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RESEARCH ARTICLE

GC-MS profiling and antimicrobial activity of *Moringa oleifera* leaf and *Citrus sinensis* peel extracts and their individual and combinational effects

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Abstract

The plant-based antimicrobials have emerged as an alternative source of medicine to synthetic antibiotics due to their cost-effectiveness, cause lesser side effects and have a broad range of activity. The present study deals with the assessment of antibacterial efficiency of ethanolic leaf extracts of *Moringa oleifera* Lam., as well as ethanolic peel extracts of *Citrus sinensis* L., against *Vibrio harveyi* (Gram-negative bacteria) and *Bacillus subtilis* (Gram-positive bacteria). The *M. oleifera* and the *C. sinensis* are widely available, inexpensive and eco-friendly botanical materials with wide range of phytochemical content. The crude extracts were obtained through Soxhlet extraction process with 70 % ethanol. The preliminary qualitative phytochemical analysis showed the presence of alkaloids, tannins, saponins, phenols, flavonoids and terpenoids. Antibacterial activity was assessed by agar well diffusion, broth microdilution (MIC/MBC) and checkerboard synergy testing methods. Findings indicated that the two extracts reduced the growth of bacteria in a dose-dependent manner. *Moringa oleifera* and *Citrus sinensis* extracts exhibited a potent antimicrobial activity against *B. subtilis* (MIC-3.125 ± 0.11 and 12.50 ± 0.00 mg/mL respectively) and *V. harvevi* (6.25 ± 0.17 and 3.125 ± 0.05 mg/mL respectively). Against *B. subtilis*, a synergistic effect was defined (fractional inhibitory concentration index (FICI) = 0.31), whereas against *V. harveyi,* an indifferent effect was observed (FICI = 1.03). Through gas chromatography-mass spectroscopy (GC-MS) analysis we were able to identify some antibacterial compounds in *M. oleifera* extract such as n-hexadecanoic acid, phytol and α-linolenic acid. Similarly, limonene, 5-hydroxymethylfurfural and phenolic derivatives in *C. sinensis*. The results support the feasibility of widely used plant resources such *as M. oleifera* and *C. sinensis* and their natural antibacterial capacity at an affordable cost and potential anti-microbial usage in food safety and disinfectant spray formulations.

Keywords: antibacterial activity; checkerboard assay; GC-MS analysis; minimal bactericidal concentration; minimal inhibitory concentration

Introduction

Plant-based extracts have been essential in traditional medicinal practices and are now being studied more extensively for potential antimicrobial agents. Medicinal plants are rich in phytochemicals that act individually or synergistically to inhibit pathogens (1). *Moringa oleifera* Lam., the “drumstick” or “miracle tree,” is known worldwide for its nutritional and therapeutic value (2). Nearly all parts of *M. oleifera* (leaves, seeds, bark) contain vitamins, minerals and bioactive compounds such as flavonoids, isothiocyanates and phenolics (2). These compounds have shown antioxidant, anti-inflammatory and antimicrobial activities (2). *Moringa oleifera* leaf extracts have shown bacteriostatic effects against Gram-positive bacteria (e.g., *Staphylococcus aureus*) and Gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*), often comparable to standard antibiotics such as streptomycin (3). In many cultures, *M. oleifera* leaves are used in folk medicines for infections and wounds, emphasizing its ethnomedicinal relevance (3).

Citrus fruits are globally important crops with peels that are rich in bioactive compounds (4). Dried citrus peels have been used in Asian traditional medicine to address digestive and respiratory problems (4). Citrus peels contain large amounts of flavanones (hesperidin, naringin), polymethoxylated flavones and essential oils (primarily limonene). These components show high antioxidant and antimicrobial activities (5). Methanolic extract or essential oil from orange and other citrus peels have also shown antibacterial activity. The phytochemical-rich citrus peel extract could be useful for developing value-added antimicrobial products (5).

Antibiotic resistance is an increasing global threat, prompting greater interest in plant-derived antimicrobials as safer alternatives to allopathic medicines (6). In this context, *M. oleifera* (drumstick tree) leaves and *Citrus sinensis* L. (orange) peels are rich in bioactive molecules that exhibit antibacterial effects (3, 5). *Vibrio harveyi* was selected as a representative Gram-negative marine/food-associated organism of relevance to aquaculture and seafood spoilage; evaluation against this strain assesses extract potential in non-clinical, food-safety and aquaculture contexts (7). But no prior study has explored the combined antimicrobial effects of these two plant extracts. This study investigates the synergistic inhibition of *Bacillus subtilis* by combining *M. oleifera* leaf and *C. sinensis* peel extracts. Such synergistic combinations may target

multiple bacterial pathways and could help overcome resistance mechanisms, supporting the notion that botanical mixtures are promising sources of new anti-infective agents (8).

Plant extract combinations are also a good method that would enhance efficacy and minimize doses (9). Synergistic effects between herbal extracts may involve activation of several pathways and overcome microbial resistance, giving a combined effect more than the sum of individual effects (9). Such interactions are quantified using checkerboard assays in which the fractional inhibitory concentration (FIC) index is calculated. While synergy has been reported for other medicinal plants, there is a paucity of data on combinations involving *M. oleifera* and *C. sinensis* extracts. This study therefore assesses the antibacterial activity of *M. oleifera* leaf extract and *C. sinensis* peel extract, both alone by agar well diffusion assay and minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination and in combination by checkerboard assays, which provide FIC indices that help to identify whether the combination is synergistic- where the combined effect is greater than the sum of individual effects, additive- where the combined effect is equivalent to the sum of individual effects, or antagonistic against representative pathogens (10). GC-MS profiling was performed to characterize the phytochemical composition of each extract, providing context for any observed biological activity (1).

Materials and Methods

Plant material and extraction

Fresh leaves of *M. oleifera* Lam. (family: Moringaceae) and peels of *Citrus aurantium* L. (syn. *Citrus sinensis*) (family: Rutaceae) were collected from Bengaluru, Karnataka, India and authenticated by the Central Ayurveda Research Institute (CARI), Bengaluru, under the Ministry of AYUSH, Government of India. The authentication reference numbers were RRCBI-19929 for *M. oleifera* and RRCBI-mus 475 for *C. sinensis* which were certified by a Research Officer (Botany). Collected plant materials were washed thoroughly under running tap water and shade-dried at room temperature (25 ± 2 °C) for 7 days and ground to a fine powder. Each powder (20 g) was subjected to hot continuous extraction using a Soxhlet apparatus with 200 mL of ethanol (solvent-to-plant material ratio: 10 mL/g) for 12 hr (11). The solvent was heated to 70 °C allowing vapor condensation into the thimble chamber containing plant material and cyclic siphoning of the extract back into the flask (11). After extraction, the solvent was removed using a rotary evaporator under reduced pressure (60 °C) to obtain concentrated crude extracts (11).

Preparation of stock solutions

Each crude dried extract was dissolved in ethanol to prepare a stock solution of 100 mg/mL (11). Working solutions were made by diluting the stock solution in Mueller-Hinton broth (MHB) for MIC and in ethanol for agar well diffusion assay. For antimicrobial assay the stock solutions were further diluted to the range of 0.195 to 100 mg/mL concentrations prepared in broth medium. Ethanol (70 %) was used as negative control (12).

Phytochemical screening

Plant extracts were screened qualitatively for secondary metabolites. *Moringa oleifera* leaf and *Citrus sinensis* peel extracts were tested for alkaloids, tannins, flavonoids, phenolics, saponins, terpenoids using standard protocols (4, 13).

Alkaloids test

Alkaloids were detected using Wagner's reagent (iodine in potassium iodide). The formation of a reddish-brown precipitate indicated the presence of alkaloids (4).

Tannins test

Tannins were identified by adding dilute ferric chloride solution to the extract. The appearance of a brownish-black or greenish coloration confirmed the presence of tannins (13).

Flavonoids test

Flavonoids were assessed using the alkaline reagent test. Addition of sodium hydroxide (NaOH) produced a yellow coloration, which disappeared upon acidification, indicating flavonoids (13).

Phenolics test

Phenolic compounds were detected by the ferric chloride test, where the development of a blue-black coloration signified the presence of phenolics (4).

Saponins test

Saponins were identified using the froth test. Vigorous shaking of the extract with distilled water resulted in the formation of a stable, persistent foam, indicating saponins (4).

Terpenoids test

Terpenoids were examined using the Salkowski reaction. The extract was mixed with chloroform followed by the careful addition of concentrated sulfuric acid (H_2SO_4); the formation of a reddish-brown or bluish-green ring at the interface confirmed the presence of terpenoids (4).

Bacterial strains and culture

The test organisms included Gram-positive and Gram-negative bacteria. *B. subtilis* MTCC 2413 was used as a Gram-positive model and *V. harveyi* MTCC 3438 as a Gram-negative model. Bacteria were subcultured and grown overnight at 37 °C. A fresh bacterial suspension was prepared each time in MHB from the streaked plate and was adjusted to McFarland 0.5 standard by adjusting the turbidity using sterile saline or broth to achieve 1.5×10^8 CFU/mL (McFarland 0.5 standard unit) before each assay (8).

Antimicrobial activity through agar well diffusion assay

Antimicrobial activity was assessed by agar well diffusion method (8). Mueller-Hinton agar plates were inoculated by spreading 100 µL of bacterial suspension (1.5×10^8 CFU/mL) uniformly across the surface. Sterile 9 mm wells were bored into the agar. Each well was filled with 50 µL of extract solution at predetermined concentrations - 100, 75, 50, 25 mg/mL in each well under aseptic conditions. Ethanol (70 %) and Streptomycin solution (1 mg/mL) were included as negative and positive controls, respectively (8). Plates were allowed to diffuse at room temperature (~20 min), then incubated at 37 °C for 18–24 hr. Zones of inhibition (clear halos) were measured (mm, with well diameter) with a ruler or caliper (8).

Antimicrobial activity through minimum inhibitory concentration (MIC) assay

The activity of the extracts on bacterial growth was determined using microdilution method. The microorganisms were grown on nutrient agar plates and incubated for 24 hr at 37 °C. Isolated colonies were then inoculated as loopful cultures into Mueller Hinton broth and incubated at 37 °C with shaking at 180 rpm overnight. The culture was then diluted to an optical density corresponding to the standard 0.5 on the McFarland scale (OD_{620} = 0.10) (12). Microtiter plate wells were filled with 100 µL of Mueller Hinton broth that had extract concentrations ranging from

0.098 mg/mL to 100 mg/mL (12). Bacterial inoculum of 100 µL (1.5×10^8 CFU/mL) was added to each well, yielding final extract concentrations (100 to 0.195 mg/mL) and 1.5×10^8 CFU/well. Growth control (broth + bacteria, no extract), positive control (Streptomycin at 1 mg/mL) and negative controls (broth only) were included (8). Plates were incubated at 37 °C for 18–24 hr. MIC was defined as the lowest concentration showing no visible turbidity. Bacterial growth (turbidity) was also quantified by measuring optical density (OD) at 595 nm.

The percentage inhibition of microbial growth was calculated using the following formula.

$$\text{Percentage Inhibition} = \left[\frac{(\text{OD of control} - \text{OD of sample})}{\text{OD of control}} \right] \times 100 \quad \text{(Eqn.1)}$$

Minimum bactericidal concentration (MBC)

The specific quantity (10 µL) of contents of each well of the microtiter plate from the MIC assay were subcultured on MHA through streaking plate method and incubated for 24 hr. MBC was calculated as the lowest concentration of the test samples, which completely inhibit the growth of microbes (8).

Checkerboard synergy assay (FICI)

Synergy between *M. oleifera* and *C. sinensis* extracts was evaluated by the checkerboard assay (10). Two-fold serial dilutions were prepared from 100 mg/mL down to 0.195 mg/mL. The extracts were combined in a 1:1 proportion in sterile 96-well microtiter plates, with *M. oleifera* concentrations arranged along the rows and *C. sinensis* along the columns, generating a full matrix of concentration combinations. Each well was inoculated with *B. subtilis* or *V. harveyi* adjusted to a McFarland 0.5 standard (≈1.5×10^8 CFU/mL) and incubated at 37 °C for 24 hr. The FICI for each combination was calculated as,

$$\text{FIC of extract A (FICA)} = \text{MIC of extract A in } \frac{\text{Combination}}{\text{Alone}} \quad \text{(Eqn. 2)}$$

$$\text{FIC of extract B (FICB)} = \text{MIC of extract B in } \frac{\text{Combination}}{\text{Alone}} \quad \text{(Eqn. 3)}$$

$$\text{FIC index} = \text{FICA} + \text{FICB} \quad (14) \quad \text{(Eqn.4)}$$

Extract A- *M. oleifera* leaf extract.

Extract B- *C. sinensis* (syn. *C. aurantium*) peel extract. According to the established criteria, FICI ≤0.50 indicates synergy, 0.50–1.00 additivity, 1.00–4.00 indifference and >4.00 antagonisms (12).

GC-MS analysis

The ethanol extract of *M. oleifera* leaves and *C. sinensis* peel was subjected to GC-MS analysis to identify its possible phytochemical constituents. The analysis was performed using Shimadzu GCMS-QP2010 ultra instrument equipped with a quadrupole mass analyzer. The oven temperature was programmed to start at 60 °C (held for 2 min), then ramped at 10 °C/min to 300 °C, where it was held for 10 min. Helium was used as the carrier gas. Mass detection was conducted using electron ionization (EI) at 70 eV, with the interface temperature set at 280 °C and ion source at 200 °C. Compound identification was done by comparison using NIST 20 mass spectral library. Only compounds with ≥ 90 % spectral match and retention index agreement were considered. All solvents were of analytical grade and samples were pre-filtered through 0.22 µm polytetrafluoroethylene (PTFE) membranes before injection. Appropriate blanks were run to confirm the absence of contaminants and ensure spectral accuracy (15).

Statistical analysis

All experiments were conducted in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using IBM SPSS Statistics. Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc test. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Qualitative phytochemical screening

Both *M. oleifera* leaves and *C. sinensis peel* extracts tested positive (+) for all 6 classes (alkaloids, tannins, phenols, flavonoids, saponins-slightly, terpenoids) (Table 1).

All these chemical constituent categories have well known antimicrobial properties, such as phenolics (flavonoids, tannins) show their activity by binding to microbial enzymes and the cell wall. Tannins can inactivate bacterial adhesins and enzymes inhibiting growth (13). Flavonoids and phenolic acids scavenge free radicals and disrupt cell membranes. Saponins form complexes with membrane sterols increasing permeability. Alkaloids inhibit DNA and RNA synthesis, or they function as enzyme inhibitors. Terpenoids (such as citrus limonoids) disrupt cell membranes and metabolic pathways. Thus the observed presence of these classes suggests inherent antibacterial potential of both extracts (16).

Table 1. Phytochemical profile of *M. oleifera* leaf and *C. sinensis* peel extracts. Presence (+) or absence (-) of each metabolite class was determined by qualitative tests

Phytochemical	*M. oleifera*	*C. sinesis*
Alkaloids	+	+
Tannins	+	+
Phenols	+	+
Flavonoids	+	+
Saponins	+	+
Terpenoids	+	+

Antibacterial activity (Zones of inhibition)

Bacillus subtilis (Gram positive bacteria)

Zone of inhibition (ZI) increased with increased concentration for all extracts. *Moringa oleifera* extract showed the potent antimicrobial activity with concentration-dependent manner (11.3 ± 0.58 mm, 12.3 ± 0.58 mm, 13.3 ± 0.58 mm, 14.3 ± 0.58 mm ZI at 25, 50, 75 and 100 mg/mL concentration respectively). Similarly, *C. sinensis* exhibited a wide array of activity with 11.6 ± 0.58 mm, 12.6 ± 0.58 mm, 13.6 ± 0.58 mm and 15.3 ± 1.15 mm ZI at the same concentrations respectively. Thus, it was found that *C. sinensis* extract is comparatively more active with highest ZI (15.3 and 14.3 mm).

Vibrio harveyi (Gram negative bacteria)

Antibacterial activity against *V. harveyi* also increased with increasing extract concentration. *Moringa oleifera* extract produced inhibition zones of 12.0 ± 0.0, 13.0 ± 1.0, 14.0 ± 1.0 and 15.0 ± 0.0 mm at 25, 50, 75 and 100 mg/mL, respectively. In comparison, *C. sinensis* extract yielded ZI values of 11.6 ± 0.58, 11.6 ± 0.58, 12.3 ± 0.58 and 14.3 ± 0.58 mm at the same concentrations. Although both extracts showed similar activity at 100 mg/mL, *M. oleifera* was slightly more active than *C. sinensis* at 75 mg/mL (Table 2).

Table 2. Agar-well diffusion assay - zones of inhibition (mean ± SD, mm; n = 3). Values are mean ± SD (n = 3). Means within a column followed by different superscript letters are significantly different (one-way ANOVA followed by Tukey's HSD, $p < 0.05$)

Extract / control	Concentration (mg/mL)	*B. subtilis* (mm)	*V. harveyi* (mm)
M. oleifera	25	11.3 ± 0.58^{d}	12.0 ± 0.00^{c}
M. oleifera	50	12.3 ± 0.58^{c}	13.0 ± 1.00^{c}
M. oleifera	75	13.3 ± 0.58^{c}	14.0 ± 1.00^{b}
M. oleifera	100	14.3 ± 0.58^{b}	15.0 ± 0.00^{b}
C. sinensis	25	11.6 ± 0.58^{c}	11.6 ± 0.58^{c}
C. sinensis	50	12.6 ± 0.58^{c}	11.6 ± 0.58^{c}
C. sinensis	75	13.6 ± 0.58^{b}	12.3 ± 0.58^{c}
C. sinensis	100	15.3 ± 1.15^{b}	14.3 ± 0.58^{b}
Streptomycin (positive control)	1	33.0 ± 0.58^{a}	23.0 ± 1.00^{a}

Values are expressed as mean ± standard deviation (SD) (n = 3). Means within the same column followed by different superscript letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test ($p < 0.05$). Reported ANOVA statistics: *M. oleifera* vs *B. subtilis*, $F(3,8) = 14.863$, $p = 0.0012$; *M. oleifera* vs *V. harveyi*, $F(3,8) = 10.000$, $p = 0.0044$; *C. sinensis* vs *B. subtilis*, $F(3,8) = 12.810$, $p = 0.0020$; *C. sinensis* vs *V. harveyi*, $F(3,8) = 14.536$, $p = 0.0013$.

Both plant extracts effectively inhibited Gram-positive and Gram-negative bacteria in a concentration-dependent manner (17). The generally higher susceptibility of *B. subtilis* compared to *V. harveyi* is consistent with the known structural differences between Gram-positive and Gram-negative bacteria, particularly the presence of an outer membrane in Gram-negative organisms that can restrict the penetration of antimicrobial molecules. Whereas Gram-positive organisms lack this barrier, rendering them more susceptible to compounds that disrupt peptidoglycan integrity or cytoplasmic membranes. Additionally, the chemical nature of the extracts that is fatty-acid derivatives in *M. oleifera* and terpene and phenolic compounds in *C. sinensis* may favor interaction with particular bacterial targets, producing organism-specific potency profiles (18). The observed antibacterial effects are likely the result of multiple bioactive molecules acting through complementary mechanisms, supporting the broad-spectrum antimicrobial potential of both *M. oleifera* leaves and *C. sinensis* peels (16).

MIC and MBC

Bacillus subtilis

Broth microdilution assays showed a clear concentration-dependent inhibition by both extracts. For *M. oleifera*, bacterial growth decreased progressively with increasing concentration and complete growth inhibition was observed at 3.125 mg/mL, which was recorded as the MIC. At this concentration, the optical density (OD 595) declined to approximately 0.01, indicating effective suppression of bacterial proliferation. Bactericidal activity was achieved at 12.5 mg/mL, confirming the MBC value. In contrast, the *C. sinensis* extract required a higher concentration to inhibit growth, with an MIC of 12.5 mg/mL and an MBC of 25 mg/mL. These findings indicate that *M. oleifera* exhibited stronger antibacterial efficacy against *B. subtilis* than *C. sinensis*.

Vibrio harveyi

Against *V. harveyi*, *M. oleifera* extract produced visibly clear wells at 6.25 mg/mL, which was identified as the MIC, while complete bactericidal activity was observed at 12.5 mg/mL. The *C. sinensis* extract demonstrated a lower MIC value of 3.125 mg/mL, evidenced by the absence of turbidity, with an MBC of 12.5 mg/mL. Overall, both extracts showed effective antibacterial activity against *V. harveyi*, with *C. sinensis* displaying slightly higher inhibitory potency at lower concentrations, whereas *M. oleifera* maintained consistent bactericidal activity across both test organisms (Table 3).

Table 3. MIC and MBC (mg/mL) of *M. oleifera* and *C. sinensis* against test bacteria

Extract	*B. subtilis* - MIC / MBC (mg/mL)	*V. harveyi* - MIC / MBC (mg/mL)
M. oleifera	3.125 ± 0.11 / 12.5 ± 0.00	6.25 ± 0.17 / 12.5 ± 0.00
C. sinensis	12.5 ± 0.00 / 25± 0.00	3.125 ± 0.05/ 12.5 ± 0.00

The percentage-inhibition profiles of *M. oleifera* leaf and *C. sinensis* peel extracts demonstrated a clear, concentration-dependent antibacterial response against both *B. subtilis* and *V. harveyi*. In *B. subtilis*, both extracts showed gradual increase in inhibitory activity (1–100 mg/mL concentrations). The inhibition pattern observed in the percentage-inhibition assays was consistent with the agar well diffusion results. At the highest tested concentration (100 mg/mL), *C. sinensis* produced slightly larger inhibition zones than *M. oleifera* (15.3 ± 1.15 mm and 14.3 ± 0.58 mm, respectively). In contrast, against *V. harveyi*, *M. oleifera* showed a stronger dose-dependent inhibitory response at intermediate concentrations (25–75 mg/mL), while both extracts achieved > 90 % inhibition at concentrations ≥ 50 mg/mL. These findings indicate that the marginally higher activity of *C. sinensis* at 100 mg/mL is likely related to enhanced diffusion in agar, whereas the steeper response of *M. oleifera* reflects greater antibacterial effectiveness at lower to intermediate concentrations (3, 5). Both *M. oleifera* and *C. sinensis* ethanolic extracts exhibited clear concentration-dependent antibacterial activity in agar diffusion and broth microdilution assays. ZI ranged from 11 to 15 mm, while MIC values fell between 3.125 and 12.5 mg/mL, which are typical for crude botanical extracts. *Moringa oleifera* showed greater potency against *B. subtilis* (MIC = 3.125 mg/mL), whereas *C. sinensis* demonstrated stronger activity against *V. harveyi* (MIC = 3.125 mg/mL), indicating organism-specific differences in susceptibility. This variation is likely attributable to differences in phytochemical composition, with fatty-acid-derived compounds contributing to the activity of *M. oleifera* and phenolic- and terpene-rich constituents influencing the antibacterial effect of *C. sinensis* (15, 19). In several comparisons, MBC/MIC ratios were ≤ 4, suggesting that bactericidal concentrations were only moderately higher than inhibitory levels. Overall, the results confirm that both extracts exert effective antibacterial action at concentrations commonly applied for crude plant extracts and highlight their complementary activity profiles against Gram-positive and Gram-negative bacteria.

Checkerboard synergy (FICI)

The checkerboard microdilution assay showed a marked synergistic interaction between *M. oleifera* and *C. sinensis* extracts against *B. subtilis*, as proven by a FICI of 0.312. FICI values ≤ 0.5 are indicative of synergism (20). When both the extracts were combined, the MIC of *M. oleifera* decreased from 3.125 mg/mL to 0.195mg/mL and the MIC of *C. sinensis* extract also reduced from 12.5 mg/mL to 3.125mg/mL indicating a complementary antibacterial interaction resulting from the combined action of bioactive molecules present in both extracts. Similar synergistic effects between plant polyphenols and essential oil components have been reported in previous studies (21, 22), supporting the hypothesis that bioactive secondary metabolites can potentiate each other's antimicrobial effects by disrupting multiple cellular targets or enhancing membrane permeability.

The interaction against *V. harveyi* was found to be indifferent as the calculated FICI of 1.031 was falling out of synergistic range. Even though MIC of *M. oleifera* extract dropped significantly (from 6.25mg/mL to 0.195mg/mL), the MIC of *C. sinensis* remained unchanged at 3.125mg/mL, resulting in an overall indifferent effect. This disparity in interaction profiles may reflect organism-specific differences in cell envelope architecture or metabolic resistance pathways, particularly between Gram-positive and Gram-negative species (23). This data highlights the potential of combining *M. oleifera* with *C. sinensis* compounds as a good strategy for the development of plant-based antimicrobial formulations especially against Gram-positive pathogens (Table 4).

GC-MS phytochemical profiling

GC-MS analysis identified several bioactive constituents in each extract. GC-MS profiling of the *M. oleifera* leaf extract revealed a lipid-dominant phytochemical composition (Table 5). The most abundant constituents were 9,12,15-octadecatrienoic acid (α-linolenic acid) and its ethyl ester, n-hexadecanoic acid, hexadecanoic acid ethyl ester and phytol (Fig. 1). These fatty acids and sterols (e.g., stigmasterol) are well documented for antimicrobial, antioxidant and anti-inflammatory activities, supporting the strong efficacy observed in antimicrobial assays (24–27). The prevalence of α-linolenic-derived compounds aligns with prior reports of *M. oleifera* leaves as a rich source of ω-3 fatty acids that disrupt microbial membranes and modulate oxidative stress (26, 28).

In contrast, the *C. sinensis* peel extract displayed a chemically diverse profile enriched in phenolics, furan derivatives and fatty acids (Table 6). Major constituents included 5-hydroxymethylfurfural (5-HMF), 2-methoxy-4-vinylphenol, 4-vinylphenol, n-hexadecanoic acid and 9, 12-octadecadienoic acid (linoleic acid) (Fig. 2)(19, 29). Phenolic derivatives such as 4-vinylphenol and 2-methoxy-4-vinylphenol have been linked to antimicrobial and quorum-sensing inhibition in citrus matrices, while 5-HMF, a product of thermal or enzymatic sugar degradation, contributes to antimicrobial and antibiofilm activity. The identified fatty acids further enhance membrane-targeted antimicrobial

Table 4. FICI calculation

Organism	MIC A (Alone) in mg/mL	MIC B (Alone) in mg/mL	MIC A in combination	MIC B in combination	FIC A	FIC B	FICI
B. subtilis	3.125 ± 0.11	12.5 ± 0.00	0.195 ± 0.01	3.125 ± 0.05	0.06	0.25	0.31
V. harveyi	6.25 ± 0.17	3.125 ± 0.05	0.195 ± 0.01	3.125 ± 0.00	0.03	1.00	1.03

Table 5. GC-MS profile of *M. oleifera* leaf extract showing retention time, relative peak abundance, compound identity and known biological activities

Retention time	%	Name of compound	Biological activities	References
4.058	1.22	1-Propanamine, 2-methyl-N-(2-methylpropylidene)-	No activity reported	-
9.484	1.28	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	Antimicrobial	(32)
9.738	1.60	1-Butanamine, 3-methyl-N-(3-methylbutylidene)-	Antimicrobial and anti-inflammatory	(33)
23.772	2.33	Dodecanoic acid	Antimicrobial, anti-inflammatory, anti-cancer, anti fungal, antioxidants	(34)
24.135	1.64	3-Methyl-4-phenyl-1H-pyrrole	Anticancer	(35)
27.332	4.71	Tetradecanoic acid	Antibacterial and antifungal	(36)
27.410	1.92	Loliolide	Antioxidant, anti-inflammatory and neuroprotective	(37)
28.197	1.36	Neophytadiene	Anti-inflammatory, antioxidant and cardioprotective	(38)
29.329	17.10	n-Hexadecanoic acid	Antibacterial and antioxidant	(39)
29.589	10.15	Hexadecanoic acid, ethyl ester	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor	(40)
30.486	3.28	Phytol	Antimicrobial and antinociceptive	(25)
30.672	14.90	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	Antibiofilm and antimicrobial	(26)
30.865	26.74	9,12,15-Octadecatrienoic acid, ethyl ester, (Z, Z, Z)-	Antibacterial, anticancer and antifungal	(24)
32.889	3.05	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Antimicrobial and insecticidal	(41)
34.166	7.19	Stigmasterol	Antimicrobial, anti-inflammatory, antioxidant and antidiabetic	(27)
34.551	1.53	13-Docosenamide, (Z)-	Antibacterial, antifungal and anticancer	(42)

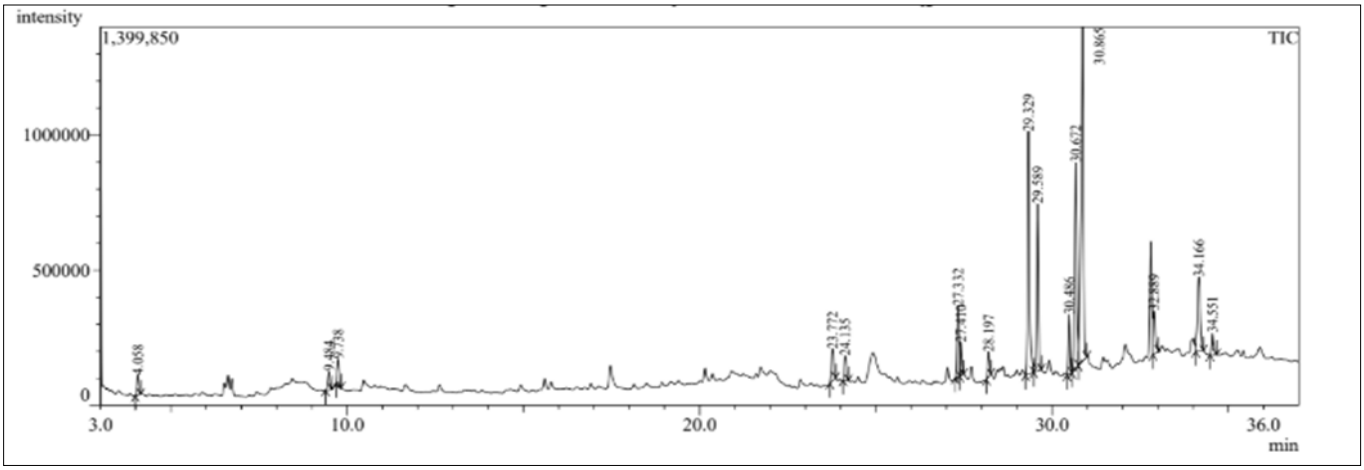

Fig. 1. GC-MS peaks in *M. oleifera* leaf extract.

Table 6. GC-MS profile of *C. sinensis* peel extract showing retention time, relative peak percentage, compound identity and reported biological activities.

Retention time	%	Name of compound	Biological activities	References
3.476	1.90	Diethoxymethyl acetate	No activity detected	-
4.508	1.74	2-Furanmethanol	Antimicrobial and antityrosinase	(43)
5.083	6.33	Methyl isobutyrate	Flavoring and fragnance	(44)
6.216	0.99	1,2-Cyclopentanedione	No activity detected	-
10.883	4.02	Maltol	Antimicrobial, antibiofilm, antivirulence and antioxidant	(45)
12.674	5.30	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Antioxidant	(46)
14.890	2.20	4-Vinylphenol	Anticancer, antioxidant, anti-elastase, anti-tyrosinase and antimicrobial	(47)
15.252	18.33	5-Hydroxymethylfurfural	Anti-quorum sensing and anti biofilm	(48)
15.755	5.67	1,2,3-Propanetriol, 1-acetate	Bacterial inhibiting effect	(49)
17.472	3.03	2-Methoxy-4-vinylphenol	Antimicrobial and anti-inflammatory	(30)
23.807	3.38	Phenol, 4-ethenyl-2,6-dimethoxy-	Antioxidant, antimicrobial and anticancer	(31)
27.331	2.22	Tetradecanoic acid	Antibacterial and antifungal	(36)
29.115	1.67	**α-**D-Glucopyranose, 4-O-beta-D-galactopyranosyl-	Inhibitor of α-glucosidase	(50)
29.333	12.93	n-Hexadecanoic acid	Antibacterial and antioxidant	(39)
30.645	24.48	9,12-Octadecadienoic acid (Z, Z)-	Antibacterial, hepatoprotective, antihistaminic, hypocholesterolemic, antieczemic, anticancer and anti-inflammatory	(51)
30.806	3.08	Octadecanoic acid	Anticancer	(52)
32.890	1.35	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Antimicrobial and insecticidal	(41)
35.274	1.39	γ -Sitosterol	Anti-diabetic, anti-angeogenic, anticancer, antimicrobial, anti-inflammatory, antidiarrhoeal and antiviral	(53)

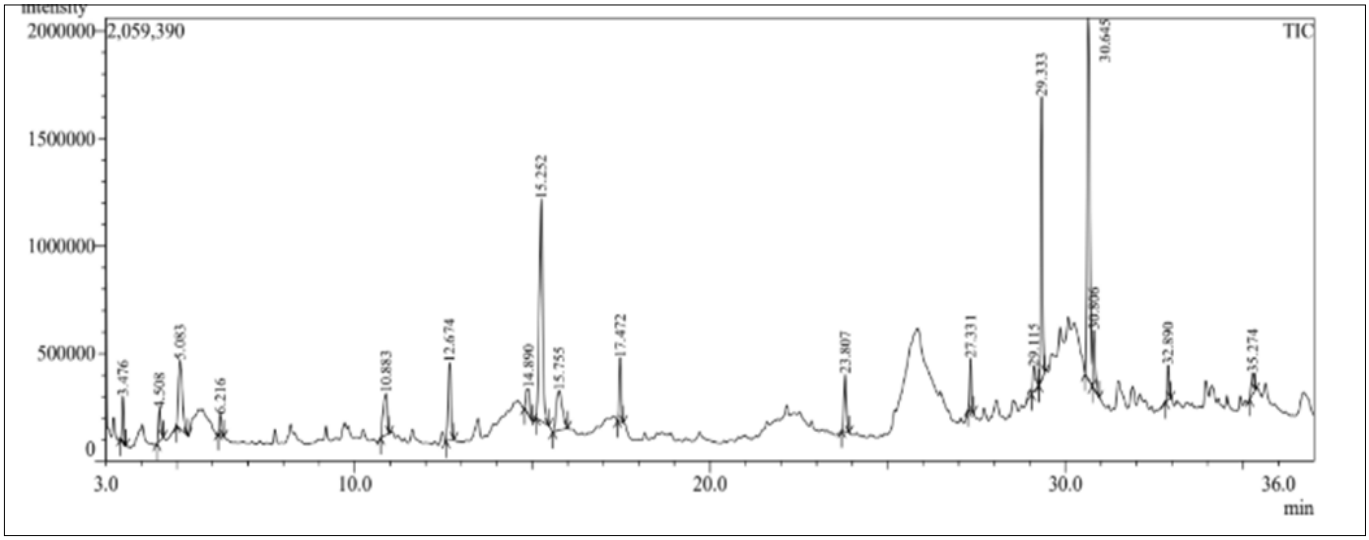

Fig. 2. Major GC-MS peaks in *C. sinensis* peel extract.

effects (30, 31).

Moringa oleifera leaf extract is primarily lipid-rich, whereas *C. sinensis* peel extract contains a higher proportion of phenolic and furan-based compounds. This compositional distinction offers a biochemical rationale for the differing antimicrobial potencies and the synergistic interactions observed in checkerboard and inhibition assays. The coexistence of membrane-active fatty acids from *M. oleifera* with phenolic antibiofilm agents from *C. sinensis* plausibly underlies the enhanced antimicrobial efficacy reported herein, corroborating previous GC-MS-based phytochemical investigations of these plant materials (1, 2, 19, 27).

Conclusion

Crude ethanolic extracts of *M. oleifera* leaves and *C. sinensis* peels exhibited statistically significant, concentration-dependent antibacterial activity against *B. subtilis* and *V. harveyi* ($p < 0.05$). Agar-well diffusion produced inhibition zones of 11.0–15.3 mm and broth microdilution gave MICs of 3.125–12.5 mg/mL. Checkerboard testing showed a synergistic interaction against *B. subtilis* (FICI = 0.31), whereas the interaction against *V. harveyi* was classified as indifferent (FICI = 1.03) according to the criteria applied (FICI ≤ 0.50 = synergy; 0.50–1.00 = additive; 1.00–4.00 = indifferent). Synergy indicates a more-than-additive enhancement of antibacterial effect when the extracts are combined, while an indifferent result indicates the combination neither enhances nor impairs activity beyond the individual effects. Overall, both extracts are effective antibacterials on their own and their combination provides a clear synergistic benefit against *B. subtilis* but not against *V. harveyi*. These findings support further work to isolate active constituents and to test formulation performance under practical conditions.

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Authors' contributions

SAG carried out methodology, investigation, data curation, original draft preparation and visualization. SPN contributed through supervision, validation and review. All authors read and approved the final manuscript.

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