

Evaluation of anti-bacterial, larvicidal, and cytotoxic properties of *Moringa oleifera* extracts

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Abstract

The emergence of antibiotic resistance has renewed interest in natural antimicrobial compounds, with *Moringa oleifera* showing particular promise due to its traditional medicinal applications. This study evaluated the anti-bacterial efficacy, phytochemical composition, and cytotoxicity of various *M. oleifera* extracts (seeds, flowers, leaves, and resin) using ethanol, acetone, and methanol as solvents. The anti-bacterial activity was assessed against six bacterial strains using the agar disc diffusion method. The ethanol extract of resin demonstrated exceptional anti-bacterial activity against *Streptococcus mutans*, achieving 76.2% effectiveness compared to the positive control. GC-MS analysis identified hydroquinone (38.46%) as the predominant compound in resin extract and benzeneacetonitrile, 4-hydroxy- (37.35%) in seed extract. Cytotoxicity testing using zebrafish embryos revealed favorable safety profiles with LC50 values of 416 mg for resin extract and 293 mg for seed extract. Additionally, larvicidal and ovicidal assessments showed significant vector control potential, with resin extract demonstrating superior efficacy (LC50 = 451.62 ppm) compared to seed extract (LC50 = 485.99 ppm). The findings suggest that *M. oleifera* extracts, particularly from resin and seeds, possess promising anti-bacterial properties with a favorable efficacy-to-toxicity ratio, warranting further investigation for potential therapeutic applications.

Keywords: *Moringa oleifera*, antimicrobial resistance, phytochemical analysis, cytotoxicity, vector control

Introduction

Moringa oleifera, often referred to as the "miracle tree," is a fast-growing species native to the Indian subcontinent that has garnered significant attention in recent years due to its remarkable nutritional and medicinal properties (Pareek *et al.*, 2023) [22]. Widely cultivated across tropical and subtropical regions, this member of the monogeneric family Moringaceae has been utilized for centuries in traditional medicine systems for its diverse therapeutic benefits (Rani *et al.*, 2018) [28].

The various parts of *M. oleifera*, including leaves, seeds, flowers, and roots, are rich in bioactive compounds such as flavonoids, phenolics, carotenoids, and glucosinolates (Dhakad *et al.*, 2019) [8]. These compounds contribute to its broad spectrum of pharmacological activities, making it a subject of intense scientific interest. In recent decades, the emergence and rapid spread of multidrug-resistant (MDR) bacterial pathogens have posed a significant threat to global public health, challenging the efficacy of conventional antibiotics and necessitating the exploration of alternative therapeutic strategies (Mulani *et al.*, 2019) [20].

This pressing issue has reinvigorated interest in plant-derived antimicrobial compounds as potential sources of novel anti-bacterial agents or adjuvants to existing treatments (Cowan 1999) [7]. Ethnobotanical research indicates that up to 80% of the population in some developing nations rely on herbal medicine for primary healthcare needs (Mohanty *et al.*, 2020) [18], underscoring the importance of investigating traditional medicinal plants such as *M. oleifera* for their potential to address

contemporary health challenges, including antimicrobial resistance (AMR).

Previous studies have demonstrated the antimicrobial potential of various *M. oleifera* extracts against a range of bacterial strains, including both Gram-positive and Gram-negative species (Anzano *et al.*, 2022) [2]. The antimicrobial properties of *M. oleifera* are attributed to its diverse phytochemical constituents, including alkaloids, tannins, saponins, and phenolic compounds (Bagheri *et al.*, 2020) [3]. These secondary metabolites can exert anti-bacterial effects through various mechanisms, such as disruption of bacterial cell membranes, inhibition of essential enzymes, and interference with quorum sensing pathways (Perry *et al.*, 2021) [24].

Despite the advent of modern antibiotics, the rise of AMR has necessitated a renewed focus on natural sources for novel antimicrobial agents or compounds that can potentiate the effects of existing antibiotics (Saha *et al.*, 2022) [29]. While traditional healers have long utilized plants for preventing or treating infectious conditions, the scientific validation of these practices is crucial for developing evidence-based phytotherapeutic approaches.

This study aims to comprehensively evaluate the phytochemical constituents, anti-bacterial activity, and cytotoxicity of *Moringa oleifera* seed, flower, leaf, and resin extracts. By investigating the potential of *M. oleifera* as a source of natural antimicrobial compounds, we seek to contribute to the ongoing efforts to combat antibiotic resistance and develop new strategies for managing bacterial infections.

Materials and methods

Plant collection

Moringa oleifera plant parts (leaves, seeds, flowers, and resin) were meticulously collected during the fruit production season on December 10, 2023. The specimens were sourced from a plant nursery located in KTC Nagar, Tirunelveli, at a latitude of 8.731970 and longitude of 77.774710. The precise geographical coordinates and timing of collection were recorded to ensure reproducibility and account for seasonal variations in phytochemical content.



Fig 1: Habitat of *Moringa oleifera* (Family: Moringaceae)

Drying process

The collected plant parts, including resin, leaves, seeds, and flowers, were dried under controlled conditions to preserve their nutritional and bioactive compounds while extending their shelf life. This process was crucial in maintaining the integrity and potency of the plant materials for subsequent extraction and analysis.

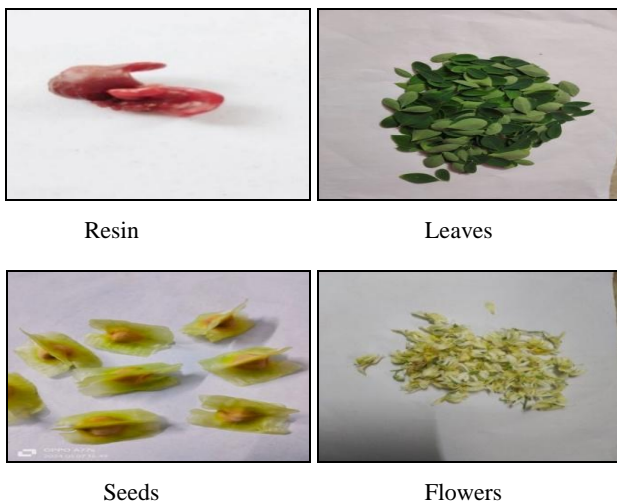


Fig 2

Powder preparation

Mature and healthy leaves were harvested, thoroughly cleaned to eliminate impurities, dried, and ground into a fine powder. Similarly, mature seeds were harvested, cleaned, dried appropriately, and milled into powder form these powders served as the starting material for solvent extraction.

Extraction method

Bioactive compounds were isolated from the powdered plant parts using various solvents, including ethanol, petroleum ether, and ethyl acetate. Each solvent extraction involved soaking the powdered material for a specified

duration to ensure optimal extraction of target phytochemicals. Solvents were chosen based on their polarity and ability to solubilize different classes of phytochemicals (Emmanuel *et al.*, 2014) [12].

Test pathogens

The anti-bacterial activity of *Moringa oleifera* extracts was tested against both gram-negative bacteria (*Enterobacter aerogenes*, *Salmonella typhimurium*, *Escherichia coli*) and gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus mutans*, *Enterococcus faecalis*).

Anti-bacterial activity

The anti-bacterial activity was evaluated using the disc diffusion method. Discs impregnated with the extracts were placed on agar plates inoculated with the test bacteria. The zones of inhibition around the discs were measured to determine the anti-bacterial efficacy of the extracts.

Larvicidal bioassay

Fourth-instar larvae of *Aedes aegypti* (n=30) were exposed to serial concentrations (1-1000 ppm) seed and resin extracts placed in 250 mL beakers with 200 mL of the test solution (WHO, 2005). Experiments were conducted in triplicate under controlled conditions ($27 \pm 2^\circ\text{C}$, $75 \pm 5\%$ RH) with 0.5% DMSO controls. Larval mortality was recorded after 24h exposure, with larvae considered dead upon showing no response to physical stimulus (Ferreira *et al.*, 2009) [13, 14].

Ovicidal bioassay

Fresh *A. aegypti* eggs ($\leq 24\text{h}$ old, n=100/replicate) were exposed to extract concentrations matching the larvicidal bioassay (1-1000 ppm). Egg hatchability was assessed after 72h, and adult emergence was monitored until completion (Pontual *et al.*, 2014) [26]. All treatments were performed in triplicate under identical environmental conditions as the larvicidal assay, with 0.5% DMSO serving as control.

Hatchability and adult formation assessment

Newly laid *Aedes aegypti* eggs (not older than 24 hours, 100 per replicate) were subjected to various concentrations of *M. oleifera* seed and resin extracts, ranging from 1 to 1000 ppm (specifically 1, 10, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ppm) in three replicates. The experiments were performed under controlled conditions of $27 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity, using 0.5% DMSO as a control (Ferreira *et al.*, 2009) [13, 14]. The rate of egg hatching was evaluated 72 hours after treatment, and the subsequent emergence of adult mosquitoes was observed until completion (Coelho *et al.*, 2009) [6]. The outcomes were reported as the percentage of eggs that hatched and the proportion of adults that successfully emerged.

Phytochemical screening

Systematic procedures were employed to identify and quantify bioactive compounds present in *Moringa oleifera*. This screening included tests for alkaloids, flavonoids, tannins, saponins, and other secondary metabolites known for their therapeutic properties (Dubale *et al.*, 2023) [9].

Spectroscopy analysis (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) was used to analyze the chemical composition of the *Moringa oleifera* extracts. This technique allowed for the

identification and quantification of volatile and semi-volatile compounds, providing insights into the bioactive constituents responsible for the anti-bacterial activity (Nabi *et al.*, 2022) ^[21].

Cytotoxicity analysis

The cytotoxicity of *Moringa oleifera* extracts was assessed through various *in vitro* assays to evaluate their impact on cell viability. These assays determined the safety and potential therapeutic window of the extracts, ensuring that their anti-bacterial properties did not come at the expense of cytotoxicity to human cells (Muhammad *et al.*, 2023) ^[19].

Correlation coefficient analysis

The correlation coefficient was used as a statistical measure to evaluate the strength and direction of the linear relationship between the concentration of *Moringa oleifera* extracts and their anti-bacterial activity. This analysis helped in understanding the dose-response relationship and optimizing the effective concentrations of the extracts for maximum anti-bacterial efficacy (Nabi *et al.*, 2022) ^[21].

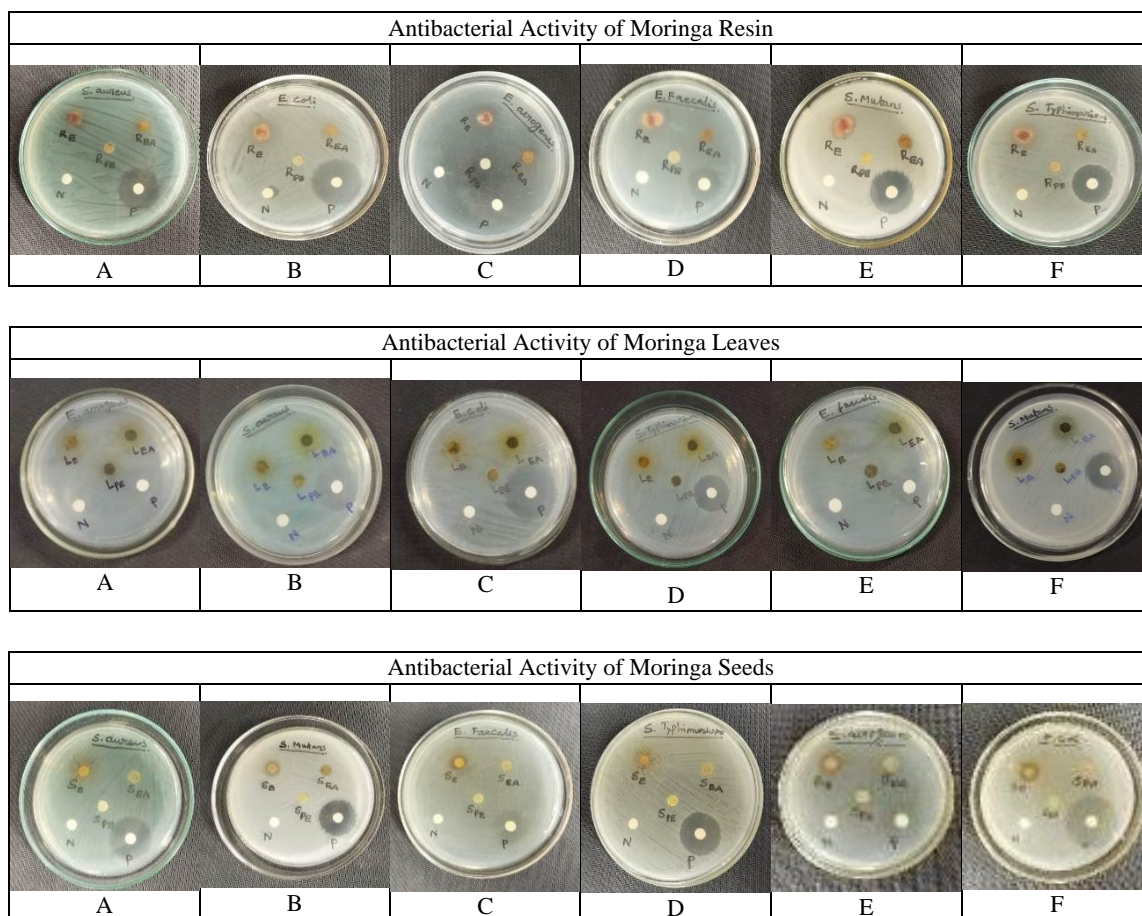
Result and discussion

Antibacterial activity

Our investigation demonstrates the notable anti-bacterial efficacy of *Moringa oleifera* extracts, with particular emphasis on those derived from resin and seeds using ethanol as the extraction solvent. These observations are consistent with prior studies by Gopalakrishnan *et al.* (2016)

^[15], which elucidated the antimicrobial properties of *Moringa oleifera*. Ethanol consistently exhibited superior extraction efficiency for anti-bacterial compounds, corroborating the findings of Abdulkadir *et al.* (2015) ^[1] regarding ethanol's effectiveness in isolating bioactive constituents. The enhanced activity observed in resin and seed extracts aligns with the research of Dzotam *et al.* (2016) on seed extract potency. Of particular note, the resin ethanol extract's efficacy against *Streptococcus mutans* (achieving 76.2% of the positive control's activity) supports the conclusions of Elgamily *et al.* (2016) ^[11] regarding *Moringa*'s potential applications in oral health. The statistically significant interaction between plant part and solvent type ($p < 0.001$) reveals a complex extraction dynamic that has not been extensively explored in previous investigations. Moreover, the broad-spectrum activity of resin extracts against all six bacterial strains tested extends the findings of Peixoto *et al.* (2011) ^[23] with leaf extracts. These results contribute to the growing body of evidence supporting *Moringa oleifera* as a potential source of natural anti-bacterial agents. However, they also underscore the necessity for further research in areas such as compound identification, potential synergistic effects with conventional antibiotics, *in vivo* studies, and the development of standardized extraction protocols. Our findings provide a foundation for the potential development of natural alternatives to address the increasing concern of antibiotic resistance, subject to further research and regulatory approval.

Fig 3: Antibacterial Activity



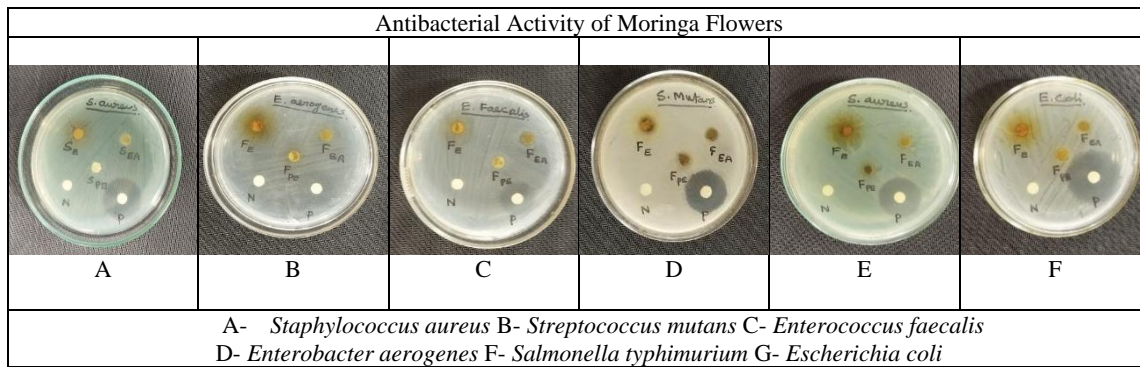


Table 1: Antibacterial activity

Bacteria	Solvent	Resin	Flower	Leaf	Seed
Staphylococcus aureus	Ethanol	8±0.1	9±0.2	8±0	8±0.5
	Ethyl Acetate	7±0	10±1	7±0.2	7±0.2
	Petroleum Ether	7±0.5	0	0	7±0.5
Streptococcus mutans	Ethanol	16 ± 0.7	12±1	15±0.5	14±1
	Ethyl Acetate	9±1	10±0.5	15±1	7±0.2
	Petroleum Ether	7±0.5	7±0	0	7±0
Enterococcus faecalis	Ethanol	14±0	7±0	0	11±0.1
	Ethyl Acetate	7±0.5	7±0	0	7±0.2
	Petroleum Ether	0	0	0	0
Enterobacter aerogenes	Ethanol	12±0.5	7±0	0	12±0.3
	Ethyl Acetate	8±0	7±0	0	0
	Petroleum Ether	0	7±0.5	0	0
Salmonella typhimurium	Ethanol	10±0	8±1	7 ± 0.4	7±0
	Ethyl Acetate	7±0	8±0.5	7 ± 0.2	7±0
	Petroleum Ether	8±0.2	7±0	7 ± 0.4	0
Escherichia coli	Ethanol	10±0.9	7±0.5	7 ± 0.4	13±0
	Ethyl Acetate	0	8±0.5	7 ± 0.2	7±0
	Petroleum Ether	8±0.2	8±0.5	7 ± 0.4	0

Larvicidal and ovicidal activity

The larvicidal and ovicidal bioassays of *Moringa oleifera* extracts demonstrated significant vector control potential, aligning with previous findings on botanical insecticides. The resin extract exhibited superior larvicidal efficacy (LC50 = 451.62 ppm, 95% CI: 426.54-476.36 ppm) compared to the seed extract (LC50 = 485.99 ppm, 95% CI: 449.22-520.7 ppm). (Table 2). This enhanced activity correlates with findings by (Ferreira *et al.*, 2009) [13, 14], who reported similar efficacy ranges for *M. oleifera* seed extracts against *Aedes aegypti* larvae. The statistical analysis confirmed result reliability with chi-square values of 31.36 (df = 16.5, p = 0.597) for resin extract, indicating good model fit.

The superior performance of the resin extract can be attributed to its rich phytochemical composition, particularly the presence of glucosinolates and isothiocyanates, as characterized by (Guevara *et al.*, 1999) [16] in their comprehensive analysis of *M. oleifera* bioactive compounds. These findings are particularly significant when compared to other botanical insecticides, with efficacy comparable to established natural vector control agents

(Coelho *et al.*, 2009) [6]. At higher concentrations, the resin extract maintained consistent efficacy, achieving LC90 and LC95 values of 760.70 ppm and 848.42 ppm respectively. Ovicidal assays revealed a pattern of effectiveness requiring higher concentrations, with the resin extract showing LC50 of 503.80 ppm (95% CI: 473.80-532.60 ppm) compared to the seed extract's 615.90 ppm (95% CI: 592.50-639.30 ppm). This differential response between larvicidal and ovicidal activities suggests distinct mechanisms of action, consistent with observations by (Pontual *et al.*, 2014) [26] regarding the selective activity of plant-derived compounds against different developmental stages of insects. The probit analysis demonstrated robust statistical validity (resin: $\chi^2 = 39.41$, df = 34.0, p = 0.992; seed: $\chi^2 = 38.82$, df = 10.0, p = 0.261). The narrow confidence intervals, particularly in larvicidal assays, indicate high reproducibility of toxic effects, supporting earlier findings on the consistency of *M. oleifera*-based biopesticides (Chumark *et al.*, 2008) [5]. The observed activity profile aligns with documented studies of plant-based vector control agents, highlighting *M. oleifera* promise as a sustainable alternative to synthetic insecticides (Prabhakar *et al.*, 2010).

Table 2: Larvicidal and Ovicidal Activity

Sample	LC50 (ppm)	CI 95% (LC50)	LC90 (ppm)	CI 95% (LC90)	LC95 (ppm)	CI 95% (LC95)	Chi	dF	p
Larvicidal									
Seed	485.99	449.22 - 520.7	869.43	819.97 - 929.92	978.5	918.76 - 1052.12	48.00	21.5	0.056
Resin	451.62	426.54 - 476.36	760.70	725.53 - 802.26	848.42	806.61 - 898.40	31.36	16.5	0.597
Ovicidal									
Seed	615.90	592.50 - 639.30	906.10	871.70 - 946.20	988.39	948.12 - 1035.90	38.82	10.0	0.261
Resin	503.80	473.80 - 532.60	901.50	859.40 - 951.12	1014.00	963.00 - 1074.00	39.41	34.0	0.992

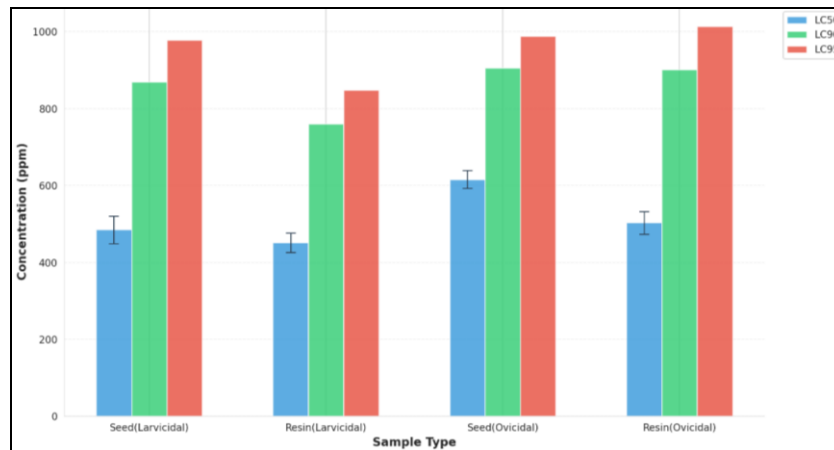


Fig 4: Comparison of LC Values across Sample types

Hatchability and adult forming

The ovicidal efficacy of *M. oleifera* extracts was further evaluated through hatchability and adult formation assessments. At low concentrations (1-10 ppm), both extracts showed minimal inhibition, with hatchability rates above 87% and adult formation exceeding 60%. However, significant dose-dependent effects emerged at higher concentrations, particularly in the range of 100-300 ppm. The seed extract reduced hatchability from 64.3% at 100 ppm to 35.3% at 300 ppm, while the resin extract showed a similar trend (73.3% to 36.6%). This aligns with findings by (Ferreira *et al.*, 2009) [13, 14] regarding the concentration-dependent effects of *M. oleifera* extracts on mosquito development.

A notable observation was the differential impact on adult formation compared to hatchability. At 300 ppm, despite hatchability rates of 35-36% for both extracts, adult formation was drastically reduced to 6.66% (seed) and 0% (resin). Complete inhibition of adult formation occurred at lower concentrations (400-500 ppm) compared to complete hatchability inhibition (1000 ppm), suggesting stronger effects on post-embryonic development. This developmental interference pattern corresponds with mechanisms reported by (Coelho *et al.*, 2009) [6] in their study of *M. oleifera* effects on *Aedes aegypti* development.

The resin extract demonstrated marginally higher efficiency at higher concentrations, achieving complete hatchability inhibition at 800 ppm compared to the seed extract, which maintained 2.23% hatchability at 900 ppm. This enhanced activity likely relates to variations in bioactive compound concentrations between plant tissues, as documented by (Pontual *et al.*, 2014) [26].

Table 3: Hatchability and Adult Forming

Concentration	Hatchability		Adult forming	
	Seed	Resin	Seed	Resin
1 ppm	97.6	95.3	86.6	70
10 ppm	93.3	87.6	70	60
100 ppm	64.3	73.3	36.6	33.3
200 ppm	46.6	47.6	30	6.66
300 ppm	35.3	36.6	6.66	0
400 ppm	36.6	28.9	0	6.66
500 ppm	26.6	17.7	0	0
600 ppm	22.2	15.5	0	3.33
700 ppm	12.2	1.1	0	3.33
800 ppm	3.33	0	0	0
900 ppm	2.23	0	0	0
1000 ppm	0	0	0	0

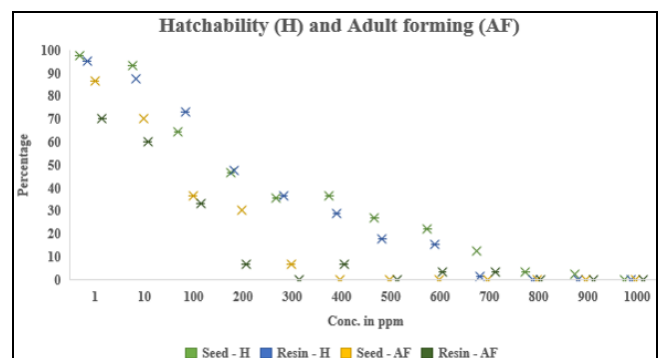


Fig 5: Spectroscopy analysis of Seed and Resin of *Moringa oleifera*

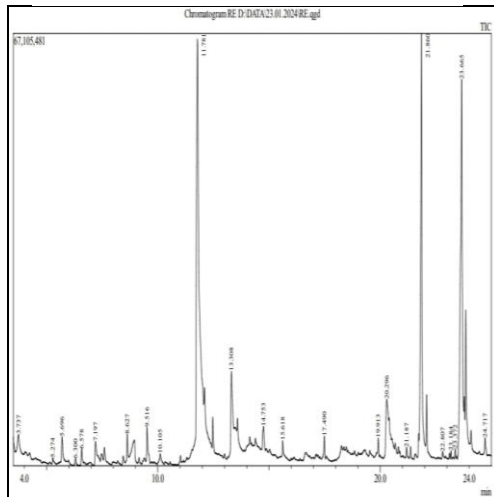
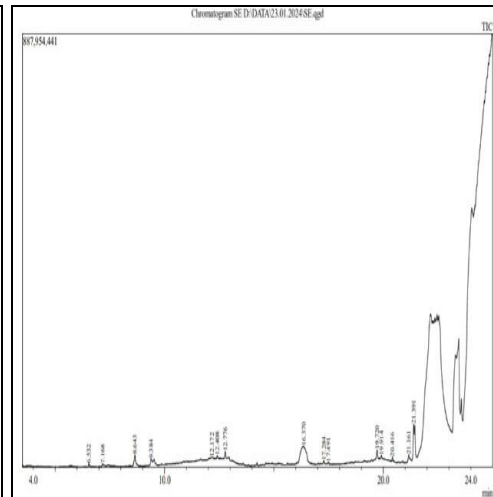
Preliminary Phytochemical Analysis

The phytochemical screening and GC-MS analysis of *Moringa oleifera* resin and seed extracts were studied for their prevalent anti-bacterial activity revealed a diverse array of bioactive compounds, consistent with findings from numerous studies on plant extracts and resins. The resin extract contained steroids, tannins, saponins, quinones, and terpenoids, while the seed extract exhibited flavonoids, quinones, cardiac glycosides, terpenoids, and coumarins. This variation in phytochemical composition between different plant parts aligns with the known diversity in plant tissues, as reported by Saxena *et al.*, (2013) [30]. Both extracts' absence of glycosides, phenols, and amino acids is noteworthy and may contribute to their specific biological activities.

GC-MS analysis

GC-MS analysis of the resin extract identified hydroquinone as the predominant compound (38.46% of total area), along with 9-octadecenoic acid (E)- (24.22%), n-hexadecanoic acid (16.61%), and 2-hydroxy-5-methylisophthalaldehyde (4.97%). The seed extract showed a different profile, with benzeneacetonitrile, 4-hydroxy- (37.35%), triacontanoic acid (26.26%), 2-methyl-3-(methylthio) furan (8.15%), and phenol, 3-methyl- (9.26%) as major compounds. The presence of hydroquinone in significant quantities aligns with findings from other studies on plant resins and extracts, where phenolic compounds often contribute to their bioactive potential (Kaur *et al.*, 2008) [17]. The identification of fatty acids and other diverse compounds is consistent with the chemical composition reported in various plant materials and contributes to the bioactive potential and therapeutic properties of these natural products (Chandana, *et al.*, 2021) [4].

Spectroscopic Analysis

GCMS chromatogram of ethanolic extract of
Moringa oleifera SeedGCMS chromatogram of ethanolic extract of
Moringa oleifera Resin

Cytotoxicity analysis

The cytotoxicity analysis of *Moringa oleifera* extracts using zebrafish embryos revealed dose-dependent effects. The ethanol extract of *Moringa oleifera* seed showed an LC50 value of 293 mg, while the resin extract had a higher LC50 of 416 mg, indicating potentially lower toxicity. These findings, combined with the strong anti-bacterial activity of the resin extract against *Streptococcus mutans* (16±0.7 mm inhibition zone, 76.2% of positive control effectiveness) and the seed extract's efficacy against *S. mutans* (14±1 mm) and *E. coli* (13±0 mm), suggest a favorable efficacy-to-toxicity ratio. The results highlight the potential of these extracts, particularly ethanol-based preparations from resin and seeds, as promising sources for developing new anti-bacterial agents, especially those targeting *S. mutans* infections.

Conclusion

This study demonstrates the significant anti-bacterial potential of *Moringa oleifera* extracts, particularly those derived from resin and seeds using ethanol as a solvent. The resin ethanol extract exhibited notable efficacy against *Streptococcus mutans*, achieving 76.2% of the positive control's effectiveness. GC-MS analysis revealed a diverse phytochemical profile, with hydroquinone, benzeneacetonitrile, and 4-hydroxy as predominant compounds in resin and seed extracts. Cytotoxicity assays using zebrafish embryos indicated a favorable efficacy-to-toxicity ratio. These findings provide a scientific basis for *M. oleifera*'s traditional medicinal applications and suggest its potential as a source of natural anti-bacterial agents. However, further research is required to elucidate mechanisms of action, potential synergistic effects with conventional antibiotics, and *in vivo* efficacy. This study contributes to the ongoing efforts to address antibiotic resistance through plant-based alternatives while emphasizing the need for standardization and clinical trials before therapeutic application.

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