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In vitro* antibacterial, antibiofilm, and cytotoxicity analysis of the interaction between *Moringa oleifera* and *Hygrophila auriculata* leaf extracts against *Proteus mirabilis

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Abstract

Background: The increasing resistance of pathogenic bacteria to antibiotics has driven interest in medicinal plants such as *Moringa oleifera* (*M. oleifera*) and *Hygrophila auriculata* (*H. auriculata*), which are inexpensive, traditionally accepted, and known for their antimicrobial, antioxidant, and nephroprotective properties.

Method: This study evaluates the antibacterial, antibiofilm, and cytotoxic potential of aqueous leaf extracts of *M. oleifera* and *H. auriculata*, individually and in combination against *Proteus mirabilis*. Leaf extracts were prepared using four different methods: boiling, steam extraction, ayurvedic decoction, and 50% ethanolic Soxhlet extraction. Antibacterial activity was assessed using agar well diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays. Biofilm inhibition was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Embryotoxicity was assessed using zebrafish embryos toxicity assay. The chemical composition of the extracts was characterized using Fourier-transform infrared spectroscopy (FTIR).

Results: The combined treatment of *H. auriculata* (decoction) and *M. oleifera* (boiled) exhibited enhanced antibacterial and antibiofilm efficacy, with reduced MIC, MBC, and MBIC₅₀ values (0.1220- 0.4883 mg/mL; 0.2441-0.9766 mg/mL; 0.6098 - 2.161 mg/mL) compared to individual treatments. When applied separately, leaf extracts of *M. oleifera* showed MIC, MBC, and MBIC₅₀ ranges of 0.2441 - 0.9766 mg/mL, 0.4883-1.9531 mg/mL, and 1.229-4.227 mg/mL, respectively, while *H. auriculata* exhibited ranges of 0.4883-0.9766 mg/mL, 0.9766-1.9531 mg/mL, and 1.029-3.322 mg/mL, respectively. Zebrafish embryo toxicity assays revealed a dose-dependent response; however, the combined treatment exhibited reduced embryotoxicity, with the highest LC₅₀ value

(1812.74 µg/mL) compared to *M. oleifera* (1527.5 µg/mL) and *H. auriculata* (1274.28 µg/mL) leaf extracts. FTIR analysis of both extracts revealed the presence of key functional groups such as hydroxyl, carbonyl, and aromatic compounds.

Conclusion: The study outlines promising antimicrobial and antibiofilm potential of leaf extracts of *M. oleifera* and *H. auriculata*, particularly when used in combination. Their synergistic activity and combined with low cytotoxicity levels suggest that these leaf extracts hold potential for development as alternative therapeutics for managing infections such as urinary tract infections where *P. mirabilis* is a causative agent in the face of growing antibiotic resistance.

Keywords: *Moringa oleifera*, *Hygrophila auriculata*, *Proteus mirabilis*, Cytotoxicity, Antimicrobial, Biofilms, Urinary Tract Infections

1.Introduction

Urinary Tract Infections (UTIs) are among the most common bacterial infections, affecting millions of people worldwide. Although several bacterial species, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus* contribute to UTIs, *P. mirabilis* is particularly problematic due to its strong urease activity and ability to form crystalline biofilms which leads to catheter blockage, and contributes to recurrent infections reducing treatment efficacy (1). In Sri Lanka, *P. mirabilis* has been identified in approximately 7.9% of pediatric UTI cases, highlighting its importance among non-*E. coli* uropathogens in local clinical settings (2). Across Southeast Asia, although comprehensive surveillance data remain limited, *P. mirabilis* is consistently reported in both community and hospital-acquired UTIs, with increased prevalence among catheterized patients (1).

63 Globally, uropathogens have developed increasing resistance to conventional antibiotics, making
64 treatment more difficult and posing a major public health burden (3). As a result, researchers are
65 more engaging in exploring plant-based alternatives with strong antibacterial properties (4).

66 Among the many medicinal plants studied for their antimicrobial capability, *Moringa oleifera* and
67 *Hygrophila auriculata* have attracted considerable scientific interest due to their unique
68 therapeutic qualities (5). *M. oleifera*, commonly referred to as the “Drumstick”, is widely used in
69 traditional medicine and valued for its nutritional and phytochemical richness. It contains a range
70 of bioactive compounds including flavonoids, alkaloids, and phenolic compounds that enhance its
71 antimicrobial activity (5). Similarly, *H. auriculata*, known in Sri Lanka as “Neeramulli”, is
72 commonly used in ayurvedic medicine for the treatment of inflammation, kidney stones, and UTIs.
73 Different extractions of *H. auriculata* have been shown to exhibit both antibacterial and
74 antioxidant effects, supporting its traditional use in treating urinary and kidney-related disorders
75 (7, 8).

76 Importantly, previous in vitro and in vivo studies have demonstrated that *M. oleifera* extracts
77 exhibit significant antibacterial and antibiofilm activity against a wide range of pathogens,
78 including Gram-negative bacteria associated with urinary tract infections (7). Toxicological
79 investigations in animal models have also indicated that different parts of *M. oleifera* (leaves,
80 seeds, and stem extracts) generally exhibit a favorable safety profile at tested doses (8).
81 Furthermore, zebrafish embryo-based studies have been employed to evaluate the developmental
82 toxicity of *M. oleifera*-derived compounds, demonstrating dose-dependent effects at higher
83 concentrations while supporting its utility for biomedical applications when used within safe

84 ranges (9). These findings provide scientific justification for further evaluation of its biological
85 activity and biosafety.

86 Similarly, *H. auriculata* has been reported to exhibit antimicrobial, antioxidant, and
87 pharmacological activities in experimental models (6, 10). Acute toxicity studies in mammalian
88 systems have indicated that its leaf extracts are generally well tolerated at high doses without
89 significant adverse effects, supporting its relative safety (11). However, despite these findings,
90 detailed developmental toxicity studies, particularly using vertebrate embryonic models, remain
91 limited, highlighting the need for further investigation into its biosafety profile (11).

92 Further, natural plant extracts have shown promise in breaking down biofilms by disrupting the
93 maturation of biofilms, making them powerful anti-biofilm agents (12). Since recent studies have
94 reported that biofilm-mediated reduced antibiotic susceptibility remarkably hinders the effective
95 treatment of *P. mirabilis* biofilm infections (13), developing therapies that target the disruption of
96 crystalline biofilm formation is crucial.

97 Thus, the present study investigated antimicrobial and antibiofilm activities of *M. oleifera* and *H.*
98 *auriculata* leaf extracts against *P. mirabilis*, while evaluating the synergistic potential of their
99 combination, bridging traditional medicinal knowledge with modern analytical strategies to
100 combat crystalline biofilm formation by *P. mirabilis*. Further, cytotoxicity of plant-derived
101 bioactive compounds was assessed *in vivo* using Zebrafish (*Danio rerio*) embryos, for their safe
102 use in therapeutic applications. Hence, the findings of this study would contribute to the
103 development of new, plant-based treatments for UTIs, addressing the growing need for sustainable
104 solutions to antimicrobial resistance.

2. Materials and Method

2.1 Plant material collection and preparation

Fresh, healthy leaves of *H. auriculata* and *M. oleifera* leaves were collected from Maharagama area in Colombo district, Sri Lanka. The plant materials were authenticated prior to use, and voucher specimens of *Moringa oleifera* Lam. and *Hygrophila auriculata* (Schumach.) Heine were deposited under accession numbers ACC 3353 and ACC 3354, respectively. Collected leaves were thoroughly washed under running tap water to remove dirt, followed by rinsing with distilled water to eliminate any surface contaminants. The leaves were shade-dried at 37 °C for four days to preserve the bioactive compounds and prevent degradation. Once dried, those were ground into a fine powder using an electric grinder and stored in airtight containers until use.

2.2 Bacterial Strains

A quality control strain of *P. mirabilis* and biofilm-forming *P. mirabilis* clinical isolates, isolated from urine specimens were obtained from the culture collection of the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura. These bacterial species were sub-cultured from freezer stocks onto freshly prepared, quality-controlled nutrient agar plates, followed by incubation at 37 °C for 24 h.

2.3 Extraction methods

The leaves of *H. auriculata* and *M. oleifera* were subjected to extraction using four different methods.

2.3.1 Method 1: Boiling extraction

A total of 40 g of dried leaf material from *H. auriculata* and *M. oleifera* was separately ground and boiled with 80 mL of distilled water, maintaining a 1:2 (w/v) ratio of leaves to water. The mixture was boiled for 30 minutes, and the resulting extract was filtered through a sterile cotton cloth. The filtrate was collected into a sterile bottle, and used directly for antibacterial testing (11).

2.3.2 Method 2: Ayurvedic decoction method

According to the traditional ayurvedic procedure, 40 g of dried leaves from each species was separately ground and mixed with 400 mL of distilled water. This mixture was boiled until the volume was reduced to approximately 50 mL. The concentrated decoction was filtered through a sterile cotton cloth, and the final filtrate was used for testing (15).

2.3.3 Method 3: Steam extraction

Forty grams (40g) of dried ground leaves were wrapped separately in sterile cotton cloth and exposed to steam at 100°C for 2 hours. After steaming, the material was squeezed under aseptic conditions to collect the juice, which was stored in a sterile container and used as the test extract (11).

2.3.4 Method 4: Soxhlet extraction (Ethanolic extract)

Dried leaves (40 g) were finely powdered separately and placed into a thimble inside a Soxhlet apparatus. Ethanol (400 mL) was used as the solvent, and extraction was carried out for 6 hours at 60°C (16). The resulting ethanol extract was concentrated using a rotary evaporator (16) under reduced pressure at 40°C to remove the solvent. The concentrated extract was collected into a sterile bottle and stored at 4°C until further analysis (14).

2.4. Antibacterial activity of crude extracts

2.4.1. Agar well diffusion assay

The antibacterial activity of the four crude extracts was analyzed using the agar well diffusion method, as described by Abdalla et al., 2016 (15). Clinical isolates of *P. mirabilis* (n=10) were used as test organisms. Bacterial suspensions were adjusted to match the 0.5 McFarland turbidity standard ($\sim 10^8$ CFU/mL) and uniformly swabbed onto Mueller-Hinton Agar (MHA, HiMedia, India) plates.

Wells of 9 mm diameter were aseptically punched into the agar using sterile pipet tips, and bottom of the wells was sealed with sterile molten MHA to prevent leakage. Each well was loaded with 100 μ L of the respective test extract. Plates were allowed to stand at room temperature for 15 minutes to allow for pre-diffusion of the extracts, and then incubated at 37°C for 24 hours. Sterile distilled water and ethanol were served as the negative controls, while gentamicin (25 mg/mL) was used as the positive control. After incubation, the diameters of the zones of inhibition were measured in millimeters. A clear zone around the well was considered indicative of antimicrobial activity.

The extract and extraction method that produced the largest inhibition zones were selected for subsequent determination of MIC, MBC, MBIC₅₀, IC₅₀, and cytotoxicity. In addition to testing the individual extracts, a synergistic combination of leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction) was also tested to determine potential enhancement in antibacterial activity. The combination was prepared by mixing equal volumes of the respective extracts that produced the largest inhibition zones (leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction)),

and subjected to the agar well diffusion assay using the same methodology described for individual extracts, along with the positive and negative controls, to validate the experimental conditions. The inoculated MHA plates were incubated at 37 °C for 24 hours, after which the diameters of the zones of inhibition were measured in millimeters.

Each experiment, including the synergistic combination of leaf extracts were performed in triplicate to ensure the reproducibility and reliability of the results.

2.4.2 Determination of Minimum Inhibitory Concentration (MIC)

Based on preliminary inhibition zone assays, the boiled *M. oleifera* leaf extract and *H. auriculata* decoction extract was selected for MIC determination. The MIC of each leaf extracts was determined using the broth microdilution method, as described in the clinical laboratory standard institution (CLSI) M27 guideline (19). A two-fold serial dilution of the extracts was prepared in Brain Heart Infusion (BHI) broth (BHI, Oxoid UK) in 96-well microtiter plates (Thermo Fisher Scientific, USA). Each well was inoculated with 100 µL of diluted extract and 100 µL of a standardized biofilm-forming *P. mirabilis* suspension ($\sim 10^8$ CFU/mL; equivalent to 0.5 McFarland). Wells containing BHI broth and bacterial suspension served as growth controls, while those with only BHI broth served as sterility controls. The plates were incubated at 37°C for 24 hours. Turbidity was assessed visually before and after incubation to preliminarily determine the MIC. After incubation, 30 µL of 0.015% resazurin solution was added to each well, and the plates were incubated for an additional 2-4 hours at 37°C. Wells that remained blue (indicating no metabolic activity and thus no bacterial growth) were considered to contain concentrations above the MIC.

To confirm the visual findings, absorbance was quantified at 570 nm using an ELISA (Enzyme linked Immunosorbent Assay) plate reader ((Thermo Fisher Scientific, Multiskan Ex,S/N 3550905398, USA) with Thermo Scientific™ SkanIt™ software. MIC was defined as the lowest concentration at which no visible growth or color change (blue) was observed and where absorbance readings supported bacterial inhibition (CLSI, 2012).

2.4.3 Determination of Minimum Bactericidal Concentration (MBC).

The leaf extracts that produced the largest inhibition zones (leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction) were used to determine Minimum Bactericidal Concentration (MBC) by plating 5 µL from each well onto fresh MHA plates. The plates were then incubated aerobically at 37°C for 24 hours. The MBC was defined as the lowest concentration of leaf extracts that resulted in no bacterial growth on the MHA surface after the incubation period (13,19).

2.4.4 Determination of Half Maximal Inhibitory Concentration (IC₅₀)

The IC₅₀ values for leaf extracts of *M. oleifera* (boiled), *H. auriculata* (decoction), and their combination against *P. mirabilis* were determined using a standard two-fold microdilution method performed in 96-well microtiter plates. Serial dilutions of each compound were prepared in BHI broth and inoculated with bacterial suspensions adjusted to a 0.5 McFarland standard. Plates were incubated at 37 °C for 24 h. Bacterial growth was measured at 570 nm using a microplate reader. The percentage of bacterial inhibition at each concentration was calculated relative to the untreated control. These inhibition percentages (Y-axis) were plotted against the log-transformed concentrations (X-axis) of each compound. Non-linear regression analysis was performed using GraphPad Prism 10 software to generate dose–response curves and calculate the IC₅₀ values.

2.5 Determination of Minimum Biofilm Inhibitory Concentration (MBIC₅₀)

Standard cell suspensions of *P. mirabilis* were prepared by adjusting the turbidity of cultures in BHI broth to 0.5 McFarland standard ($\sim 10^8$ CFU/mL). A volume of 100 μ L from each suspension was dispensed into the wells of sterile 96-well flat-bottomed microtiter plates and incubated at 37°C for 48 hours to allow biofilm formation. At the 24-hour mark, the medium was replenished with an equal volume of fresh BHI broth. After incubation, mature biofilms were gently washed twice with sterile phosphate-buffered saline (PBS) (PBS, Oxoid UK) to remove non-adherent cells. Biofilm treatment was performed by adding 100 μ L of the test extract solutions in a two-fold serial dilution ranging from 500 mg/mL to 0.2441 mg/mL. The leaf extracts tested included *M. oleifera* (boiled), *H. auriculata* (decoction), and their combination. Growth control wells consisted of BHI broth and bacterial suspension without test compounds, while sterility controls contained broth only. Blank control wells contained broth and test extracts without bacterial inoculation to account for any background interference. All plates were incubated at 37°C for 24 hours (16).

Subsequent treatment, wells were washed three times with 200 μ L of sterile PBS to remove planktonic cells. MTT assay was conducted by adding 50 μ L of 1 mg/mL MTT solution to each well, followed by incubation at 37°C for 4 hours. The MTT solution was then discarded, and 100 μ L of dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) was added to solubilize formazan crystals. Absorbance was recorded at 570 nm using a microtiter plate reader (Thermo Fisher Scientific, Multiskan Ex, S/N 3550905398, USA). The Minimum Biofilm Inhibitory Concentration required to inhibit 50% of the biofilm biomass (MBIC₅₀) was calculated relative to untreated controls using GraphPad Prism 6.0.1.

2.6 Cytotoxicity to detect the toxic effect to the cellular components

231 The zebrafish (*Danio rerio*) embryotoxicity assay was conducted in accordance with
232 internationally accepted OECD No. 236 ethical guidelines. Under Directive 2010/63/EU of the
233 European Parliament, zebrafish embryos and larvae are not considered protected animals until the
234 stage of independent feeding 120 hours post-fertilization (hpf). Accordingly, experiments
235 conducted within the first 96 hpf are considered to involve pre-protected developmental stages
236 which do not require ethical approval. Since embryotoxic and developmental effects were assessed
237 only up to 96 hpf in the present study, ethical approval was not required (OECD TG 236).

238 Crude leaf extracts of *M. oleifera* (boiled), and *H. auriculata* (decoction) were initially dissolved
239 in sterile distilled water to prepare a stock solution of 10000 µg/mL for each extract. Two-fold
240 serial dilutions were then performed to obtain a concentration series of 10000 µg/mL, 5000 µg/mL,
241 2500 µg/mL, 1250 µg/mL, 625 µg/mL, 312.5 µg/mL, 156.3 µg/mL, 78.12 µg/mL, 39.06 µg/mL
242 and 19.53 µg/mL. A separate dilution series was prepared using a synergistic combination of leaf
243 extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction) over the same concentration range
244 to assess combined embryotoxic effects. Embryo media alone was used as negative control, while
245 3,4-dichloroaniline (3,4-DCA) was used as the positive control. Zebrafish (*Danio rerio*) embryos
246 at 24 hours post-fertilization (24hpf) were selected under a microscope, and only healthy, fertilized
247 embryos were used for the assay. The exposure experiment was conducted using 24-well plates,
248 with 20 embryos placed in each well. All treatments, including controls, were performed in
249 triplicate. The plates were incubated at $28 \pm 1^{\circ}\text{C}$ and the embryos and larvae were observed daily
250 for four days (96 hours) using a microscope. Analyzed endpoints included embryo and larval
251 mortality, coagulation, absence of heartbeat, delayed hatching, tail detachment, somite formation,
252 eye and otolith development, skeletal deformities, and edema (both pericardial and yolk sac). All
253 observations were recorded daily (17).

2.7 Calculation of Fractional Inhibitory Concentration Index (FICI)

Using the previously reported MICs, the mean FICI was calculated to assess potential synergistic interactions (20). The FICI was calculated using the following equation:

$$\Sigma FICI = FIC (Hygrophila auriculata decoction) + FIC (Moringa oleifera boiled)$$

$$FIC = \frac{MIC \text{ of plant extract in combination}}{MIC \text{ of tested plant extract individually}}$$

This index was used to assess the potential synergy between the compounds where,

- $\Sigma FICI < 1$ indicates synergy
- $\Sigma FICI = 1$ indicates an additive effect
- $\Sigma FICI > 1$ indicates antagonism

2.8 FTIR Analysis

FTIR spectroscopy was employed to identify the functional groups present in the aqueous leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction). Dried powder samples were subjected to FTIR analysis using a Shimadzu IR Prestige-21 spectrophotometer (Kyoto, Japan). The infrared spectra were recorded over the range of 4000–500 cm^{-1} , allowing the detection of characteristic absorption peaks corresponding to various chemical bonds and functional groups within the phytochemical constituents of each extract.

2.9 Data Analysis

OriginPro 9.0 and GraphPad Prism 9.0 were used to generate clear, quality graphs for calculating IC_{50} and $MBIC_{50}$. The LC_{50} values were calculated using probit regression analysis to estimate the concentration causing 50% lethality.

3. Results

3.1 Agar well diffusion assay

All tested *P. mirabilis* strains, including the quality control strain and ten clinical isolates (PM1–PM10), were susceptible to leaf extracts of *M. oleifera* and *H. auriculata*. The inhibition zones were varied according to extraction method and the isolate. The leaf extracts of *M. oleifera* showed inhibition zones ranging from 12.3 ± 0.94 mm to 26.0 ± 0.00 mm, whereas *H. auriculata* extracts exhibited zones between 12.3 ± 0.47 mm and 25.0 ± 0.00 mm across the tested isolates.

The QC strain showed relatively higher susceptibility to *M. oleifera* boiled extract (20.0 ± 0.00 mm) compared to *H. auriculata* (17.3 ± 1.41 mm). Among the clinical isolates, variability in sensitivity was observed, with some isolates (PM3, PM4, PM8, and PM10) showing comparatively higher inhibition zones for *H. auriculata* decoction extracts. Boiled extracts of *M. oleifera* demonstrated strong antibacterial activity against several isolates, including PM2 (23.7 ± 0.94 mm), PM5 (23.3 ± 0.47 mm), PM8 (23.3 ± 0.47 mm), and PM9 (15.7 ± 0.94 mm), whereas steam extracts generally produced smaller zones or no detectable inhibition for certain isolates. In contrast, decoction extracts of both plant species consistently showed moderate to high antibacterial activity across multiple isolates. No inhibition zones were observed for the negative controls, confirming that the observed antibacterial effect was attributable solely to the plant extracts. Gentamicin, used as the positive control, showed the largest zones of inhibition across all

292 strains (22.7 ± 0.47 mm to 47.3 ± 0.94 mm), confirming the susceptibility of the tested isolates
 293 and validating the assay (Table 1).

294

295 Table 1: Zones of inhibition of *M. oleifera* and *H. auriculata* extracts against *P. mirabilis*

Strain		Inhibition zone diameter (Mean $\pm$ SD mm)						
	Extract	Boiled	Steamed	Decoction	Ethanollic	SDW	Ethanol	Gentamicin
		extract	extract	extract	extract	(NC)	(NC)	(PC)
PM	<i>M. oleifera</i>	20.0 $\pm$ 0.0	14.7 $\pm$ 0.4	17.3 $\pm$ 0.94	-	-	-	47.3 $\pm$ 0.94
QC		0	7					
strain	<i>H.</i>	17.3 $\pm$ 1.4	15.3 $\pm$ 0.9	14.7 $\pm$ 0.94	-	-	-	38.0 $\pm$ 1.41
	<i>auriculata</i>	1	4					
PM 1	<i>M. oleifera</i>	16.7 $\pm$ 0.8	15.0 $\pm$ 0.0	13.3 $\pm$ 0.47	-	-	-	22.7 $\pm$ 0.47
		1	0					
	<i>H.</i>	24.0 $\pm$ 0.9	13.3 $\pm$ 0.9	19.3 $\pm$ 0.81	-	-	-	29.3 $\pm$ 0.94
	<i>auriculata</i>	4	4					
PM 2	<i>M. oleifera</i>	23.7 $\pm$ 0.9	15.3 $\pm$ 0.9	20.0 $\pm$ 0.81	-	-	-	32.3 $\pm$ 0.47
		4	4					
	<i>H.</i>	16.0 $\pm$ 0.0	14.7 $\pm$ 0.4	20.0 $\pm$ 0.00	-	-	-	36.7 $\pm$ 0.81
	<i>auriculata</i>	0	7					
PM 3	<i>M. oleifera</i>	19.7 $\pm$ 0.9	12.3 $\pm$ 0.9	17.7 $\pm$ 0.94	-	-	-	29.7 $\pm$ 0.47
		4	4					

	<i>H.</i>	19.3±0.9	21.3±0.4	24.0±0.47	-	-	-	24.7±0.47
	<i>auriculata</i>	4	7					
PM 4	<i>M. oleifera</i>	16.0±0.4	15.3±0.9	15.3±0.94	-	-	-	36.7±0.81
		7	4					
	<i>H.</i>	20.7±0.8	17.3±0.4	24.7±0.94	-	-	-	39.0±0.00
	<i>auriculata</i>	1	7					
PM 5	<i>M. oleifera</i>	23.3±0.4	15.0±0.0	16.3±0.94	-	-	-	36.7±0.94
		7	0					
	<i>H.</i>	22.3±0.8	14.3±0.4	17.7±0.94	-	-	-	35.3±0.47
	<i>auriculata</i>	1	7					
PM 6	<i>M. oleifera</i>	20.3±0.4	-	18.7±0.94	-	-	-	37.3±0.47
		7						
	<i>H.</i>	21.7±0.9	15.0±0.0	19.7±0.94	-	-	-	37.0±0.00
	<i>auriculata</i>	4						
PM 7	<i>M. oleifera</i>	21.7±0.9	-	-	-	-	-	39.0±0.00
		4						
	<i>H.</i>	12.3±0.4	16.7±0.9	17.7±0.94	-	-	-	34.7±0.94
	<i>auriculata</i>	7	4					
PM 8	<i>M. oleifera</i>	23.3±0.4	15.7±0.9	22.7±0.94	-	-	-	29.7±0.94
		7	4					
	<i>H.</i>	23.7±0.9	15.0±0.0	25.0±0.00	-	-	-	34.3±0.47
	<i>auriculata</i>	4	0					

PM 9	<i>M. oleifera</i>	15.7±0.9	-	26.0±0.00	-	-	-	25.0±0.00
		4						
	<i>H.</i>	13.7±0.9	-	21.0±0.00	-	-	-	27.7±0.94
	<i>auriculata</i>	4						
PM 10	<i>M. oleifera</i>	-	21.3±0.4	13.7±0.94	-	-	-	34.0±0.00
			7					
	<i>H.</i>	22.0±0.0	14.7±0.9	24.7±0.94	-	-	-	26.7±0.94
	<i>auriculata</i>	0	4					

PM: *P. mirabilis*, QC: Quality control, SD: Standard deviation, -; No zone of inhibition, SDW-
Sterile Distilled Water, NC-Negative Control, PC-Positive Control

Among the extraction methods, boiled and decoction leaf extracts generally produced larger zones of inhibition compared to steam extracts. Accordingly, the boiled leaf extract of *M. oleifera* and the decoction of *H. auriculata*, which exhibited the highest antimicrobial activity, were selected for subsequent experiments (determination of their individual and combination effects, using MIC, MBC, MBIC₅₀, IC₅₀, and cytotoxicity).

The combined extract of *M. oleifera* (boiled) and *H. auriculata* (decoction) demonstrated consistent and strong antibacterial activity against all tested strains, with zones of inhibition ranging from 20.33 ± 1.24 mm to 26.66 ± 0.47 mm. The highest inhibitory activity was recorded against PM7 (26.66 ± 0.47 mm), followed by PM6 (25.00 ± 0.00 mm) and PM3 (24.00 ± 0.00 mm). No inhibition was observed for the negative control, confirming that the antibacterial effect was attributable to the combined leaf extracts. The combined leaf extracts produced inhibition zones that were comparable to or greater than those of gentamicin for several isolates, indicating

enhanced antibacterial activity when leaf of extracts *M. oleifera* (boiled) and *H. auriculata* (decoction) were used together (Table 2).

Table 2: Zones of inhibitions of combined leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction) against *P. mirabilis*

Test Strain	Inhibition zone diameter (Mean $\pm$ SD mm)		
	Combined extracts of <i>H. auriculata</i> (decoction) and <i>M.</i> <i>oleifera</i> (boiled),	SDW (NC)	Gentamicin (PC)
PM QC Strain	25.00 $\pm$ 0.81	-	23.00 $\pm$ 0.81
PM 1	20.33 $\pm$ 1.24	-	26.33 $\pm$ 0.94
PM 2	20.66 $\pm$ 0.94	-	22.00 $\pm$ 0.00
PM 3	24.00 $\pm$ 0.00	-	23.00 $\pm$ 0.00
PM 4	23.66 $\pm$ 0.47	-	24.66 $\pm$ 1.24
PM 5	21.66 $\pm$ 0.47	-	24.33 $\pm$ 0.94
PM 6	25.00 $\pm$ 0.00	-	21.00 $\pm$ 0.00
PM 7	26.66 $\pm$ 0.47	-	24.66 $\pm$ 1.24
PM 8	24.33 $\pm$ 1.24	-	25.66 $\pm$ 1.24
PM 9	23.00 $\pm$ 0.00	-	22.00 $\pm$ 0.00
PM 10	23.66 $\pm$ 1.24	-	23.00 $\pm$ 0.00

PM: *P. mirabilis*, QC: Quality control, SD: Standard deviation, -; No zone of inhibition, SDW-
Sterile Distilled Water, NC-Negative Control, PC-Positive Control

319

3.2 Determination of MIC and MBC

The MIC and MBC values of leaf extracts of *H. auriculata* (decoction), *M. oleifera* (boiled), and
their combined treatment are presented in Table 3.

Both individual extracts exhibited notable antibacterial activity against all tested isolates. The MIC
values for *H. auriculata* leaf extract (decoction) ranged from 0.4883 to 0.9766 mg/mL, while *M.*
oleifera (boiled) showed MIC values between 0.2441 and 0.9766 mg/mL. Correspondingly, MBC
values ranged from 0.9766 to 1.9531 mg/mL for leaf extract of *H. auriculata* (decoction) and
0.4883 to 1.9531 mg/mL for *M. oleifera* leaf extract (boiled).

The combined treatment of *H. auriculata* and *M. oleifera* extracts demonstrated enhanced
antibacterial efficacy, with reduced MIC values ranging from 0.1220 to 0.4883 mg/mL and MBC
values between 0.2441 and 0.9766 mg/mL across the tested strains. Notably, several isolates (PM2,
PM3, PM5, PM8, and PM10) including the reference strain, exhibited the lowest MIC (0.1220
mg/mL) and MBC (0.2441 mg/mL) values when treated with the combined extracts.

Table 3: MIC and MBC values (mg/mL) of leaf extracts of *H. auriculata* (decoction), *M. oleifera*
(boiled) and their combined treatment (1:1 ratio) against *P. mirabilis*

Test Strain	MIC (mg/mL)			MBC (mg/mL)		
	<i>H.</i>	<i>M.</i>	Combined	<i>H.</i>	<i>M.</i>	Combined
	<i>auriculata</i>	<i>oleifera</i>	treatment	<i>auriculata</i>	<i>oleifera</i>	treatment

PM QC	0.9766	0.4883	0.1220	1.9531	0.9766	0.2441
Strain						
PM 1	0.9766	0.4883	0.4883	1.9531	0.9766	0.9766
PM 2	0.9766	0.2441	0.1220	1.9531	0.4883	0.2441
PM 3	0.4883	0.2441	0.1220	0.9766	0.4883	0.2441
PM 4	0.4883	0.4883	0.2441	0.9766	0.9766	0.4883
PM 5	0.9766	0.9766	0.1220	1.9531	1.9531	0.2441
PM 6	0.9766	0.4883	0.2441	1.9531	0.9766	0.4883
PM 7	0.9766	0.2441	0.2441	1.9531	0.4883	0.4883
PM 8	0.4883	0.2441	0.1220	0.9766	0.4883	0.2441
PM 9	0.4883	0.4883	0.2441	0.9766	0.9766	0.4883
PM 10	0.4883	0.2441	0.1220	0.9766	0.4883	0.2441

335 PM: *P. mirabilis*, QC: Quality control, MIC: Minimum Inhibitory Concentration, MBC: Minimum
336 Bactericidal Concentration

337 3.3 Determination of Half-maximal Inhibitory Concentration (IC₅₀)

338 The IC₅₀ values represent a key pharmacodynamic parameter reflecting the concentration required
339 to inhibit a specific target by 50%. A lower IC₅₀ value typically suggests that an extract is effective
340 at lower concentrations, which is important for reducing potential toxicity and minimizing the
341 necessary dose in practical applications (19). In this study, the calculated IC₅₀ values aligned well
342 with MIC and MBC data, strengthening the validity of the observations and confirming the relative
343 effectiveness of each extract.

Across all tested strains, leaf extract of *H. auriculata* (decoction), consistently demonstrated lower IC_{50} values than *M. oleifera* (boiled), indicating higher antibacterial potency when used alone. The IC_{50} values of *H. auriculata* ranged approximately from 0.84 to 2.61 mg/mL, whereas *M. oleifera* exhibited higher IC_{50} values, ranging from 1.79 to 4.16 mg/mL, suggesting comparatively weaker inhibitory activity.

The combination of *H. auriculata* (decoction) and *M. oleifera* (boiled) resulted in a marked reduction in IC_{50} values for the majority of isolates, indicating enhanced antibacterial efficacy through combined application. Combination IC_{50} values were generally lower than those observed for either extract alone, with several isolates (including QC strain, PM3, PM6, and PM10) exhibiting IC_{50} values below 1 mg/mL. This leftward shift in the dose-response relationship suggests that lower concentrations of the combined extracts were sufficient to achieve 50% growth inhibition, highlighting a potential synergistic or additive interaction between the two leaf extracts (Table 4, Figure 1).

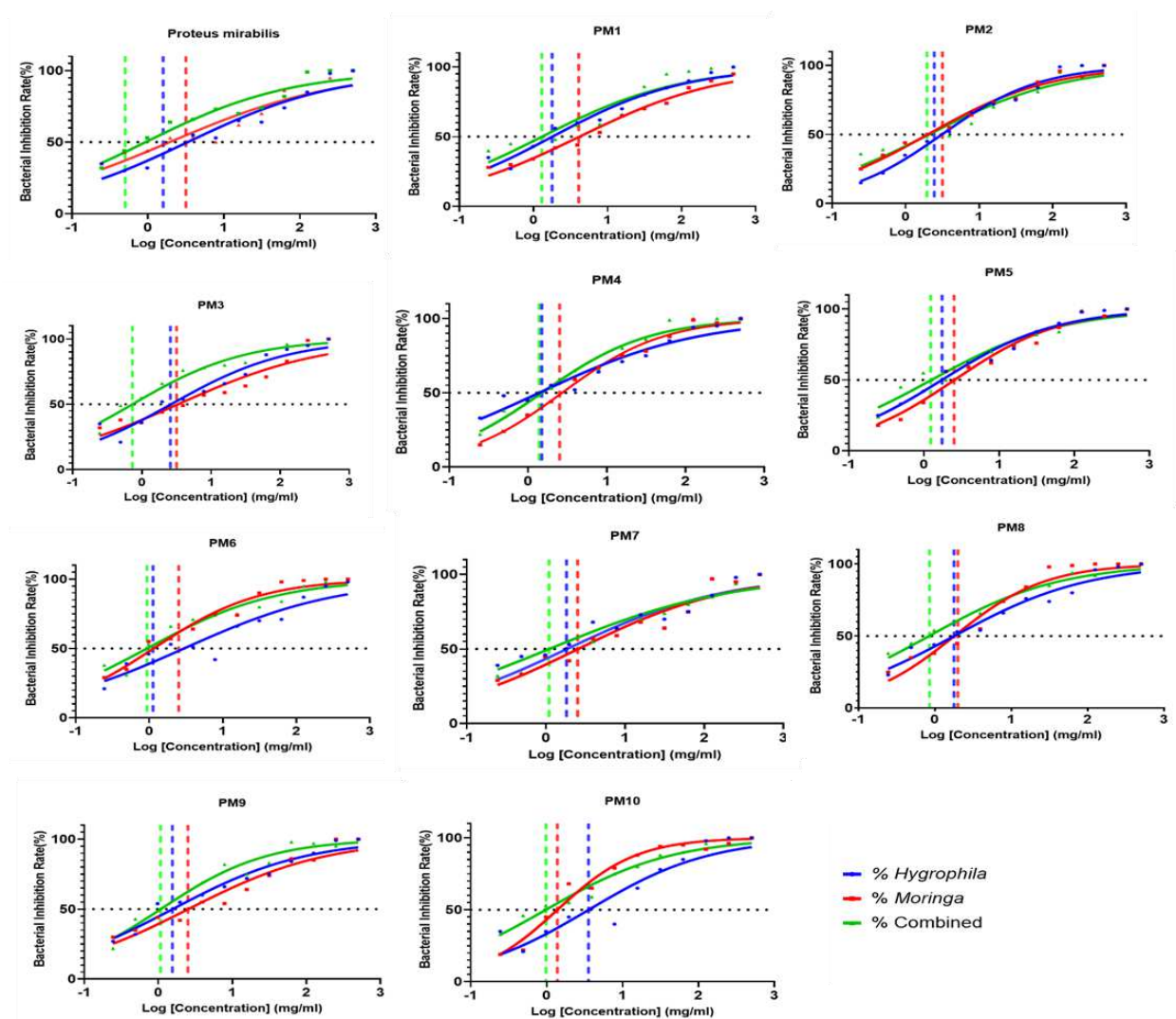


Figure 1: Half maximal inhibitory concentration (IC₅₀) of leaf extracts of *H. auriculata* (decoction) (blue) and *M. oleifera* (boiled) (red), and their combination (green)

Data points represent the mean $\pm$ standard deviation of triplicate measurements. IC₅₀ values for each extract against bacterial strains were calculated using nonlinear regression analysis in GraphPad Prism.

3.4 Determination of Minimum Biofilm Inhibitory Concentration (MBIC₅₀)

The MBIC₅₀ is the minimal dosage of an antimicrobial drug necessary to diminish biofilm biomass by 50% relative to untreated controls, as determined by biofilm quantification assays (20). Both individual plant extracts demonstrated the ability to inhibit biofilm formation, with MBIC₅₀ ranging from 1.036 to 3.843 mg/mL. *H. auriculata* (decoction) generally exhibited lower MBIC₅₀ than *M. oleifera* (boiled) across most isolates, indicating comparatively stronger antibiofilm activity. However, the combined treatment consistently resulted in reduced MBIC₅₀ values, ranging from 0.6098 to 2.161 mg/mL, compared to their individual extracts.

Particularly, several isolates, including PM3, PM4, PM6, PM9, and PM10, showed markedly lower MBIC₅₀ under the combined treatment, with the lowest value observed for PM3 (0.6098 mg/mL) (Table 4). All treatments exhibited a concentration-dependent increase in biofilm inhibition.

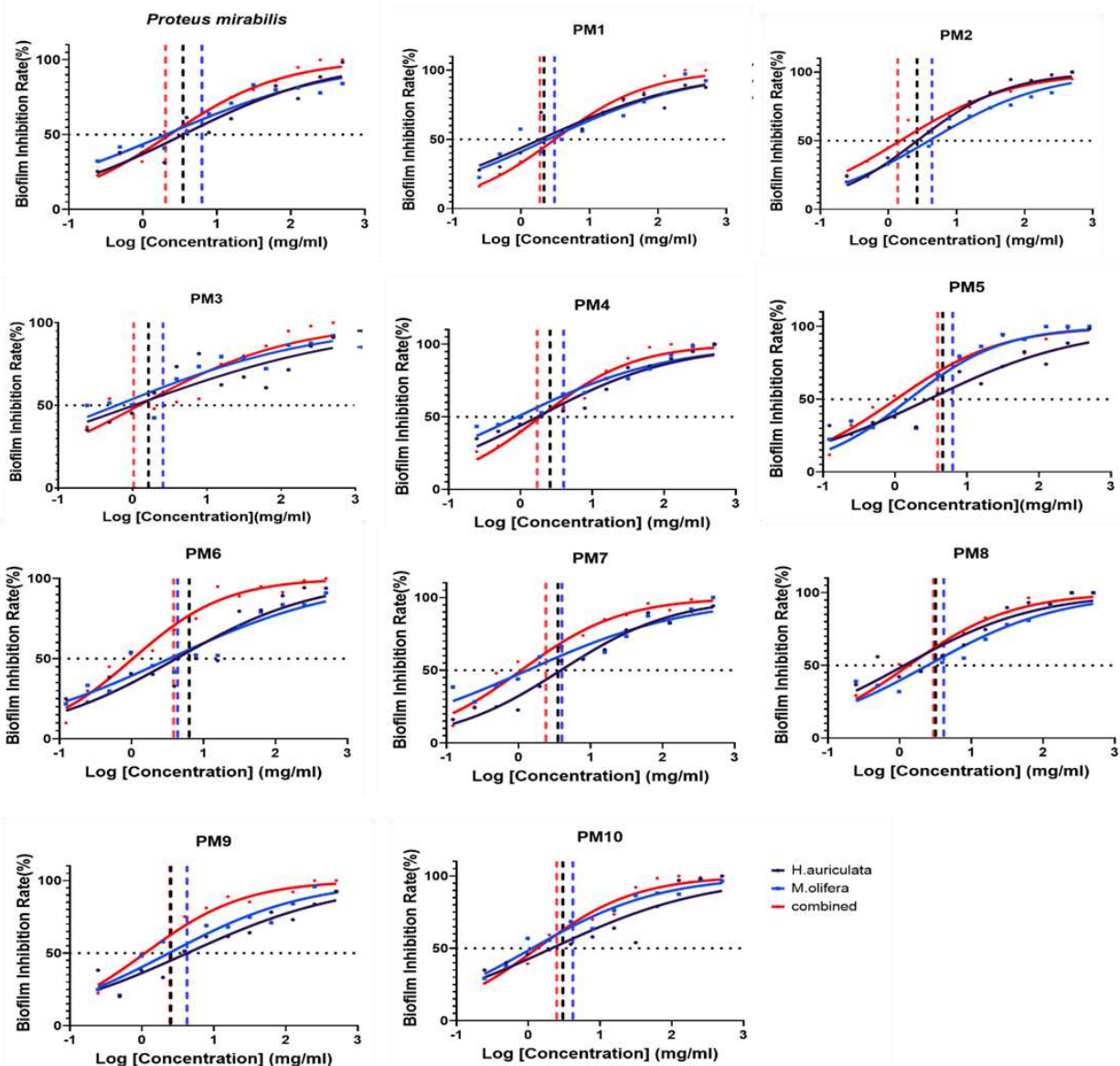


Figure 2: Minimum Biofilm inhibitory Concentration (MBIC₅₀) of leaf extracts of *H. auriculata* (decoction) (black), and *M. oleifera* (boiled) (blue), and their combination (red).

Data points represent the mean $\pm$ standard deviation of triplicate measurements. MBIC₅₀ values for each extract against bacterial strains were calculated using nonlinear regression analysis in GraphPad Prism.

Table 4: MBIC₅₀ and IC₅₀ (mg/mL) of leaf extracts of *H. auriculata* (decoction), *M. oleifera* (boiled), and their combined treatment against *P. mirabilis*

Test Strain	MBIC ₅₀ (mg/mL)			IC ₅₀ (mg/mL)		
	<i>H.</i>	<i>M.</i>	Combination	<i>H.</i>	<i>M.</i>	Combination
	<i>auriculata</i>	<i>oleifera</i>		<i>auriculata</i>	<i>oleifera</i>	
PM QC	2.299	3.498	2.029	1.920	3.315	0.9283
Strain						
PM 1	1.891	3.100	2.161	1.788	4.158	1.296
PM 2	2.636	3.922	1.488	2.07	3.106	1.994
PM 3	1.036	1.229	0.6098	2.614	3.286	0.724
PM 4	1.713	1.879	0.9023	1.493	2.701	1.382
PM 5	1.029	2.921	1.557	1.732	2.547	1.257
PM 6	3.322	4.129	1.036	1.142	2.953	0.929
PM 7	1.082	3.843	1.319	1.097	2.530	1.835
PM 8	1.151	2.429	1.324	0.836	1.792	1.780
PM 9	2.350	4.227	1.096	1.078	2.535	1.560
PM 10	1.318	2.094	1.118	0.970	3.572	1.380

PM: *P. mirabilis*, QC: Quality control, MBIC₅₀: Minimum Biofilm Inhibitory Concentration, IC₅₀: Half Maximal Inhibitory Concentration

3.5 Cytotoxicity of leaf extracts of *H. auriculata* (decoction) and *M. oleifera* (boiled)

A clear dose-dependent embryotoxic trend was observed with *H. auriculata* decoction. At the highest concentrations tested (10000, 5000, and 2500 µg/mL), embryos exhibited pronounced

developmental defects including coagulation, yolk sac edema, skeletal deformities, delayed hatching, lack of tail detachment, and impaired somite formation. At the highest concentration (10000 µg/mL), the most foremost defects were egg coagulation (34%) and yolk sac edema (15%). At 5000 µg/mL, egg coagulation (28%) and yolk sac edema (12%) remained the most frequent abnormalities, while at 2500 µg/mL, yolk sac edema (13%) and egg coagulation (25%) predominated.

At intermediate concentrations (1250 - 78.12 µg/mL), the total number and severity of defects gradually decreased, however minor issues such as coagulation, delayed hatching, yolk sac edema, and incomplete tail detachment were observed. At the lowest concentrations (39.06 - 19.53 µg/mL), severe defects were largely absent, with only occasional yolk sac edema and minor somite formation impairment, reflecting substantially reduced cytotoxicity. Particularly, at 78.12 µg/mL, *H. auriculata* exposure resulted in only 2% of embryos with coagulation, 1% with tail detachment defects, and 5% with yolk sac edema. In contrast, negative control embryos displayed normal development at all time points, confirming that the observed effects were treatment-dependent. The LC₅₀ value of *H. auriculata* extract was 1274.28 µg/mL, representing the highest cytotoxicity among the individual treatments (Figure 3(a)(b)).

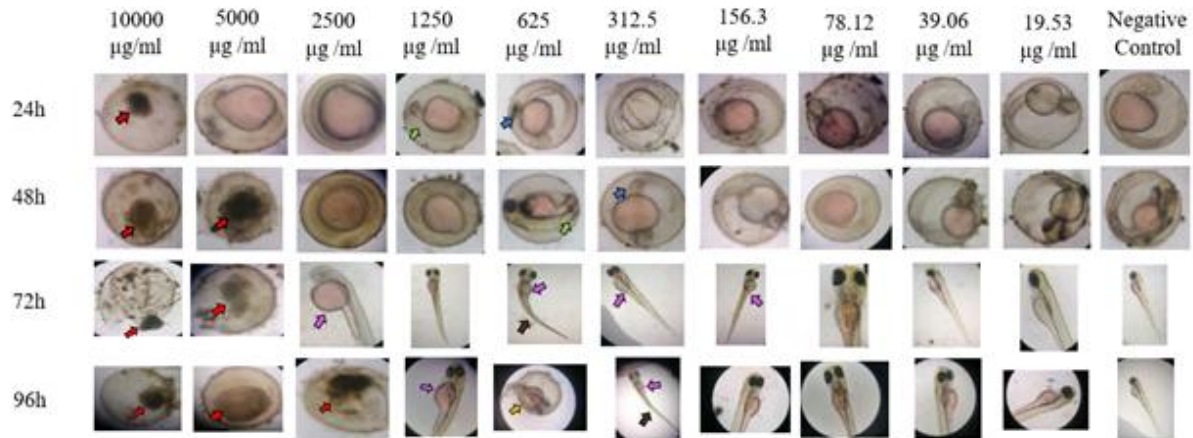


Figure 3(a): Embryotoxic effects of leaf extract of *H. auriculata* (decoction) on zebrafish embryos.

Zebrafish embryos were exposed to varying concentrations (10000-19.53 µg /mL) of leaf extracts of *H. auriculata* (decoction) and monitored at 24, 48, 72 and 96 hours post fertilization. Microscopic images show abnormalities such as coagulation (**red**), edema (**purple**), skeletal deformities (**brown**), delayed hatching (**yellow**), lack of tail detachment (**green**), and impaired somite formation (**blue**) at increasing concentrations.

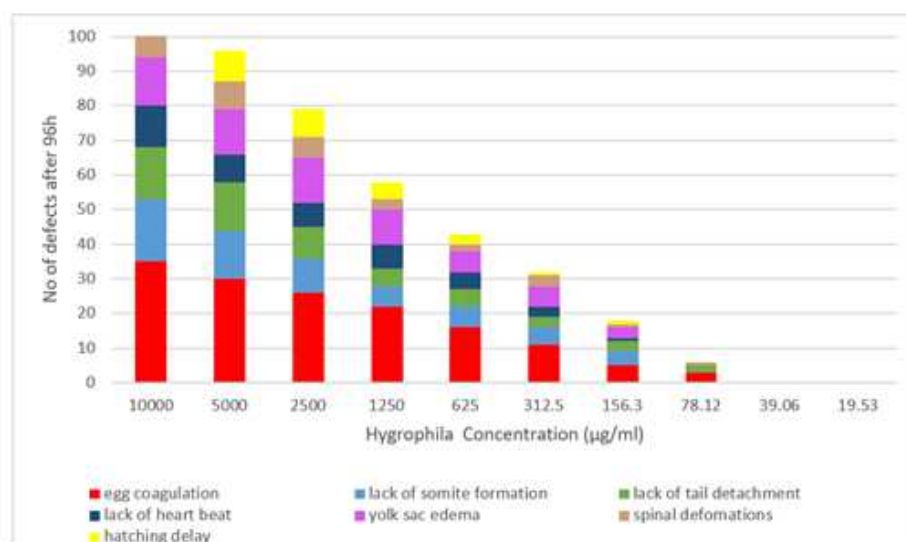


Figure 3(b): Observed apical points and developmental defects in embryos treated with leaf extract of *H. auriculata* (decoction) at 96 hpf

Similarly, embryos exposed to leaf extract of *M. oleifera* (boiled) extract displayed teratogenic abnormalities including apical point formation, delayed development, and abnormal morphogenesis. These effects were most prominent at concentrations $\geq 2500 \mu\text{g/mL}$, where structural deformities and developmental delays were consistently observed. At $10000 \mu\text{g/mL}$, the most pronounced defects were egg coagulation (35%) and lack of somite formation (18%). At $5000 \mu\text{g/mL}$, egg coagulation (30%) and yolk sac edema (13%) were the most prominent, while at $2,500 \mu\text{g/mL}$, egg coagulation (26%) and yolk sac edema (13%) predominated. At intermediate concentrations ($1,250 - 625 \mu\text{g/mL}$), egg coagulation remained the most frequent defect, with minor contributions from yolk sac edema and tail detachment. At lower concentrations ($312.5 - 78.12 \mu\text{g/mL}$), defects were minimal, with egg coagulation being the only noticeable abnormality, and no abnormalities were observed at $39.06 - 19.53 \mu\text{g/mL}$. The LC_{50} value for *M. oleifera* extract was calculated as $1527.5 \mu\text{g/mL}$, indicating a moderate level of cytotoxicity (Figure 4(a)(b)).

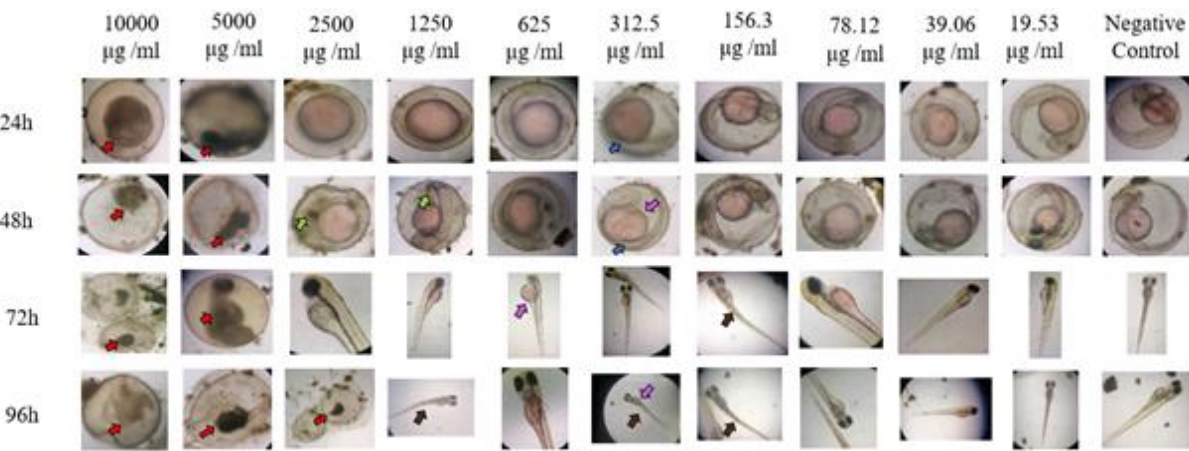


Figure 4(a): Embryotoxic effects of leaf extract of *M. oleifera* (boiled) on zebrafish embryos.

Zebrafish embryos were exposed to varying concentrations (10000 - 19.53 µg/mL) of leaf extract of *M. oleifera* (boiled) and monitored at 24, 48, 72 and 96 hours post fertilization. Microscopic images show abnormalities such as coagulation (red), edema (purple), skeletal deformities (brown), delayed hatching (yellow), lack of tail detachment (green), and impaired somite formation (blue) at increasing concentrations.

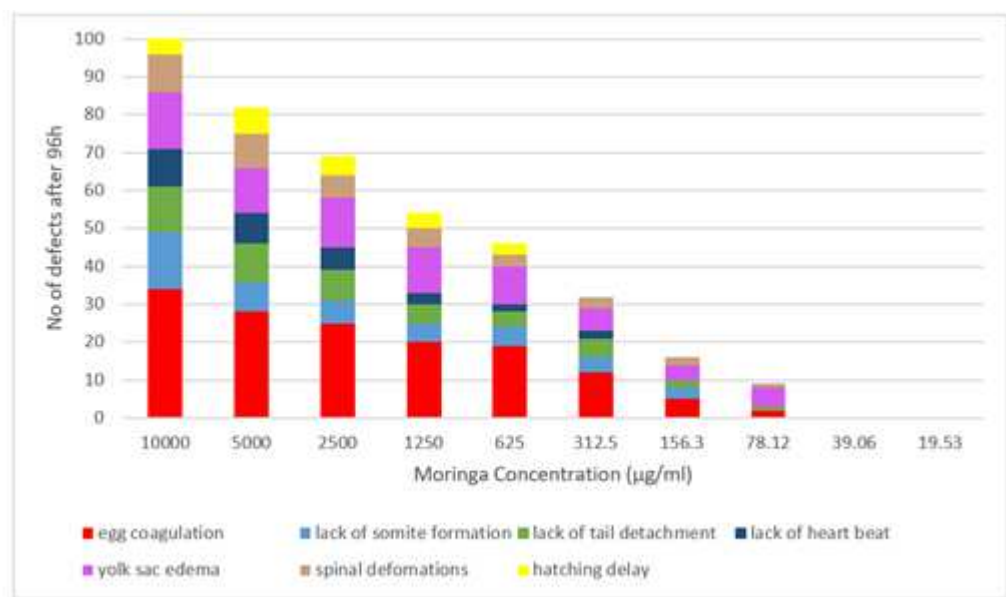


Figure 4(b): Observed apical points and developmental defects in embryos treated leaf extract of *M. oleifera* (boiled) at 96 hpf

When both leaf extracts were applied in combination, zebrafish embryos exhibited a broader range of developmental abnormalities compared to individual treatments. At the highest concentration (10000 µg/mL), the most prominent defects were egg coagulation (40%) and spinal deformations (12%), accompanied by yolk sac edema (14%), lack of somite formation (16%), delayed hatching (10%), tail detachment failure (10%), and absence of heartbeat (10%). At 5000 and 2500 µg/mL, egg coagulation (34 - 30%) and yolk sac edema (10-9%) remained the most frequent abnormalities,

while other defects such as spinal deformities, delayed hatching, and lack of tail detachment were also observed but to a lesser extent.

At intermediate concentrations (1,250 - 625 $\mu\text{g/mL}$), egg coagulation (22 - 18%) and yolk sac edema (9 - 10%) continued to be the predominant defects, with minor contributions from spinal deformities and delayed hatching. Lower concentrations (312.5 - 78.12 $\mu\text{g/mL}$) caused minimal abnormalities, mostly limited to egg coagulation and yolk sac edema, whereas no defects were observed at 39.06 - 19.53 $\mu\text{g/mL}$. Coagulated embryos at higher doses lacked heartbeat and movement, often showing early tail degeneration, indicative of embryonic lethality. The observed reduction in cytotoxicity at lower concentrations suggests a possible interaction or partial synergistic effect between the extracts, although this synergy was insufficient to offset the marked toxicity observed at higher combined concentrations. The combined treatment of *M. oleifera* (boiled) and *H. auriculata* (decoction) showed an LC_{50} value of 1812.74 $\mu\text{g/mL}$, indicating comparatively lower cytotoxicity than the individual extracts (Figure 5(a)(b)).

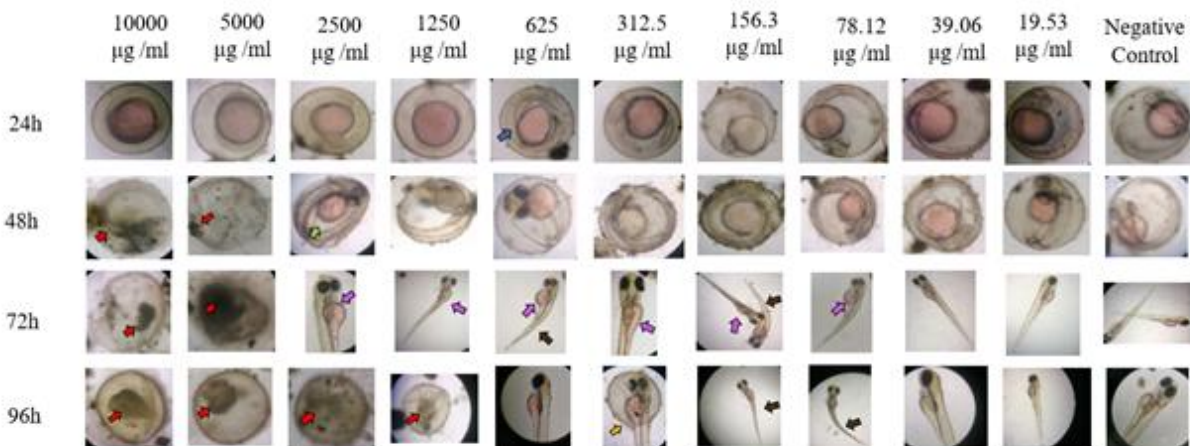


Figure 5(a): Embryotoxic effects of combined leaf extracts of *H. auriculata* (decoction) and *M. oleifera* (boiled) on zebrafish embryos.

Zebrafish embryos were exposed to varying concentrations (10000-19.53 $\mu\text{g}/\text{mL}$) of leaf extracts of *H. auriculata* (decoction) and *M. oleifera* (boiled) and monitored at 24, 48, 72 and 96 hours post fertilization. Microscopic images show abnormalities such as coagulation (**red**), edema (**purple**), skeletal deformities (**brown**), delayed hatching (**yellow**), lack of tail detachment (**green**), and impaired somite formation (**blue**) at increasing concentrations.

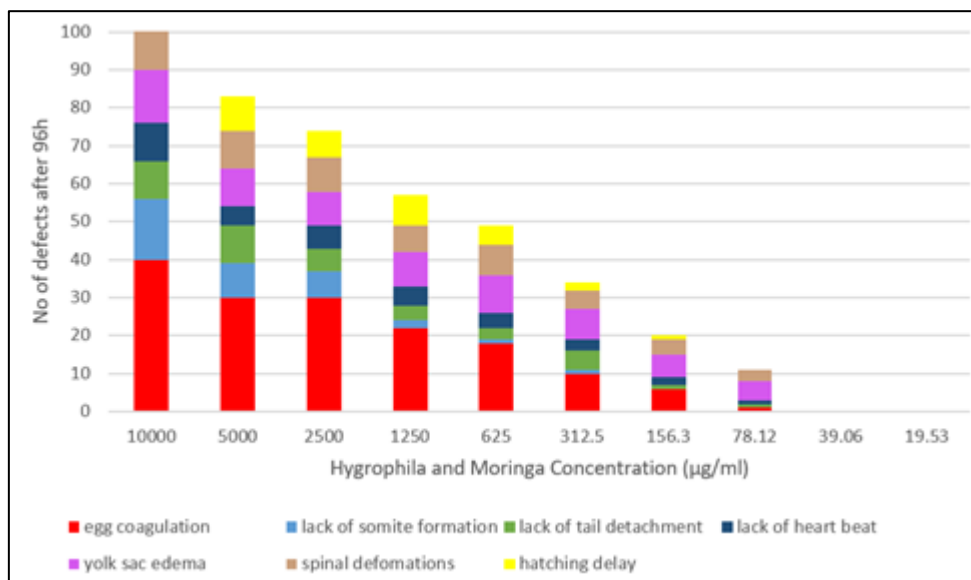


Figure 5(b): Observed apical points and developmental defects in embryos treated with combined leaf extract of *H. auriculata* (decoction) and *M. oleifera* (boiled) at 96 hpf

The LC_{50} values determined from zebrafish embryo toxicity assays were higher than the concentrations required for antibacterial and antibiofilm activity, indicating a wide safety margin. *H. auriculata* (decoction) exhibited an LC_{50} of 1274.28 $\mu\text{g}/\text{mL}$, *M. oleifera* (boiled) 1527.5 $\mu\text{g}/\text{mL}$, and the combined extracts 1812.74 $\mu\text{g}/\text{mL}$. MIC values fell between 0.4883 to 0.9766 mg/mL for *H. auriculata* (decoction) and 0.2441 to 0.9766 mg/mL for *M. oleifera* (boiled), while MBC values ranged from 0.9766 to 1.9531 mg/mL and 0.4883 to 1.9531 mg/mL, respectively. Anti-biofilm activity, expressed as MBIC_{50} , varied from 1.036 to 4.479 mg/mL for individual extracts and

decreased to 0.6098 - 2.161 mg/mL when used in combination. These findings confirm that the antibacterial and antibiofilm effects of *H. auriculata* (decoction) and *M. oleifera* (boiled) leaf extracts are achieved at concentrations well below those causing embryotoxicity.

3.6 Evaluation of the Fractional Inhibitory Concentration Index (FICI) to determine interactions between leaf extracts of *H. auriculata* (decoction) and *M. oleifera* (boiled)

To further validate the combined antibacterial efficacy of *H. auriculata* (decoction) and *M. oleifera* (boiled) extracts, the Fractional Inhibitory Concentration Index (FICI) was determined. It is a quantitative measure used to classify the interaction between antimicrobial agents, identifying whether their combined effect is synergistic, additive or antagonistic.

In this study, *P. mirabilis* strains including PM2, PM3, PM4, PM5, PM6, PM8, PM9, and PM10, demonstrated synergistic interactions, with FICI values ranging from 0.248 to 0.998 (Table 5). This indicates that combining both plant extracts substantially improved their antibacterial performance compared to individual treatments. The observed synergy may be attributed to complementary phytochemicals present in *H. auriculata* (e.g., flavonoids, alkaloids) and *M. oleifera* (e.g., isothiocyanates, phenolics), which could act on multiple bacterial targets or enhance membrane permeability for bioactive compounds (10,23).

Remarkably, *P. mirabilis* clinical isolates PM5 and the QC strain showed the strongest synergistic interaction (FICI = 0.248). Additive effects were observed for isolate PM1 (FICI = 1.000), suggesting that while the extracts maintain their individual antibacterial effects, no additional enhancement occurs upon combination. This may reflect limited complementarity between their active compounds for these specific strains. Only one isolate (PM7) exhibited an antagonistic

interaction (FICI = 1.249). This could result from inhibitory interference between certain bioactive constituents, reduced compound uptake, or competitive inhibition-findings consistent with occasional antagonistic effects reported in plant-plant combination studies using complex extract mixtures.

Table 5: FICI values of *H. auriculata* and *M. oleifera* extracts against *P. mirabilis*

Test Strain	FIC Values		FICI	Effect
	<i>H. auriculata</i>	<i>M. oleifera</i>		
PM QC Strain	0.124	0.124	0.248	Synergistic
PM 1	0.500	0.500	1.000	Additive Effect
PM 2	0.124	0.499	0.623	Synergistic
PM 3	0.249	0.499	0.748	Synergistic
PM 4	0.499	0.499	0.998	Synergistic
PM 5	0.124	0.124	0.248	Synergistic
PM 6	0.249	0.499	0.748	Synergistic
PM 7	0.249	1.000	1.249	Antagonistic
PM 8	0.250	0.499	0.749	Synergistic
PM 9	0.499	0.499	0.998	Synergistic
PM 10	0.249	0.499	0.748	Synergistic

PM: *P. mirabilis*, QC: Quality control, FICI: Fractional Inhibitory Concentration Index

3.7 FTIR Analysis

The FTIR spectrum of the *M. oleifera* (boiled) leaf extract (Figure 6(a)) displayed prominent absorption bands corresponding to functional groups known to influence antimicrobial activity. A

sharp band at 3313.17 cm^{-1} was observed in the far-infrared region, possibly linked to skeletal vibrations of plant metabolites. The strong peak at 2920.93 cm^{-1} corresponded to aliphatic C–H stretching vibrations, suggesting the presence of fatty acids or long-chain hydrocarbons. An intense band at 1734.10 cm^{-1} indicated C=O stretching of ester, aldehyde, or carboxylic functional groups, while the absorption at 1603.10 cm^{-1} was assigned to aromatic C=C or conjugated C=O stretching, characteristic of flavonoids and phenolic compounds. The peak at 1422 cm^{-1} represented C–H bending or O–H bending vibrations, further supporting the presence of phenolic groups. Additionally, the band at 1017.05 cm^{-1} corresponded to C–O stretching of alcohols, ethers, or glycosides, whereas the peak at 655.81 cm^{-1} was attributed to out-of-plane ring bending vibrations of aromatic compounds.

Similarly, the FTIR spectrum of *H. auriculata* (decoction) leaf extract (Figure 6(b)) exhibited absorption peaks consistent with antimicrobial bioactive compounds. A strong and broad band at 3692.79 cm^{-1} corresponded to O–H stretching vibrations, typical of hydroxyl groups in alcohols and polyphenols. Another broad absorption at 3263.56 cm^{-1} was also attributed to hydrogen-bonded O–H groups, confirming polyphenolic content. Peaks at 2911.62 cm^{-1} and 2852.71 cm^{-1} were due to asymmetric and symmetric C–H stretching of aliphatic $-\text{CH}_2$ groups, suggesting the presence of terpenoids or fatty acids. A sharp absorption band at 1724.80 cm^{-1} corresponded to C=O stretching of esters or carboxylic groups, while the band at 1603.10 cm^{-1} indicated aromatic C=C stretching. Additional bands at 1325.25 cm^{-1} and 1601.24 cm^{-1} were attributed to C–N or C–O stretching vibrations, while the band at 790.69 cm^{-1} corresponded to out-of-plane bending of aromatic rings.

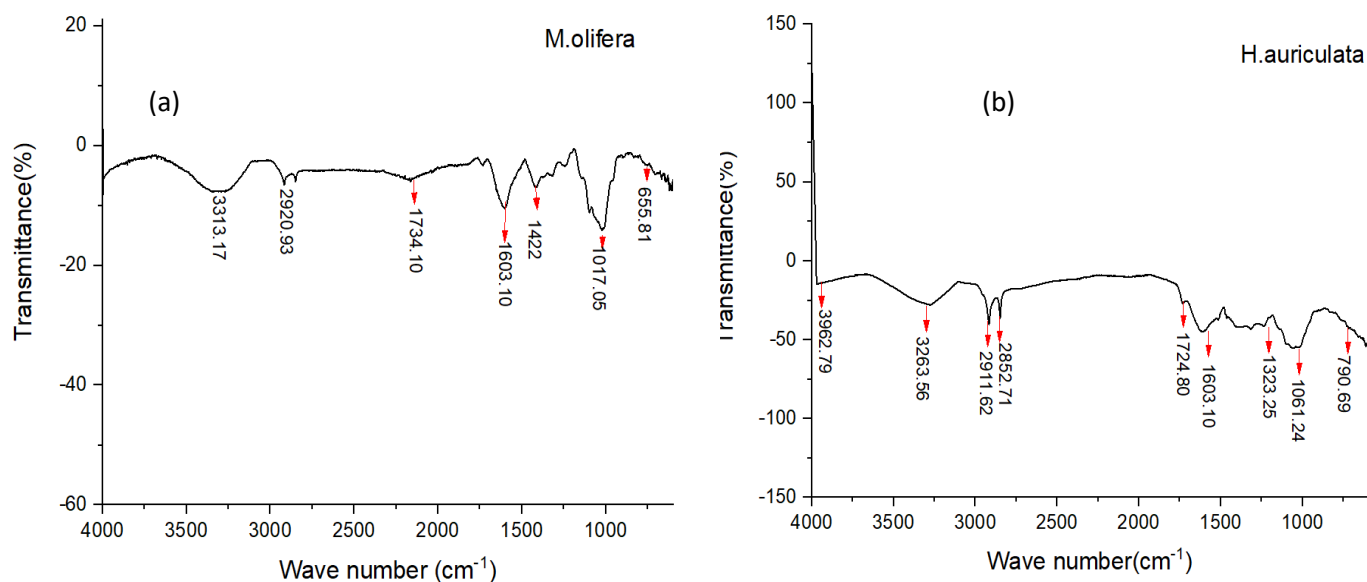


Figure 6: Fourier Transform Infrared spectra of (a) *M. oleifera* (decoction) and (b) *H. auriculata* (boiled)

4. Discussion

Medicinal plants offer a promising source of bioactive compounds with therapeutic potential and generally fewer side effects compared to synthetic antibiotics. In this study, the antibacterial, antibiofilm, and toxicity profiles of aqueous leaf extracts of *M. oleifera* and *H. auriculata*, individually and in combination (boiled and decoction, respectively) were evaluated against biofilm-forming *P. mirabilis* isolates. The findings showed that both leaf extracts possess antimicrobial activity, with enhanced efficacy when used in combination. The choice of solvent and extraction method majorly influences the spectrum and concentration of bioactive compounds, thereby affecting antimicrobial potency (24). Variations in antibacterial activity between extracts also reflect the influence of plant part used, extraction temperature, solvent polarity, and inherent phytochemical diversity (25).

553 Among the extraction methods evaluated, decoction and boiling consistently yielded the strongest
554 antibacterial activity, whereas 50% ethanolic-Soxhlet extracts showed no inhibitory effect. This
555 suggests that the active phytoconstituents responsible for antibacterial activity are predominantly
556 water-soluble and thermally stable. These findings were aligned with previous studies indicating
557 that aqueous extraction methods, such as boiling and decoction, successfully extract polar
558 compounds like tannins and saponins, which disrupt bacterial membrane integrity (Sikkema et al.,
559 1995). Both plant species manifest crucial antibacterial activity against *P. mirabilis*, despite their
560 complex outer membrane composed of lipopolysaccharides (LPS) that serve as permeability
561 barriers (27,28).

562 Plant derived molecules can overcome these barriers through several mechanisms. Flavonoids
563 such as quercetin and rutin disrupt bacterial membranes, increasing permeability and inducing cell
564 lysis by causing structural abnormalities and leakage of intracellular contents (27). Tannins
565 contribute by inactivating enzymes involved in cell wall synthesis or binding to the peptidoglycan
566 layer, making bacteria more susceptible to osmotic lysis and altering membrane fluidity and
567 potential (29,28). These mechanisms are analogous to the action of β -lactam antibiotics, which
568 inhibit penicillin-binding proteins responsible for peptidoglycan cross-linking (30).

569 Hydro-alcoholic solvents are effective in extracting a wide range of plant derived molecules (33).
570 However, recent articles have highlighted that relatively low phytochemical yield from *M. oleifera*
571 leaf extracts using Soxhlet extraction, highlighting variability depending on plant material and
572 extraction conditions (32). In the present study, 50% ethanolic-Soxhlet extracts showed limited
573 antibacterial activity, possibly due to inefficient extraction of highly polar compounds or
574 degradation of heat-sensitive bioactive compounds under prolonged heating (35). Enzyme-assisted

575 extraction (EAE) methods have been proposed to overcome these limitations by breaking down
576 plant cell walls and releasing bound plant compounds, resulting in enhanced yield and bioactivity
577 (34). Furthermore, higher ethanol concentrations ($\geq 70\%$) have been recommended to maximize
578 phenolic extraction (35).

579 Even though both extracts displayed varying levels of activity, *M. oleifera* boiling extract stood
580 out for its productivity, especially against several clinical *P. mirabilis* strains. The lowest MIC
581 observed was 0.2441 mg/mL (e.g., PM2, PM3, PM10), reinforcing its potential as a broad-
582 spectrum antibacterial agent. Similarly, Husnan et al., 2016 (35) reported MIC values for aqueous
583 *M. oleifera* extracts ranging from 0.652 to 5.265 mg/mL against various pathogens, which
584 correspond well with the current discoveries.

585 In contrast, the decoction of *H. auriculata* showed moderate antibacterial activity, with MIC values
586 ranging from 0.4883 to 0.9766 mg/mL. Although less potent than *M. oleifera*, it still disclosed
587 bactericidal activity, indicating the presence of active compounds that warrant further
588 investigation. When examining MBC values, our observations revealed that *M. oleifera* required
589 lower concentrations to achieve bactericidal effects compared to *H. auriculata*. Remarkably, the
590 combined extract of *M. oleifera* (boiled) and *H. auriculata* (decoction) showed even greater
591 potency, achieving an MBC as low as 0.2441 mg/mL and MIC values as low as 0.1220 mg/mL.
592 This evidence is in line with previous studies demonstrating enhanced antibacterial efficacy
593 through synergistic plant combinations, which may work by targeting multiple bacterial pathways
594 or enhancing bioactive compound bioavailability (36). These findings suggest a possible
595 synergistic interaction between secondary metabolites in *M. oleifera* and *H. auriculata*, enhancing
596 their collective efficacy against *P. mirabilis*.

The observed synergy was further supported by the FICI, where most isolates demonstrated synergistic interactions ($\Sigma\text{FICI} < 1$). Synergistic plant-plant combinations are known to enhance antimicrobial efficacy by targeting multiple bacterial pathways simultaneously, increasing membrane permeability, or facilitating intracellular uptake of active compounds (37). Such mechanisms are advantageous against *P. mirabilis*, which is associated with recurrent and catheter-related UTIs.

The IC_{50} values further supported the MIC and MBC results, demonstrating that the combined extract achieved 50% inhibition of bacterial growth at substantially lower concentrations than the individual treatments. Importantly, the requirement of lower effective concentrations indicates that the antimicrobial activity of the combination is achieved at doses that are less likely to exert toxic effects. Lower IC_{50} values are generally associated with greater efficacy at minimal dosages, which is critical for reducing cytotoxicity and enhancing therapeutic applicability (38). In the present study, the IC_{50} values of the combined extracts (0.724-1.994 mg/mL) were markedly lower than those of the individual treatments, indicating enhanced antimicrobial potency while supporting the safety of the products at the corresponding MIC levels.

Biofilm forming *P. mirabilis* poses a remarkable challenge in clinical settings, as biofilms protect bacterial communities from antibiotics and host immune responses, often leading to persistent infections. In this study, *H. auriculata* (decoction) and *M. oleifera* (boiled) leaf extracts conveyed distinct anti-biofilm properties, with MBIC_{50} values extended from 1.036 to 4.479 mg/mL when used individually. However, when applied in combination, the MBIC_{50} values were consistently lower, falling between 0.6098 to 2.161 mg/mL, indicating synergistic interactions that enhanced biofilm inhibition. Furthermore, the MBIC_{50} values observed in the present study are consistent

with previous reports demonstrating that, *M. oleifera* extract had broad-spectrum antimicrobial action against a variety of microorganisms including *Pseudomonas aeruginosa*, *Candida albicans*, and *Staphylococcus aureus* (39). This reinforces the therapeutic potential of *M. oleifera* in combating various pathogens. Given the well-known ability of *P. mirabilis* to form robust crystalline biofilms, the enhanced inhibitory effect of the combined extracts is particularly promising and may involve disruption of quorum sensing or biofilm extracellular matrix components.

Zebrafish embryos, a widely accepted model for developmental toxicity studies due to their transparency, rapid embryogenesis, and genetic similarity to humans (42,43). According to the results obtained, both extracts exhibited dose-dependent embryotoxic effects, with higher concentrations causing pronounced developmental abnormalities. The calculated LC₅₀ values were 1527.5 µg/mL for *M. oleifera* (boiled), 1274.28 µg/mL for *H. auriculata* (decoction) and 1812.74 µg/mL for the combined treatment, indicating that 50% embryo mortality occurred only at relatively high concentrations. In contrast, no observable developmental toxicity was detected at concentrations ≤19.53 µg/mL for the individual extracts and ≤39.06 µg/mL for the combined treatment, confirming a clear margin between toxic and non-toxic doses. The antibacterial activity of the extracts was achieved at much lower concentrations, with MIC values ranging from 0.2441-0.9766 mg/mL for *M. oleifera* (boiled) and 0.4883 - 0.9766 mg/mL for *H. auriculata* (decoction), while corresponding MBC values ranged from 0.4883 - 1.9531 mg/mL and 0.9766 - 1.9531 mg/mL, respectively. Importantly, these MIC and MBC concentrations remain substantially below the LC₅₀ thresholds derived from zebrafish embryos, demonstrating that the antibacterial and antibiofilm effective doses fall within a non-toxic range. Collectively, this comparison supports the selection of concentrations ≤19.53 µg/mL for individual extracts and ≤39.06 µg/mL for the

combined treatment as safe for further *in vitro* and preliminary *in vivo*. These results indicate that, while both plant extracts exhibit embryotoxicity at higher concentrations, the combined formulation appears less toxic, as reflected by its highest LC₅₀ value. This observation is particularly encouraging, suggesting that synergistic interactions may enhance antimicrobial efficacy while modulating toxicity. The strong consistency between MIC, MBC, IC₅₀, and MBIC₅₀ values strengthens the internal validity of the study and confirms that the enhanced activity of the combined extract is consistent across multiple antimicrobial endpoints. Although these experiments were conducted under *in vitro* conditions and evaluated only against *P. mirabilis*, the findings provide initial cytotoxicity and safety insights. Further studies involving a broader spectrum of clinically relevant pathogens and long-term biocompatibility testing in mammalian models will be necessary to confirm therapeutic applicability.

The presence of functional groups, including hydroxyls, carbonyls, aliphatic chains, aromatic rings, and ether linkages, in both extracts is indicative of phytochemicals such as phenolics, flavonoids, terpenoids, and alkaloids. These bioactive compounds are widely recognized for their antimicrobial mechanisms, including disruption of microbial cell membranes, enzyme inhibition, and interference with nucleic acid synthesis (42,43,18). Therefore, the FTIR spectra confirms that both *M. oleifera* (boiled) and *H. auriculata* (decoction) contain a rich diversity of functional groups associated with antimicrobial activity.

5. Conclusion

This study highlights those aqueous leaf extracts of *M. oleifera* (boiled) and *H. auriculata* (decoction), particularly when used in combination, exhibits good antibacterial and antibiofilm activity against *P. mirabilis* at low MIC and MBC values, while remaining non-toxic at effective

concentrations. The FTIR spectra confirmed the presence of bioactive functional groups in both leaf extracts, including phenolic and flavonoid-associated moieties, which likely contribute to their antimicrobial properties. These findings support the therapeutic potential of plant-based treatments as viable alternatives to synthetic antibiotics.

List of abbreviations

MIC - Minimum Inhibitory Concentration

MBC - Minimum Bactericidal Concentration

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

MBIC₅₀ - Minimum Biofilm Inhibitory Concentration

FICI - Fractional Inhibitory Concentration Index

FTIR - Fourier-transform infrared spectroscopy

IC₅₀ - Half Maximal Inhibitory Concentration

Hpf - hours post-fertilization

PM - *P. mirabilis*,

QC - Quality control

LPS - lipopolysaccharides

Declarations

Ethics approval and consent to participate:

The study involved only *in vitro* experiments using clinical bacterial isolates obtained from the culture collection of the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. No experiments were conducted directly on human participants or human tissue. According to national regulations and institutional guidelines, ethical approval was not required for *in vitro* studies using anonymized bacterial isolates.

Clinical trial registration number: Not applicable. (An *in vitro* experimental study. Not a clinical trial)

Consent for publication: Not applicable.

Availability of data and materials: All data supporting the findings are included within the manuscript.

Competing interests: The authors declare that they have no competing interests.

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