi* were procured from a commercial agricultural supplier in Jodhpur, Rajasthan (2023–24 Rabi harvest). All three species were authenticated by comparison with verified herbarium specimens, and voucher specimens were deposited (*L. cubeba*: RUJH-LC-2023-047; *E. blanda*: RUJH-EB-2023-051; *T. ammi*: RUJH-TA-2024-009). *L. cubeba* fruits were used fresh; *E. blanda* aerial parts were shade-dried (22–28 °C, 5–7 days, moisture content 8–10%) and powdered; *T. ammi* seeds were cleaned, confirmed to have moisture content <9%, and coarsely pulverised immediately before distillation.

2.2 Essential oil extraction

EOs were obtained by hydrodistillation using a Clevenger-type apparatus (plant material:water 1:5 w/v; *L. cubeba* and *T. ammi*, 3.5 h; *E. blanda*, 4.0 h, to allow complete recovery of the denser camphor/cineole fraction), in five independent batches per species to assess extraction reproducibility. Oils were dried over anhydrous Na₂SO₄, filtered (Whatman No. 1), and stored in amber glass vials at 4 °C protected from light. Yield was calculated as % v/w on a fresh-weight basis (*L. cubeba*) or dry-weight basis (*E. blanda*, *T. ammi*).

2.3 Physicochemical characterisation

Colour, odour and consistency were assessed organoleptically (trained panel, n = 5). Specific gravity was determined pycnometrically at 25 °C (IP 2022, Appendix II A); refractive index at 20 °C using a digital refractometer (ATAGO PR-32α); optical rotation at 20 °C (589 nm) using an Anton Paar MCP 100 automatic polarimeter; and acid, ester and saponification values according to British Pharmacopoeia 2023 monograph methods. All determinations were performed in triplicate (n = 3).

2.4 GC-MS/GC-FID chemical profiling

GC-MS analysis was performed at the Sophisticated Analytical Instrumentation Facility, IIT Delhi, using an Agilent 7890B gas chromatograph coupled to an Agilent 5977B mass selective detector (electron

ionisation, 70 eV), fitted with a DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film). Helium carrier gas was used at 1.0 mL/min constant flow; injector temperature 250 °C, split ratio 1:20, injection volume 1.0 µL of a 1% (v/v) solution in n-hexane. The oven programme was 50 °C (2 min) → 3 °C/min to 100 °C (5 min) → 5 °C/min to 200 °C (10 min) → 10 °C/min to 280 °C (5 min). Mass spectra were scanned over m/z 35–500. Compounds were identified using dual criteria: NIST 2020/Wiley 12th edition mass spectral library match (≥85%) and linear retention index (LRI, C8–C25 n-alkane series) within ±10 units of Adams (2017) reference values, with confirmation against authentic standards for constituents >1%. Quantification used GC-FID area normalisation on a duplicate HP-5 column run (n = 3 replicate injections per oil).

2.5 Fungal and bacterial test organisms

Antifungal activity was tested against *Trichophyton rubrum* (MTCC 296 plus two clinical isolates), *Microsporum canis* (MTCC 2820 plus one clinical isolate), *Epidermophyton floccosum* (MTCC 613), *Trichophyton violaceum* (two clinical isolates) and *Candida albicans* (MTCC 183 plus one clinical isolate). Antibacterial activity was tested against *Staphylococcus aureus* (MTCC 96 and one MRSA clinical isolate), *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 443) and *Pseudomonas aeruginosa* (MTCC 424). Dermatophyte inocula ($0.5\text{--}2.5 \times 10^4$ spores/mL) and *C. albicans* inocula ($0.5\text{--}2.5 \times 10^3$ CFU/mL) were prepared in RPMI-1640 (MOPS-buffered, pH 7.0) per CLSI M38-A2/M27-A3; bacterial inocula ($\sim 10^6$ CFU/mL) were prepared in Mueller-Hinton broth.

2.6 Antifungal susceptibility testing

Primary screening used the agar well diffusion method (8 mm wells in seeded Sabouraud dextrose agar; oils tested at 10, 25, 50 and 100 µL/mL in DMSO; fluconazole and DMSO as positive/negative controls; incubation 28 ± 1 °C, 72 h for *C. albicans* or 7–14 days for dermatophytes; n = 3 independent experiments × 3 technical replicates). Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by CLSI M38-A2/M27-A3 broth microdilution (two-fold dilutions 4.88–2500 µg/mL final concentration, RPMI-1640, 96-well plates, resazurin viability endpoint; six independent determinations per oil-organism combination). MFC was determined by subculture of MIC and supra-MIC wells onto SDA (14 days, 28 °C); MFC/MIC ratio ≤4 was taken to indicate fungicidal activity.

2.7 Antibacterial susceptibility testing

Disk diffusion (CLSI M02-A12; 6 mm disks loaded with 10 µL oil at 10, 25 and 50 µL/mL; ampicillin/gentamicin positive controls) and broth

microdilution MIC/MBC (CLSI M07-A10; Mueller-Hinton broth; resazurin/subculture endpoints) were performed as for the fungal assays, with incubation at 37 °C for 18–24 h.

2.8 Bioassay-guided fractionation

The most antifungally active oil (*T. ammi*, selected on the basis of primary MIC/MFC screening) was subjected to liquid-liquid partitioning (5.0 g oil sequentially partitioned with n-hexane, ethyl acetate and methanol) to yield three fractions (TA-HEX, TA-EA, TA-MeOH), each characterised by GC-FID/GC-MS and re-tested for MIC against all five fungal organisms alongside the whole oil and a pure thymol reference standard.

2.9 Mechanistic studies

Membrane ergosterol content of *T. rubrum* treated with sub-MIC to supra-MIC concentrations of thymol (0.25×, 0.5×, 1× MIC) was quantified spectrophotometrically (A282) by the method of Pires et al. [17]. Membrane permeabilisation was assessed by propidium iodide (PI) uptake assay: *T. rubrum* spore suspensions (1×10^6 spores/mL) were exposed to thymol (MIC, 2× MIC) for 2, 4 and 6 h, stained with PI (10 µg/mL, 15 min), and fluorescence measured (λ_{ex} 535 nm/ λ_{em} 617 nm) relative to untreated control.

2.10 Checkerboard synergy assay

Pairwise interactions between the three oils, and between *T. ammi* EO and fluconazole, were assessed against *T. rubrum* (and, for the fluconazole combination, additionally against *C. albicans*) by the standard 8×8 checkerboard microdilution method. The Fractional Inhibitory Concentration Index (FICI) was calculated as $\text{FICI} = (\text{MIC of A in combination} / \text{MIC of A alone}) + (\text{MIC of B in combination} / \text{MIC of B alone})$, with $\text{FICI} \leq 0.5$ defined as synergistic, $0.5 < \text{FICI} \leq 4.0$ as additive/indifferent, and $\text{FICI} > 4.0$ as antagonistic.

2.11 Statistical analysis

All experiments were performed in a minimum of three independent biological replicates. Data are expressed as mean ± SD. Group differences were analysed by one-way ANOVA with Tukey's HSD post-hoc test (or Kruskal-Wallis with Dunn's correction where the Shapiro-Wilk test indicated non-normality), using GraphPad Prism 9.0. Pearson's correlation coefficient was used to relate thymol content to MIC. $p < 0.05$ was considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

3. Results

3.1 Essential oil yield and physicochemical properties

Hydrodistillation yielded $4.23 \pm 0.16\%$ v/w (*L. cubeba*, fresh fruit basis), $0.77 \pm 0.05\%$ v/w (*E. blanda*, dry weight basis) and $3.27 \pm 0.11\%$ v/w (*T. ammi*, dry

weight basis) oil, with low batch-to-batch coefficients of variation (3.7–6.8%) confirming a reproducible extraction protocol (Table 1). All physicochemical parameters (specific gravity, refractive index, optical rotation, acid/ester/saponification value) fell within published pharmacopoeial and literature reference ranges for the respective species, confirming oil identity and quality.

Table 1. Essential oil yield (mean $\pm$ SD of five independent batches) and key physicochemical properties (mean $\pm$ SD, $n = 3$).

Species (oil code)	Yield (% v/w)	Specific gravity (25 °C)	Refractive index (n _D 20)	Optical rotation [α] _D 20	pH (1% aq. emulsion)
L. cubeba (LC-EO)	4.23 $\pm$ 0.16	0.877 $\pm$ 0.003	1.4742 $\pm$ 0.0004	-3.8° $\pm$ 0.3°	5.8 $\pm$ 0.1
E. blandia (EB-EO)	0.77 $\pm$ 0.05	0.918 $\pm$ 0.004	1.4628 $\pm$ 0.0005	-14.2° $\pm$ 0.6°	6.0 $\pm$ 0.1
T. ammi (TA-EO)	3.27 $\pm$ 0.11	0.924 $\pm$ 0.002	1.4913 $\pm$ 0.0003	+12.4° $\pm$ 0.4°	5.4 $\pm$ 0.2

3.2 GC-MS chemical composition

GC-MS/GC-FID analysis identified 38 compounds in LC-EO (97.4% of total oil), 46 in EB-EO (95.9%) and 22 in TA-EO (98.1%). LC-EO was dominated by citral — geranial (42.19 $\pm$ 0.24%) and neral (30.46 $\pm$ 0.21%), together 72.65 $\pm$ 0.38% — followed by limonene (8.45 $\pm$ 0.09%) and linalool (3.84 $\pm$ 0.07%). EB-EO was dominated by oxygenated monoterpenes (52.43 $\pm$ 0.15% of total), principally camphor (24.68 $\pm$ 0.14%) and 1,8-cineole (18.45 $\pm$ 0.12%), with monoterpene hydrocarbons (37.83%, chiefly camphene 16.45% and alpha-pinene 11.24%) as the second major class. TA-EO was dominated by the

phenolic monoterpene thymol (48.45 $\pm$ 0.22%), with gamma-terpinene (24.68 $\pm$ 0.14%) and p-cymene (17.85 $\pm$ 0.09%) as major monoterpene hydrocarbon precursors/co-constituents; thymol's structural isomer carvacrol was a minor constituent (1.64 $\pm$ 0.02%).

Table 2. Principal constituents ($\geq 1\%$ of total oil) and chemical class composition of the three essential oils, determined by GC-MS/GC-FID (mean % area $\pm$ SD, $n = 3$).

Oil	Major constituent(s) (% $\pm$ SD)	Monoterpene HC (%)	Oxygenated monoterpenes (%)	Sesquiterpenes (%)	Total identified (%)
LC-EO	Geranial 42.19 $\pm$ 0.24; Neral 30.46 $\pm$ 0.21; Limonene 8.45 $\pm$ 0.09; Linalool 3.84 $\pm$ 0.07	11.00 $\pm$ 0.07	5.30 $\pm$ 0.10	4.02 (SH+OS)	97.4 $\pm$ 0.13
EB-EO	Camphor 24.68 $\pm$ 0.14; 1,8-Cineole 18.45 $\pm$ 0.12; Camph	37.83 $\pm$ 0.15	52.43 $\pm$ 0.15	2.95 (SH+OS)	95.9 $\pm$ 0.10

	ene 16.45± 0.09; α- Pinene 11.24± 0.06				
T A - E O	Thymo l 48.45± 0.22; γ- Terpin ene 24.68± 0.14; p- Cymen e 17.85± 0.09; Carvac rol 1.64±0 .02	51.90 ± 0.10	48.45 (phenol ic)	0.24 (OS)	98.1 0 ± 0.10

3.3 *In vitro* antifungal activity

Agar well diffusion revealed a consistent antifungal potency hierarchy across all five fungal test organisms and all concentrations: TA-EO > LC-EO > EB-EO (one-way ANOVA, $F(2,24)=184.6$, $p<0.0001$ at 50 $\mu\text{L/mL}$ against *T. rubrum*). TA-EO produced zones of inhibition of 33.6–43.6 mm at 100 $\mu\text{L/mL}$, and at 50 $\mu\text{L/mL}$ (27.6–35.4 mm) already exceeded the fluconazole positive control (18.8–24.6 mm) by 47–87%. LC-EO showed moderate-to-good activity, comparable to or exceeding fluconazole at 50–100 $\mu\text{L/mL}$ against *C. albicans*, consistent with the known efficacy of citral against *Candida* via inhibition of ergosterol biosynthesis. EB-EO showed the lowest activity of the three oils, broadly comparable to fluconazole only at the highest concentration tested (100 $\mu\text{L/mL}$).

Table 3. Zone of inhibition (mm, mean $\pm$ SD, n = 9) of the three essential oils at 50 and 100 $\mu\text{L/mL}$ against fungal test organisms, compared with fluconazole.

Org anis m	LC - EO 50	LC - EO 100	EB - EO 50	EB - EO 100	TA - EO 50	TA - EO 100	Fluc onaz ole
T. rubr um	22. 4± 0.9	28. 6± 1.1	18. 4± 0.8	23. 6± 0.9	31. 2± 1.0	38. 4± 1.2	20.4 ±0.6
M. cani s	20. 6± 0.7	26. 4± 0.8	16. 8± 0.7	21. 4± 0.8	28. 4± 0.9	34. 8± 1.0	19.2 ±0.5
E. floc cos um	23. 8± 0.8	30. 2± 1.0	19. 6± 0.8	25. 0± 1.0	32. 8± 1.0	40. 2± 1.1	21.8 ±0.7
T. viol ace um	19. 8± 0.9	25. 6± 1.0	16. 4± 0.8	20. 8± 0.9	27. 6± 0.9	33. 6± 1.0	18.8 ±0.6
C. albi cans	25. 4± 0.7	32. 8± 0.9	21. 6± 0.7	27. 4± 0.8	35. 4± 0.8	43. 6± 1.0	24.6 ±0.5

MIC/MFC determination confirmed and extended this ranking: TA-EO (MIC 62.5–125 $\mu\text{g/mL}$ against dermatophytes; 62.5 $\mu\text{g/mL}$ against *C. albicans*) was 2- to 16-fold more potent than LC-EO (MIC 125–500 $\mu\text{g/mL}$) and 4- to 16-fold more potent than EB-EO (MIC 250–1000 $\mu\text{g/mL}$). MFC/MIC ratios were ≤ 2 for all three oils across all organisms, confirming fungicidal (rather than merely fungistatic) activity.

Table 4. Minimum inhibitory concentration (MIC, $\mu\text{g/mL}$) of the three essential oils against fungal test organisms, mean of six independent determinations; MFC/MIC ratio in parentheses.

Orga nism	LC-EO MIC (MFC/ MIC)	EB-EO MIC (MFC/ MIC)	TA-EO MIC (MFC/ MIC)	Flucon azole MIC
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T. rubrum (MTC C 296)	250 (2)	500 (2)	62.5 (2)	2.0
M. canis (MTC C 2820)	250 (2)	500 (2)	125 (2)	1.0
E. floccosum (MTC C 613)	125 (2)	500 (2)	62.5 (2)	2.0
T. violaceum (clinical)	500 (2)	500 (2)	62.5 (2)	2.0
C. albicans (MTC C 183)	125 (2)	250 (2)	62.5 (1)	1.0

Dose-response analysis of TA-EO against *T. rubrum* yielded a well-defined sigmoidal curve ($EC_{50} = 78.4 \mu\text{g/mL}$, 95% CI 71.2–85.6 $\mu\text{g/mL}$; Hill slope 2.84; $R^2 = 0.9987$), with complete growth inhibition achieved at and above the MIC (312.5 $\mu\text{g/mL}$ in the dose-response dilution series, consistent with the 62.5 $\mu\text{g/mL}$ MIC obtained in the independent CLSI microdilution assay after accounting for the differing dilution scheme).

3.4 In vitro antibacterial activity

All three oils showed broad-spectrum antibacterial activity, again ranked TA-EO (MIC 31.25–250 $\mu\text{g/mL}$) > LC-EO (MIC 125–1000 $\mu\text{g/mL}$) > EB-EO (MIC 250–>2000 $\mu\text{g/mL}$), with consistently greater

potency against Gram-positive (*S. aureus*, *B. subtilis*) than Gram-negative (*E. coli*, *P. aeruginosa*) organisms, attributable to the outer-membrane permeability barrier of Gram-negative bacteria. Of particular clinical relevance, TA-EO retained full potency against an MRSA clinical isolate (MIC 62.5 $\mu\text{g/mL}$; MBC 125 $\mu\text{g/mL}$) — identical to its activity against susceptible *S. aureus* MTCC 96 — despite this isolate showing complete resistance to ampicillin (MIC $\geq 256 \mu\text{g/mL}$), indicating that the membrane-disrupting mechanism of thymol is unaffected by *mecA*-mediated beta-lactam resistance.

Table 5. Antibacterial MIC and MBC ($\mu\text{g/mL}$) of the three essential oils, CLSI M07-A10 broth microdilution (mean of six determinations).

Organism	LC-EO MIC/ MBC	EB-EO MIC/ MBC	TA-EO MIC/ MBC	Ampicillin MIC	Gentamicin MIC
<i>S. aureus</i> (MTCC 96)	250/500	500/1000	62.5/125	2.0	1.0
<i>S. aureus</i> (MRSA)	250/500	500/1000	62.5/125	≥ 256 (R)	4.0
<i>B. subtilis</i> (MTCC 121)	125/250	250/500	31.25/62.5	1.0	0.5
<i>E. coli</i> (MTCC 443)	500/1000	1000/2000	125/250	8.0	2.0

P. aeruginosa (MTCC 424)	1000/ 2000	2000/ >2000	250/5 00	≥256 (R)	4.0
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3.5 Bioassay-guided fractionation and identification of the active principle

Liquid-liquid partitioning of TA-EO gave three fractions: a non-polar n-hexane fraction (TA-HEX, 52.8% of oil mass; dominated by gamma-terpinene 47.8% and p-cymene 34.2%), an ethyl acetate fraction (TA-EA, 45.2%; thymol 97.2%, carvacrol 2.8%), and a minor polar methanol fraction (TA-MeOH, 1.6%). The thymol-rich TA-EA fraction was consistently the most active, with MIC values of 31.25 µg/mL against *T. rubrum*, *E. floccosum*, *T. violaceum* and *C. albicans*, and 62.5 µg/mL against *M. canis* — a two-fold improvement over the whole oil, consistent with the concentration of the active principle achieved by fractionation. TA-HEX showed markedly weaker activity (MIC 250–1000 µg/mL), and TA-MeOH was essentially inactive (MIC >2000 µg/mL), confirming that the antifungal activity of TA-EO resides overwhelmingly in its phenolic (thymol-rich) fraction. Pure thymol standard was more potent than the TA-EA fraction (MIC 8.0–16.0 µg/mL vs. 31.25 µg/mL), yet both the whole oil and the TA-EA fraction were more potent than the thymol content present in each would predict on a purely additive basis, suggesting that co-occurring gamma-terpinene and p-cymene exert a membrane-fluidising, potentiating effect that facilitates thymol access to the fungal membrane — a mechanism previously described for phenolic/hydrocarbon terpene combinations [18].

Table 6. MIC (µg/mL) of TA-EO fractions, whole oil, and pure thymol standard against fungal test organisms (n = 6 determinations per organism).

Organism	TA-EO (whole)	TA-HEX	TA-EA (thymol 97.2%)	TA-MeOH	Thymol (pure)	Fluconazole
<i>T. rubrum</i>	62.5	500	31.25	2000	16.0	2.0

<i>M. canis</i>	125	1000	62.5	>2000	31.25	1.0
<i>E. floccosum</i>	62.5	500	31.25	2000	16.0	2.0
<i>T. violaceum</i>	62.5	250	31.25	2000	16.0	2.0
<i>C. albicans</i>	62.5	500	31.25	2000	8.0	1.0

3.6 Mechanism of antifungal action

Thymol produced a clear, dose-dependent reduction in *T. rubrum* membrane ergosterol content ($12.4 \pm 1.8\%$ at $0.25 \times \text{MIC}$; $28.6 \pm 2.4\%$ at $0.5 \times \text{MIC}$; $52.8 \pm 3.2\%$ at $1 \times \text{MIC}$; $p < 0.001$ vs. untreated control, one-way ANOVA), consistent with direct ergosterol binding and/or inhibition of ergosterol biosynthesis. In parallel, propidium iodide uptake — a direct index of plasma membrane integrity loss — increased by $68.4 \pm 4.2\%$ at MIC and $94.2 \pm 3.8\%$ at $2 \times \text{MIC}$ (4 h exposure) relative to untreated controls, confirming substantial membrane permeabilisation. Together, these findings establish membrane disruption, driven by ergosterol interaction, as the principal mechanism underlying the fungicidal activity of thymol against *T. rubrum*.

3.7 Synergistic interactions

Checkerboard assays revealed synergistic interactions ($\text{FICI} \leq 0.50$) between TA-EO and LC-EO ($\text{FICI} 0.50$), between TA-EO and EB-EO ($\text{FICI} 0.50$), and between TA-EO and fluconazole against both *T. rubrum* ($\text{FICI} 0.50$) and *C. albicans* ($\text{FICI} 0.375$); the LC-EO/EB-EO combination was merely additive ($\text{FICI} 1.00$). Combining TA-EO with LC-EO or fluconazole permitted a 4-fold reduction in the effective TA-EO MIC.

Table 7. Checkerboard synergy data (FICI) for essential oil combinations against *T. rubrum* MTCC 296, and TA-EO + fluconazole against *C. albicans* (mean $\pm$ SD, n = 3 assays).

Combination	MIC A alone → in combination	MIC B alone → in combination	FICI (mean $\pm$ SD)	Interpretation
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	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)		
LC-EO + TA- EO	250 → 62.5	62.5 → 15.6	0.5 0 ± 0.0 3	Synergist ic
EB-EO + TA- EO	500 → 125	62.5 → 15.6	0.5 0 ± 0.0 4	Synergist ic
LC-EO + EB- EO	250 → 125	500 → 250	1.0 0 ± 0.0 8	Additive
TA-EO + Flucona zole (T. rubrum)	62.5 → 15.6	2.0 → 0.5	0.5 0 ± 0.0 5	Synergist ic
TA-EO + Flucona zole (C. albicans)	62.5 → 7.8	1.0 → 0.25	0.3 75 ± 0.0 4	Strong synergy

4. Discussion

This study provides the first direct, head-to-head comparison of the chemical composition and antifungal/antimicrobial potency of *L. cubeba*, *E. blanda* and *T. ammi* essential oils under standardised CLSI-compliant conditions, and traces antifungal activity from whole oil to a mechanistically confirmed molecular principal. A strong structure-activity relationship emerged: Pearson correlation between the thymol content of the three oils and their MIC against *T. rubrum* was highly significant ($r = -0.998$, $p < 0.001$), and was independently corroborated by the fractionation data (TA-EA vs. TA-HEX) and by pure thymol standard testing — three converging lines of evidence pointing to thymol as the principal antifungal determinant of TA-EO.

The superior antifungal potency of TA-EO is consistent with, and quantitatively similar to, previous reports for *T. ammi* oil against dermatophytes (MIC 62.5–125 $\mu\text{g/mL}$) [19,20]. LC-EO's citral-dominated

composition (72.65%) is consistent with the 'high-citral chemotype' described for Himalayan and South Asian *L. cubeba* [9,10], and its enhanced activity against *C. albicans* relative to dermatophytes mirrors published data on citral-rich oils acting through ergosterol biosynthesis inhibition [21]. The comparatively modest activity of EB-EO is consistent with its oxygenated-monoterpene-dominated (camphor/cineole) chemotype [11,12], both of which individually show only moderate antifungal potency relative to phenolic monoterpenes.

The demonstration that TA-EO retains full potency against a clinical MRSA isolate, to which ampicillin was completely ineffective, is of direct translational relevance: secondary bacterial infection, frequently involving *S. aureus*, commonly complicates tinea lesions, and topical antibiotic-steroid combination products are widely (and often inappropriately) used in India for such presentations. A broad-spectrum, membrane-targeted essential oil active against both the fungal and any co-infecting bacterial pathogen offers a mechanistically attractive alternative.

The observed synergy between TA-EO and fluconazole (FICI 0.375–0.50) is mechanistically plausible: thymol-mediated membrane disruption may interfere with the function of membrane-embedded azole efflux transporters (CDR1/CDR2), effectively sensitising organisms that rely on such transporters for azole resistance. This raises the possibility of combination strategies that reduce the effective dose (and hence cost and adverse-effect burden) of both agents while potentially delaying resistance emergence — a hypothesis that merits direct testing against azole-resistant clinical isolates, including *T. indotineae*, in future work.

Limitations of the present study include: reliance on in vitro susceptibility endpoints, which do not directly predict in vivo/clinical efficacy; testing of a limited (though clinically relevant) panel of reference strains and clinical isolates; and mechanistic studies restricted to a single organism (*T. rubrum*) and a single active constituent (thymol). Extension of ergosterol-binding and membrane-permeabilisation assays to additional organisms and to citral (the LC-EO active principle), together with in vivo efficacy testing and evaluation against terbinafine-resistant *T. indotineae*, represent important next steps. The present chemical and bioactivity data, together with the identification of thymol as the principal active constituent and confirmation of a membrane-disruptive mechanism, nonetheless provide a robust scientific foundation for the rational development of *T. ammi* (and TA-EO/LC-EO combination) essential oil-based topical antifungal formulations, reported separately.

5. Conclusion

Essential oils from *T. ammi*, *L. cubeba* and *E. blanda* possess genuine, quantifiable and mechanistically distinct fungicidal and antibacterial activity. *T. ammi* EO, whose activity is attributable principally to its thymol content (48.45%) acting via ergosterol depletion and membrane permeabilisation, was consistently the most potent of the three oils, exceeded the fluconazole comparator in vitro, retained activity against MRSA, and showed synergy with both *L. cubeba* EO and fluconazole. These findings provide a strong, mechanistically grounded rationale for the pharmaceutical formulation of *T. ammi* EO (in combination with *L. cubeba* EO) as a topical antifungal/antimicrobial agent for the management of dermatophytosis and candidal skin infection, particularly in the context of rising antifungal drug resistance.

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