

In Vitro Antibacterial Activity and Chemical Composition of Solvent Fractions from Male Papaya Flowers Against Pathogenic Bacteria

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ABSTRACT: The objective of this research was to study the phytochemistry and antibacterial potential of solvent fractions from flowers of male papaya (*Carica papaya* L.) obtained from Vietnam. The dried flowers were extracted with ethanol and then partitioned between two solvents of different polarities, namely *n*-hexane and ethyl acetate (EtAC). The chemical makeup of each fraction was analyzed by gas chromatography-mass spectrometry (GC-MS). The antimicrobial effect on two clinically important bacterial strains — *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) was investigated using the agar well diffusion assay and broth microdilution technique to determine the minimum inhibitory concentration (MIC). It was found using GC-MS analysis that the *n*-hexane fraction was characterized by the presence of fatty acids, particularly linoleic acid (52.99%) and palmitic acid (30.97%), while the EtAC fraction contained various medicinal compounds such as benzyl β -D-glucoside (23.66%), campesterol (19.21%), and stigmaterol (19.15%). Both fractions exhibited significant, concentration-dependent antibacterial effects. At 200 μ g/mL, the crude extract produced a maximum zone of inhibition (Zoi) of 18.5 mm; the EtAC fraction yielded the lowest MIC of 25.0 μ g/mL against *P. aeruginosa*. These findings confirm that male papaya flowers are a promising source of bioactive compounds for developing natural antibacterial agents.

KEYWORDS: Male papaya flower, *Carica papaya* L., GC-MS, antibacterial activity, MIC, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

1. INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most pressing threats to global public health, severely compromising the effectiveness of existing clinical therapies [1]. Bacterial AMR was associated with an estimated 4.95 million deaths worldwide in 2019, of which 1.27 million were directly attributable to resistant infections [2]. Among the most clinically important opportunistic pathogens, *Staphylococcus aureus* — a leading cause of bacteremia, skin and soft-tissue infections, and infective endocarditis [3] — and *Pseudomonas aeruginosa* — a Gram-negative nosocomial pathogen equipped with a diverse arsenal of resistance-nodulation-division (RND) efflux pump systems [4] — have each evolved sophisticated multidrug resistance mechanisms that markedly diminish the efficacy of newer-generation antibiotics. This situation creates an urgent need to identify and develop novel plant-derived antibacterial compounds with potent antimicrobial activity and a favorable safety profile [5].

Vietnam possesses exceptionally rich medicinal plant resources, among which the male papaya tree (*Carica papaya* L., family Caricaceae) is widely distributed. All major parts of the plant — leaves, fruit, and seeds — have been documented to possess significant biological activities [6]. Flowers from the male papaya tree are traditionally used in medicinal practices to treat colds, coughs, and sore throats [7]. Recent studies have shown that the extract from this species has significant antioxidant and cytoprotective activities [8]. However, research specifically focused on the polarity-based fractionation of extracts from male papaya flowers, along with their structural identification for antibacterial activity, remains scarce [9].

In light of this, the current study was intended to: (i) fractionate male papaya flower extracts using *n*-hexane and ethyl acetate; (ii) identify and quantify the chemical constituents of each fraction by GC-MS coupled with the NIST17 spectral library; and (iii) evaluate both qualitative (Zoi) and quantitative (MIC) antibacterial activity against *P. aeruginosa* and *S. aureus*, thereby providing thereby a scientific foundation for the probable utilization of the male papaya flowers in pharmacy.

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2. MATERIALS AND METHODS

2.1. Plant material and preparation of solvent fractions

Male flowers of papaya (*Carica papaya* L.) were obtained from plants grown in Vietnam (Figure 1). The fresh flowers were washed, oven-dried at 40 °C, ground to a fine powder, and repeatedly macerated in 70% ethanol at room temperature. The combined ethanolic filtrates (Whatman No. 1 filter paper) were concentrated under reduced pressure using a rotary evaporator to yield the crude extract. The crude extract was then partitioned by sequential liquid–liquid extraction with *n*-hexane (non-polar) and ethyl acetate (EtAC, medium-polar), following a polarity-guided fractionation scheme comparable to that reported for other medicinal plants [10]. Each solvent fraction was recovered by complete removal of solvent under reduced pressure. Stock solutions for biological assays were prepared at 200 µg/mL in DMSO ($\leq 1\%$ v/v). The plant material used in this study is illustrated in Figure 1.



Figure 1. Male papaya (*Carica papaya* L.) flowers used as plant material in this study. The inflorescences bear clusters of small, white, tubular flowers characteristic of the staminate (male) plant.

2.2. GC-MS analysis

Gas chromatography–mass spectrometry (GC-MS) was performed on an Agilent 7890B/5977B system equipped with a capillary column (HP-5MS, 30 m × 0.25 mm × 0.25 µm) using helium as the carrier gas. The relative content (%) of each identified compound was determined from the peak areas in the total ion chromatogram (TIC). Compound identification was performed by comparing their mass spectra with the NIST17 library (>85% similarity). Since assignment was based solely on spectral library match, without the use of injection of known standards for verification and/or retention index, all the identifications done herein must be considered provisional.

2.3. Agar well diffusion assay

Suspensions of bacteria *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 with 0.5 McFarland density were applied evenly to the surface of Mueller-Hinton agar [11]. Circular holes (6.0 mm diameter) were drilled in the agar plates and filled with 50 µL of each fraction at concentrations of 100 µg/mL and 200 µg/mL. A positive control (standard antibiotics: ampicillin/ciprofloxacin) and a negative control (DMSO) were used. The plates were incubated for 24 hours at 37 °C, and the ZOI diameters were measured with calipers. All experiments were conducted in triplicate; data are presented as mean ± standard deviation (SD).

2.4. Minimum inhibitory concentration (MIC) determination

The MICs were obtained using the broth microdilution technique with 96-well polystyrene microplates according to CLSI M07 guidelines [12–14], and interpretative and quality control criteria were based on CLSI M100 [12–14]. The extracts were serially diluted twofold from an initial concentration of 200 µg/mL. Wells were inoculated with bacterial suspensions at a final concentration of about 5×10^5 CFU/mL, with TTC added as a metabolic viability index [15]. Viable and metabolically active cells reduce TTC to the red formazan, while inhibited wells remain colorless; hence, the MIC was defined as the lowest concentration

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at which the well remained colorless after incubation at 37 °C for 24 hours. All experiments were carried out in three separate replications.

3. RESULTS AND DISCUSSION

3.1. GC-MS phytochemical profiling

GC-MS analysis demonstrated that the two fractions exhibited distinct phytochemical profiles depending on the polarity of the extraction solvent.

The chromatogram of total ions for the *n*-hexane fraction is shown in Figure 2.

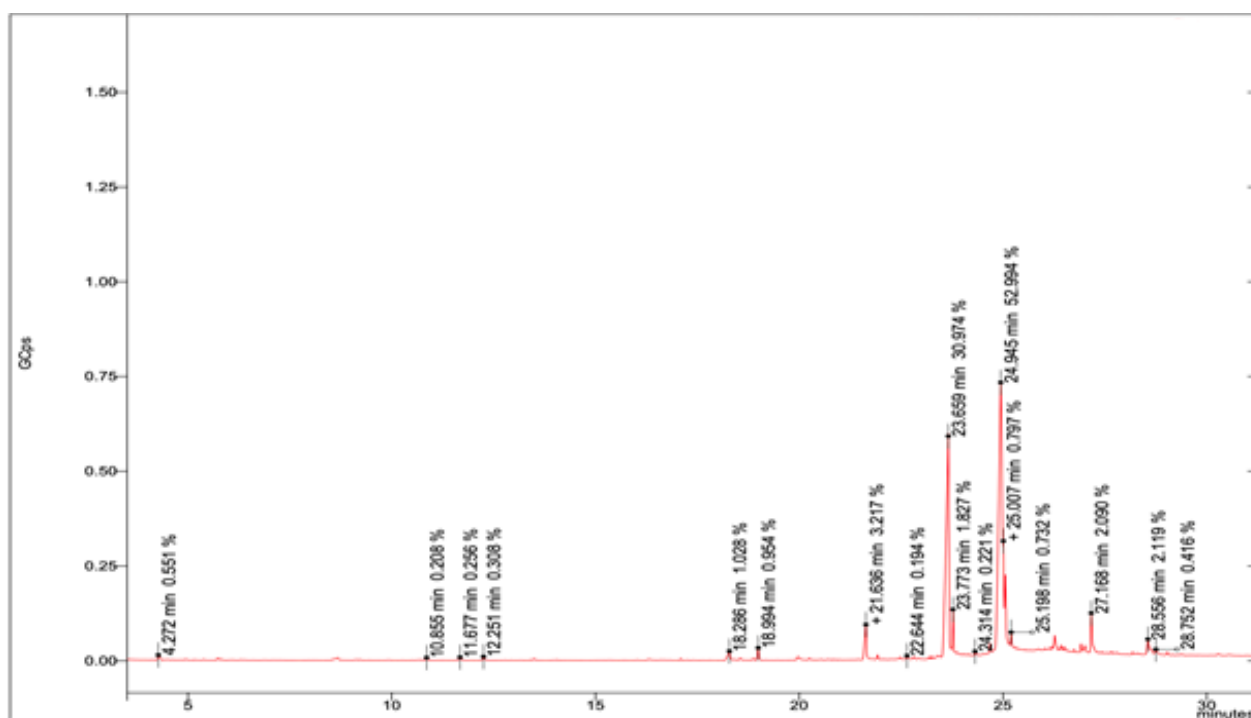


Figure 2. GC-MS total ion chromatogram (TIC) of the *n*-hexane fraction from male papaya (*Carica papaya* L.) flowers. Ordinate: signal intensity (GCps); Abscissa: retention time (min).

The TIC of the *n*-hexane fraction (Figure 2) showed effective separation from 0 to 30 minutes with a stable baseline and clearly resolved peaks. Prominent peak groups were observed at a retention time of 23-25 minutes, indicating the presence of two major components. The most intense peak at 24.945 min corresponded to linoleic acid (52.99%), whereas the second most intense peak was at 23.659 min, corresponding to palmitic acid (30.97%). Together, these two essential fatty acids accounted for roughly 84% of the total extractable material in this non-polar fraction, which is biologically important as a source of surfactant-like lipids [16].

Apart from the two major peaks, there was a significant minor compound present on the chromatogram, which included myristic acid (3.22%) appearing at 21.636 min, along with several minor peaks that occurred within the earlier eluting range (4 – 13 min), including 4.272 min (0.55%), 10.855 min (0.21%), and 12.251 min (0.31%). The long, hydrophobic hydrocarbon chains in linoleic acid, which has two double bonds, and in palmitic acid make lipids very lipophilic to bacterial phospholipid bilayer membranes, thereby forming the physicochemical foundation of the antimicrobial action observed in subsequent experiments [16, 17].

The accompanying chromatogram of the EtAC fraction is shown in Figure 3.

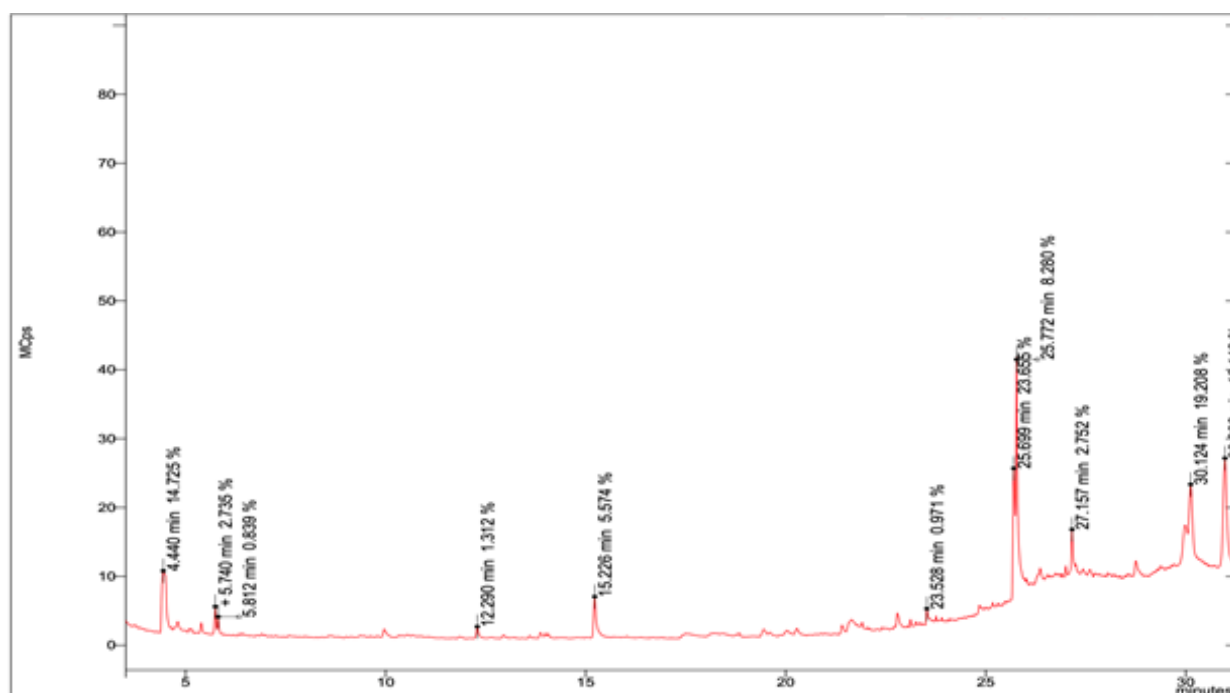


Figure 3. GC-MS total ion chromatogram (TIC) of the ethyl acetate (EtAC) fraction from male papaya (*Carica papaya* L.) flowers. Ordinate: signal intensity (MCps); Abscissa: retention time (min).

Compared with the non-polar fraction, the EtAC fraction exhibited a diverse chemical composition range of 0 to 31 minutes (Figure 3). The most meaningful characteristics were three sharp peaks clustered together towards the end of the elution process. The peak at 25.699 min was identified as benzyl β -D-glucoside (23.66%), the major component in the fraction. Previous research has shown that aryl β -D-glucosides, such as benzyl β -D-glucoside, have been identified from this species, thereby independently confirming this structure [18]. Peaks that followed at 30.124 min and 30.880 min were attributed to the phytosterols campesterol (19.21%) and stigmasterol (19.15%), respectively. These three pharmacologically active substances together comprised more than 62% of the total organic substance in the fraction. Additional peaks for support were observed at 4.440 min (14.73%), 15.226 min (5.57%), and 25.772 min (8.28%), further confirming the chemical complexity of this medium-polarity fraction [8].

The co-occurrence of a rigid steroid ring scaffold (phytosterols) and a flexible glycoside moiety in the EtAC fraction suggests a complementary mechanism of action directed primarily at intracellular enzymes and cell wall biosynthesis — acting in a complementary manner with the membrane-disrupting activity of the fatty acids in the *n*-hexane fraction. This quantitative compositional basis for the observed bioactivity is corroborated by the MIC data presented in Table 2 and Figure 5.

3.2. Antibacterial activity by agar well diffusion

Zones of inhibition recorded after 24 h incubation for both test strains are summarised in Table 1, and representative plate photographs are shown in Figures 4a and 4b.

Table 1. Zones of inhibition (mm) of the crude extract of male papaya flowers against two pathogenic strains.

Test strain	Sample	100 μ g/mL	200 μ g/mL	Positive control	Negative control
<i>Pseudomonas aeruginosa</i>	Crude extract	16.0 $\pm$ 0.5	18.5 $\pm$ 0.5	25.0 $\pm$ 0.5	0
<i>Staphylococcus aureus</i>	Crude extract	17.0 $\pm$ 0.4	18.0 $\pm$ 0.5	26.0 $\pm$ 0.4	0

Note: Zol diameters include the initial well diameter (6.0 mm) and are expressed as mean $\pm$ SD from *n* = 3 independent replicates.

The inhibition zone produced against *P. aeruginosa* is illustrated in Figure 4a.

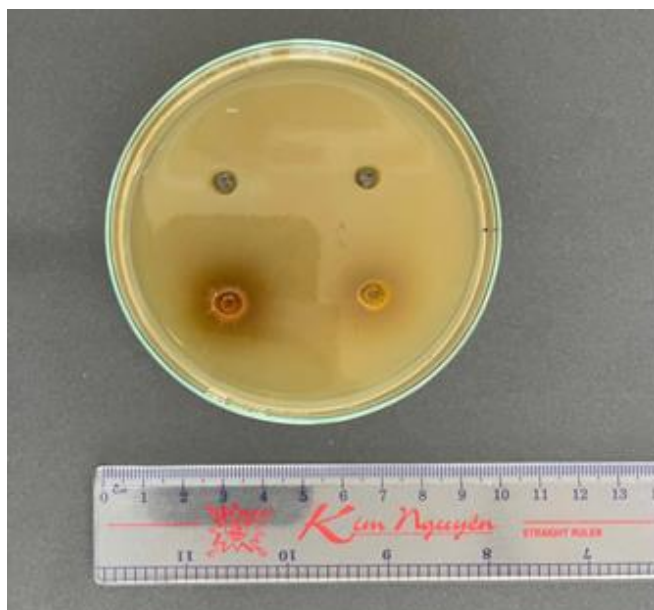


Figure 4a. Agar well diffusion assay against *Pseudomonas aeruginosa* (Gram-negative). Lower-left well: 200 $\mu\text{g/mL}$; lower-right well: 100 $\mu\text{g/mL}$; upper wells: controls. Ruler placed below plate for scale.

A concentration-dependent antibacterial effect against *P. aeruginosa* is clearly observed in Figure 4a. The bioactive components penetrated effectively through the agar matrix at a concentration of 200 $\mu\text{g/mL}$ (lower-left well), thereby forming a clearly visible inhibition zone with a ZOI of 18.5 mm. With the 100 $\mu\text{g/mL}$ (bottom-right well), decreasing the concentration to half resulted in a ZOI of 16.0 mm, with an indistinct boundary due to the smaller concentration gradient over the diffusion distance. Neither control was inhibited, indicating that the DMSO solvent lacked intrinsic antibacterial activity.

Despite *P. aeruginosa* being known for its resistant lipopolysaccharide (LPS)-coated outer membrane and the MexAB-OprM multidrug efflux pump system [4, 19], the plant extract still produced observable zones of inhibition. The effect is mediated through a two-stage process: the fatty acids in the n-hexane extract diffuse directly into the phospholipid bilayer of the outer membrane, thereby increasing fluidity [16]; upon membrane disruption, the phytosterols and glycosides in the EtAC extract enter the cell and act on intracellular targets [10]. The equivalent plate for *S. aureus* is shown in Figure 4b.

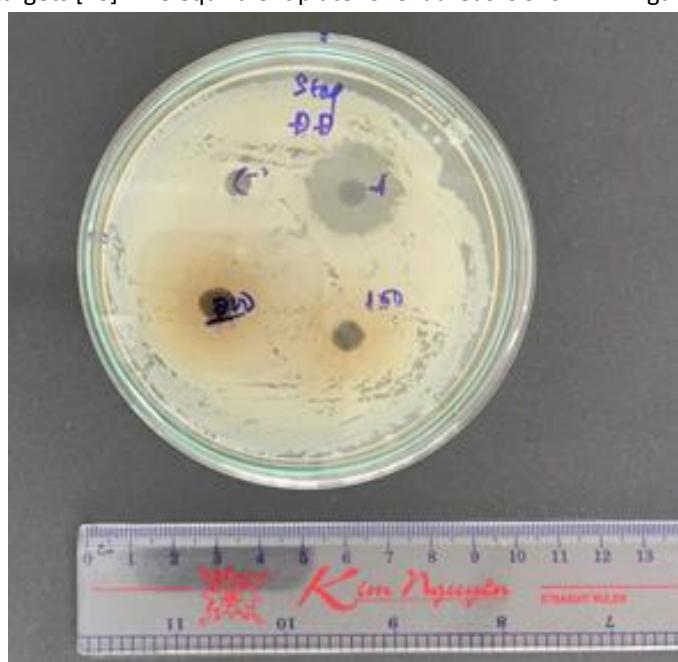


Figure 4b. Agar well diffusion assay against *Staphylococcus aureus* (Gram-positive). Label on lid: "Stap ĐĐ" (abbreviation for *S. aureus* — papaya). Lower-left well: 200 $\mu\text{g/mL}$; lower-right well: 100 $\mu\text{g/mL}$; upper wells: negative and positive controls.

With respect to *S. aureus* (Figure 4b), there was a ZOI measured at 18.0 mm for the 200 $\mu\text{g/mL}$ concentration well and 17.0 mm. Compared with the sharply delineated inhibition zones against *P. aeruginosa*, the inhibition zones exhibited a typical “hazy” or

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diffuse nature with relatively indistinct outer margins. The above difference in morphology is attributed to the dissimilarities in cell envelope structures: *S. aureus* has a thick peptidoglycan wall (20-80nm), which is physically perme, facilitating passive diffusion of the hydrophobic long-chain fatty acids, such as linoleic acid (52.99%) and palmitic acid (30.97%), through the cell wall, thus ensuring their broad diffusion throughout the agar plate [20].

As the bioactive components diffuse outward through the large inhibition zone (17.0-18.0 mm), the molecular concentration becomes less dense in the radial direction: the fatty acids cease to act bactericidally and instead adopt a bacteriostatic role, whereby they induce asymmetric distortion of the lipid bilayer. As such, *S. aureus* experiences leakage of intracellular material (K^+ ions, nucleotides), failure to transport across the cell membrane, and cell division ceases, thus explaining the formation of hazy zones seen at both concentrations. This is further supported by the presence of campesterol (19.21%) and stigmasterol (19.15%) in the EtAC fraction, which have been reported to cause structural destabilization of bacterial cell membranes [10]. Membrane disruption may allow the entry of benzyl β -D-glucoside (23.66%) into cells; however, intracellular targets for benzyl β -D-glucoside have not been explored in the current study and remain to be identified.

3.3. Minimum inhibitory concentration (MIC)

Table 2 shows MIC values obtained through the broth microdilution method. Colorimetric results obtained using the 96-well microplate are shown in Figure 5.

Table 2. MIC values ($\mu\text{g/mL}$) of solvent fractions from male papaya flowers.

Test strain	<i>n</i> -Hexane fraction	EtAC fraction
<i>Pseudomonas aeruginosa</i> (Gram-negative)	50.0 $\mu\text{g/mL}$	25.0 $\mu\text{g/mL}$
<i>Staphylococcus aureus</i> (Gram-positive)	100.0 $\mu\text{g/mL}$	50.0 $\mu\text{g/mL}$

A color gradient across the wells of the microplate due to concentration-dependent inhibition is shown in Figure 5.

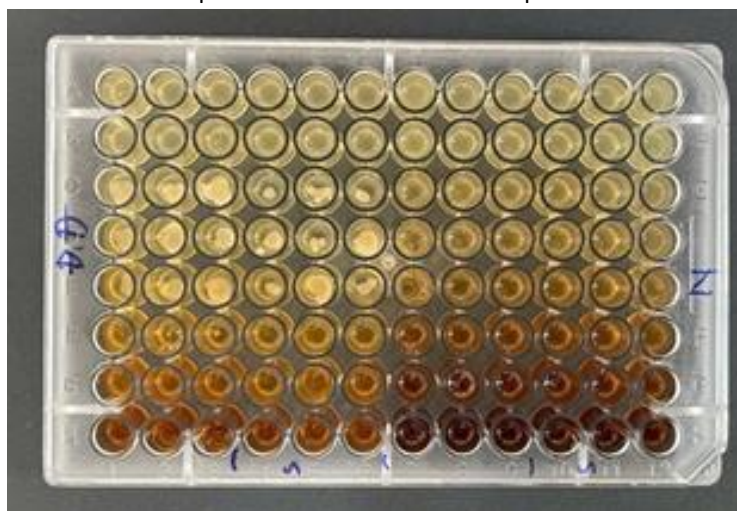


Figure 5. Broth microdilution assay in a 96-well microplate for MIC determination. A continuous color gradient from clear yellow (rows A–E, high concentration) to deep reddish-brown (rows F–H, low concentration) is evident as extract concentration decreases. Label on plate: "DD" (papaya).

The colorimetric results presented in Figure 5 clearly indicate the presence of a concentration-dependent antibacterial effect from rows A to H:

- **Rows A–E (high concentrations, 200→25 $\mu\text{g/mL}$):** wells were still clear yellow. At these concentrations, there were sufficient biologically active compounds to inhibit or kill bacteria, rendering their cellular dehydrogenases inactive and unable to reduce the TTC reagent.
- **Rows F–H (low concentrations, < 25 $\mu\text{g/mL}$):** a progressive development from red-brown to deep red was noticed. The viable bacteria were still metabolically active and thus reduced the colorimetric agent to its colored formazan form, thereby proving that sub-inhibitory levels of extracts were present.

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● **Color transition point — MIC:** MIC was determined to be the concentration of the last well containing clear liquid before discoloration: 25.0 µg/mL for the EtAC fraction versus *P. aeruginosa* and 50.0 µg/mL versus *S. aureus*, whereas for the *n*-hexane fraction, MIC values were found to be 50.0 and 100.0 µg/mL, respectively.

The following quantitative results support and explain the morphology of the hazy region shown in Figure 4b. Phytosterols such as campesterol and stigmasterol, along with benzyl β-D-glucoside, interact directly with bacteria in the aqueous environment of the microplate well, without hindrance from the agar gel matrix in the diffusion assay. This unrestricted availability enables the bioactive compounds to bind to membrane-binding sites and inhibit the respiratory chain at lower concentrations (25.0-50.0 µg/mL), thereby explaining the lower MICs of the EtAC fraction compared to the *n*-hexane fraction in both bacterial strains. These values fall within the spectrum of antibacterial activity reported earlier for *C. papaya* flower extracts [9]; however, direct quantitative comparisons are hindered by the different extraction solvents and assay procedures used in previous studies.

3.4. Structure–activity relationship and proposed mechanism of antibacterial action

The link between the chemical profiles as assessed through GC-MS (Figures 2 and 3) and the biological observations (Figures 4a, 4b, and 5; Tables 1 and 2) can be best described by a hierarchical, multitarget mode of action where the physiological compartment is selectively targeted and destroyed in two stages.

Tier one is characterized by the disruption of membranes by long-chain fatty acids of the *n*-hexane fraction. Linoleic acid (52.99%) and palmitic acid (30.97%) serve as powerful biological surfactants [16], wherein their charged carboxyl head-groups interact electrically with the hydrophilic phospholipid heads present on the bacterial membrane, whereas their hydrophobic hydrocarbon tails embed within the hydrophobic lipid bilayer core [17]. This molecular intercalation causes asymmetric membrane fluidity, disordered lipid bilayer packing, and denaturation of the membrane proteins responsible for electron transport and oxidative phosphorylation [16]. The immediate effects include inhibition of ion-pumping ATPases, intracellular solute loss (K⁺ ions, nucleotides), and disruption of the transmembrane electrochemical potential [21]. While these membrane-targeting effects occur simultaneously, inhibition of enoyl-acyl carrier protein reductase (FabI) by unsaturated fatty acids such as linoleic acid has been shown to block *de novo* fatty acid synthesis, providing another mechanism for intervention [22, 23]. In *P. aeruginosa*, which exhibits a tightly cross-linked outer membrane containing LPS, these fatty acids disrupt the lipopolysaccharide's hydrophobic core, causing outer membrane permeabilization [19].

Phytosterols and glycosides from the EtAC fraction act in the second tier to target intracellular sites utilizing the first-tier membrane compromise. Since campesterol (19.21%) and stigmasterol (19.15%) have structural similarities to membrane lipids, they are inserted into the phospholipid bilayer, thereby affecting membrane rigidity and permeability [10]. This perturbation of the membrane would lead to the formation of a passive transport pathway by which benzyl β-D-glucoside (23.66%) would enter the cytoplasm; no molecular target for this glucoside was studied here; thus, the second step is suggested rather than proved. This complementary synergy between the surface-disruption stage (*n*-hexane fraction) and the intracellular inactivation phase (EtAC fraction) is responsible for the broad-spectrum antibacterial efficacy seen against Gram-positive and Gram-negative bacteria [24]. The following needs to be stressed: The two fractions were tested independently; no checkerboard analysis or FIC testing was performed, so the interaction between them is inferred from their chemistry rather than from direct measurements.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

This research project has systematically determined the phytochemical profile and *in vitro* antibacterial activities of solvent fractions of male papaya (*Carica papaya* L.) flower extracts (Figure 1) collected from Vietnam. GC-MS analysis (Figures 2 and 3) successfully identified a clear divergence in chemical composition: the *n*-hexane fraction was dominated by linoleic acid (52.99%) and palmitic acid (30.97%); the EtAC fraction was enriched in benzyl β-D-glucoside (23.66%), campesterol (19.21%), and stigmasterol (19.15%).

Biological evaluation (Table 1, Table 2, Figures 4a–5) demonstrated potent, concentration-dependent inhibitory activity against *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), with ZoI values reaching 18.5 mm and a minimum MIC of 25.0 µg/mL (EtAC fraction vs *P. aeruginosa*). The findings are consistent with a tiered, complementary mechanism — membrane disruption by fatty acids, followed by the action of phytosterols and glycosides on intracellular targets — although this mechanism remains a working hypothesis, as fraction combinations were not tested and the intracellular targets were not identified. Taken together, the results establish male papaya flowers as a promising phytomedicinal resource for developing novel, naturally derived antibacterial agents.

To fully realize the therapeutic potential of these findings, the following research directions are recommended:

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- **Isolation and purification of bioactive compounds:** application of flash column chromatography and semi-preparative HPLC to isolate individual pure compounds from both fractions, followed by structural elucidation by NMR and HR-MS to confirm the identity of the principal antibacterial markers.
- **Cytotoxicity assessment and in vivo evaluation:** cytotoxicity testing using MTT on healthy mammalian cell cultures, and infection studies in animal models for defining safety profiles, pharmacokinetics, and dose-response studies.
- **Molecular-level mechanistic studies:** morphological changes in bacterial cell surface by using SEM/TEM analysis, estimation of intracellular protein, DNA, and RNA content leaked from cells, and docking studies to find enzyme binding sites for major bioactive molecules.
- **Pharmaceutical formulation development:** study on encapsulation using nanotechnology and biopolymer hydrogel matrices from biopolymers with flower extract for use in antibacterial wound dressings, topical antimicrobial agents, and natural food preservatives.

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