Research Article

Preliminary *in vitro* investigation of *Litsea petiolata* extracts for sustainable control of bacterial leaf blight in rice caused by *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Litsea petiolata, locally called Tham Mung, is widely distributed across tropical and subtropical regions of Asia, and North and South America. This plant is notable for its ecological significance and cultural relevance. Renowned for its rich secondary metabolites, especially phenolic compounds, *L. petiolata* has been studied for its medicinal and antimicrobial properties. This study investigates the phenolic content and antibacterial activity of ethanol and hexane extracts from the leaves and stems of *L. petiolata*, specifically targeting *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the bacterium responsible for bacterial leaf blight in rice. High-performance liquid chromatography identified key phenolic compounds, including vanillic acid in the leaves and 4-hydroxybenzoic acid in the stems. The antibacterial efficacy of these extracts was evaluated using clear zone assays, minimum inhibitory concentration, and minimum bactericidal concentration testing. The results indicated that the ethanol extract from the leaves exhibited the most potent antibacterial activity, significantly inhibiting the growth of *Xoo*. These findings suggest that *L. petiolata* holds considerable potential as a source of natural antibacterial agents, offering an environmentally sustainable alternative to chemical treatments for managing bacterial diseases of rice.

Keywords: *Litsea petiolata,* Bacterial leaf blight disease, Antibacterial activity, Plant extract, Biological control

Introduction

Litsea petiolata, known locally in Thailand as "Tham Mung," is a species of the Lauraceae family valued for its medicinal and culinary uses. In Southern Thailand, the young leaves are traditionally consumed as a vegetable, highlighting their role in both diet and culture [1]. Beyond its dietary role, *L. petiolata* is rich in secondary metabolites, including phenolic compounds known for their antioxidant, antimicrobial, and anti-inflammatory activities [2].

Several species of the *Litsea* genus have demonstrated significant antimicrobial properties. For example, *L. cubeba* essential oils exhibit broad-spectrum antibacterial activity, particularly against Gram-positive pathogens, due to their high terpenoid and phenolic content [3]. Similarly, *L. glutinosa* extracts have been reported to effectively inhibit bacterial growth, with phenolics and flavonoids as major contributors to their bioactivity [4].

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a devastating disease that affects rice crops worldwide, leading to significant yield losses [5]. The development of effective, sustainable management strategies for this pathogen is a critical priority for agriculture. Natural plant-derived compounds, such as phenolics, have emerged as promising eco-friendly alternatives to synthetic antimicrobial agents due to their bioactivity and lower environmental impact [6].

Despite its known bioactivity, the phenolic composition and antibacterial potential of *L. petiolata* against *Xoo* remain unexplored. This study uses solvents with varying polarities to examine the phenolic composition and antibacterial properties of *L. petiolata* extracts obtained from different plant parts (leaves and stems). By identifying key phenolic compounds and assessing their effects on *Xoo* we aim to establish *L. petiolata* as a natural antibacterial agent and contribute to sustainable agricultural disease management solutions.

Materials and methods

Plant material

Fresh leaves and stems of *L. petiolata* were collected from Na Bon District, Nakhon Si Thammarat Province, Thailand (latitude 8°15'58.9"N, longitude 99°32'19.5"E) (Figure 1). The plant materials were separated, thoroughly washed under running tap water, and dried in a hot air oven at a temperature not exceeding 50 °C. The dried materials were ground into a fine powder for further analysis.

Figure 1. *Litsea petiolata* used in this study.

Extraction of samples

Twenty grams of the powdered plant material were macerated in a stoppered 250 mL Erlenmeyer flask containing 100 mL of either absolute ethanol or hexane. The mixture was left at room temperature for 72 h with frequent agitation. The extract was filtered using Whatman No.1 filter paper, and the solvent was removed using a rotary evaporator. Residual solvent traces were eliminated by storing the extract in a vacuum desiccator. The dried extract was weighed to calculate the yield and stored for further experiments.

Determination of phenolic compounds

Preparation of standard solutions and samples

Standard powders of gallic acid, 4-hydroxybenzoic acid, catechin, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, rutin, and quercetin were procured from Sigma-Aldrich (St. Louis, MO, USA). A mixed standard solution was prepared in methanol and diluted to achieve final concentrations ranging from 0.625–10 µg/mL. For the extracts, 20 mg of each dried residue was dissolved in methanol, filtered through a 0.45 µm nylon membrane, and analyzed in triplicate using HPLC. The results were expressed as milligrams per 100 g dry weight (mg/100 g DW).

Chromatographic conditions

HPLC analysis of phenolic compounds was conducted using a Shimadzu HPLC system (Kyoto, Japan) with quaternary pumps (10ATVP), an online degasser (DGU-14A), and a UV-Vis detector (SPD-10AV). Separation was achieved on an Inertsil ODS-3 C18 column (250 mm × 4.6 mm, 5 µm) coupled with a Supelguard™ C18 pre-column. The mobile phase consisted of acetonitrile (solvent A) and 0.5% (v/v) formic acid in water (solvent B). The gradient elution program was started with 5% solvent A (0–2 min), gradually

increased to 15% (2–10 min), held at 15% (10–15 min), then raised to 23% (15–20 min) and maintained (20–30 min). It increased to 60% (30–35 min), held (35–40 min), and returned to 5% (40–50 min) for re-equilibration. The flow rate was set to 1 mL/min, and detection was performed at 280 nm. The column temperature was maintained at 35 °C, and the injection volume was 20 µL.

Determination of antibacterial activity

Bacterial strain

Xoo was obtained from the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand. The culture was maintained on nutrient agar (NA) at 4 °C with periodic sub-culturing. A bacterial suspension was cultured on tryptone soy agar (TSA) at 37 °C for 24 h, standardized to McFarland No. 0.5 (1.5×10^8 CFU/mL) [7], and used as inoculum for all experiments.

Agar well diffusion assay

Mueller Hinton agar (MHA) plates were inoculated with the bacterial suspension. Wells (0.8 mm diameter) were prepared using a sterile cork borer. Each well was filled with 50 µL of plant extract dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) at 100 mg/mL. A 10% (v/v) DMSO solution served as the negative control. Plates were incubated at 37 °C for 24 h, and the inhibition zones were measured in millimeters. The antimicrobial index was calculated using the formula:

$$\text{The antimicrobial index} = (D_b - D_a)/D_a$$

Where D_b is the inhibition zone diameter, and D_a is the well diameter without any antimicrobial agent.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined via broth microdilution with resazurin as an indicator [8]. Serial two-fold dilutions of the extracts (0.05–100 mg/mL) were prepared in 96-well microtiter plates containing MHA. Each well received 100 µL of bacterial suspension. Plates were incubated at 37 °C for 24 h, followed by the addition of 30 µL of 0.01% (w/v) resazurin. The color changed from blue to pink indicated bacterial growth. The MIC is defined as the lowest concentration of the extract at which no color change occurs.

Determination of minimum bactericidal concentration (MBC)

From wells showing no growth during the MIC assay, 10 µL was streaked onto NA plates and incubated at 37 °C for 24 h. The MBC is defined as the lowest concentration of extract that yielded no bacterial colonies.

Statistical analysis

All analyses were conducted in triplicate, and the results are presented as the mean ± standard deviation. The one-way analysis of variance (ANOVA) was performed to assess statistical differences between the means, followed by Tukey's HSD post hoc test. The significance level of $p \leq 0.05$ was considered statistically significant. All statistical analyses were carried out using Minitab 22.1 software (Systat Software, Inc., Chicago, IL, USA).

Results and discussion

Extraction yield

The extraction yield varied depending on both the solvent and the plant part. Ethanol, a polar solvent, produced significantly higher yields than hexane. The highest yield (10.41 ± 0.82%) was obtained from the ethanolic leaf extract, followed by the stem extract (2.19 ± 0.50%). In contrast, hexane yielded 3.60 ± 0.57% from the leaf and 0.88 ± 0.21% from the stem. These results are consistent with previous studies indicating that solvent polarity substantially influences extraction efficiency [9]. Polar solvents such as ethanol generally extract a higher quantity of bioactive compounds than non-polar solvents like hexane, likely due to the predominance of hydrophilic phytochemicals containing electronegative functional groups that enhance solubility in polar solvent [10].

Phenolic composition of *L. petiolata* extracts

The phenolic content of *L. petiolata* extracts varied significantly depending on the plant part and the extraction solvent used (Table 1). The ethanolic extract of leaves exhibited notably higher concentrations of total phenolic compounds compared to hexane extracts. HPLC chromatograms highlighting distinct phenolic profiles for each extract are shown in Figure 2.

Table 1 Phenolic content (mg/100 g DW) in crude extracts of *L. petiolata*

Peak	Phenolic compound	Rt (min)	Leaf		Stem	
			Ethanol	Hexane	Ethanol	Hexane
1	Gallic acid	9.50	ND	ND	ND	ND
2	4-Hydroxybenzoic acid	18.69	ND	ND	15.52 ± 1.95	ND
3	Catechin	19.37	6.07 ± 0.76 [a]	0.04 ± 0.01 [b]	0.24 ± 0.05 [b]	ND
4	Vanillic acid	21.80	153.34 ± 26.20 [a]	0.10 ± 0.02 [b]	ND	ND
5	Caffeic acid	22.28	18.34 ± 2.85 [a]	0.02 ± 0.01 [b]	2.25 ± 0.39 [b]	ND
6	Syringic acid	23.18	16.76 ± 2.64 [a]	ND	2.03 ± 0.35 [b]	ND
7	*p*-Coumaric acid	27.52	ND	ND	ND	ND
8	Ferulic acid	31.07	ND	0.09 ± 0.02 [a]	ND	0.02 ± 0.01 [b]
9	Rutin	33.85	8.67 ± 1.80 [a]	ND	2.18 ± 0.26 [b]	ND
10	Quercetin	38.70	ND	3.85 ± 0.21 [a]	ND	0.65 ± 0.11 [b]

ND= Not detected

Data are the means of three replicates ± standard deviation. Different letters in the same row indicate significant differences (Turkey's test $p \leq 0.05$).

Among the quantified phenolic compounds, vanillic acid was the most abundant in the ethanolic extract of leaves (153.34 ± 26.20 mg/100 g DW), followed by caffeic acid and syringic acid. In contrast, the hexane extracts exhibited lower total phenolic content, consistent with their limited ability to dissolve polar molecules. Notably, hexane leaf extracts contained detectable levels of lipophilic phenolic compounds such

as quercetin (3.85 ± 0.21 mg/100 g DW) and small amounts (≤ 0.1 mg/100 g DW) of catechin, vanillic acid, caffeic acid, and ferulic acid.

Stem extracts also displayed distinct phenolic profiles. Ethanolic stem extracts were dominated by 4-hydroxybenzoic acid (15.52 ± 1.95 mg/100 g DW), while compounds like caffeic acid, syringic acid, and rutin were present in lower concentrations compared to the leaf extracts. For hexane stem extracts, quercetin was the main phenolic compound (0.65 ± 0.11 mg/100 g DW), with ferulic acid detected in trace amounts (< 0.1 mg/100 g DW). In the present study, certain compounds such as gallic acid, and *p*-coumaric acid were not detected in all extracts of *L. petiolata*, which highlights its unique phenolic composition.

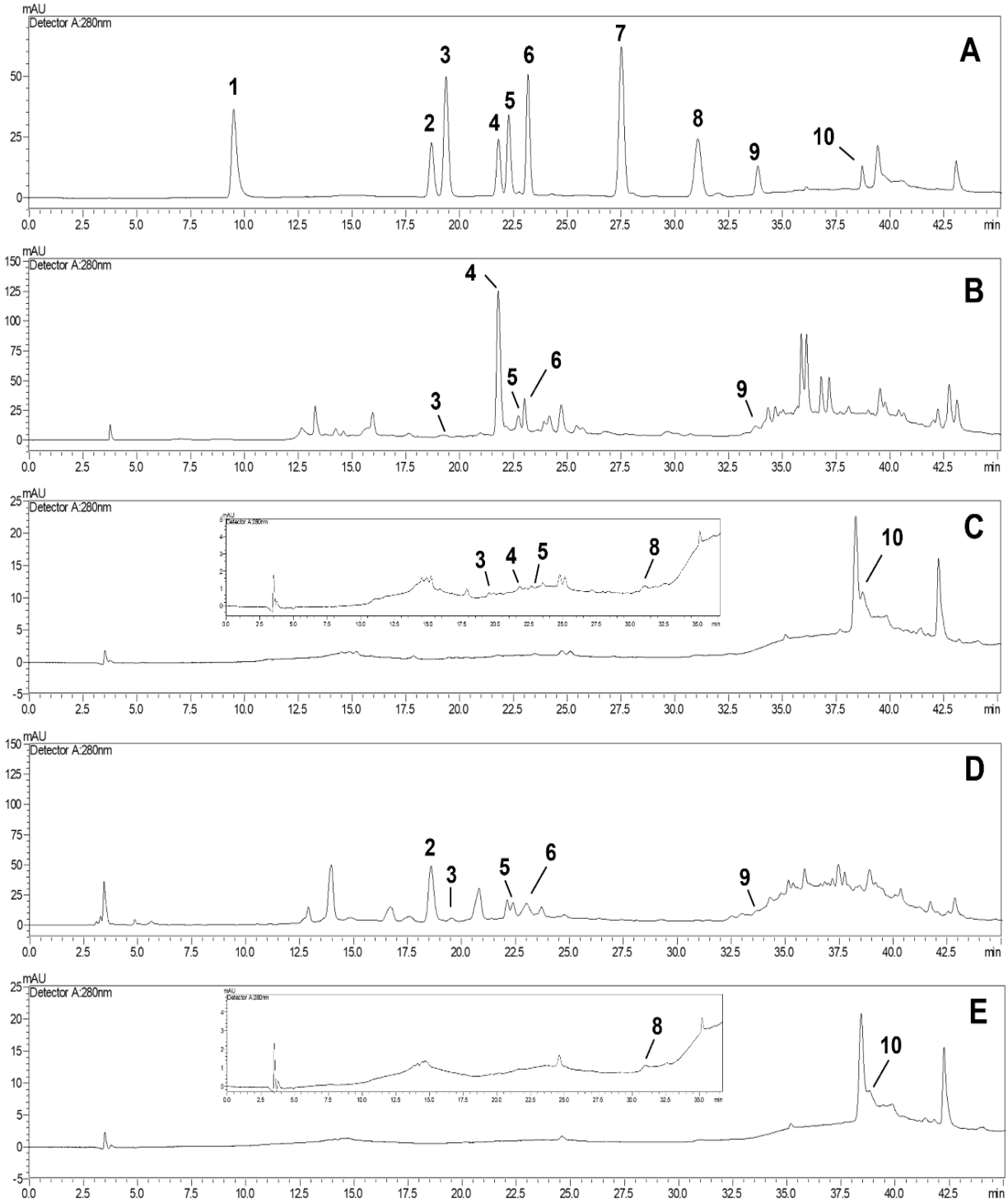

Figure 2. HPLC chromatogram of phenolic compounds detected at 280 nm in *L. petiolata* crude extracts. (A) Mixture of solvent standards (10 µg/mL each), (B) leaves extracted with ethanol, (C) leaves extracted with hexane, (D) stems extracted with ethanol, and (E) stems extracted with hexane. Peaks: (1) gallic acid, (2) 4-hydroxybenzoic acid, (3) catechin, (4) vanillic acid, (5) caffeic acid, (6) syringic acid, (7) *p*-coumaric acid, (8) ferulic acid, (9) rutin, and (10) quercetin.

This study differs from the findings reported by Sangsila et al. [11], who identified the main components in leaf extracts of *L. petiolata* collected from Chanthaburi Province (Eastern Thailand) as (+)-catechin hydrate (555–839 mg/100 g DW), followed by chlorogenic acid (105–169 mg/100 g DW), *p*-coumaric acid (64–105 mg/100 g DW), and gallic acid (29–51 mg/100 g DW). In contrast, this study found vanillic acid to be the major component in ethanolic leaf extracts of *L. petiolata* collected from Nakhon Si Thammarat Province (Southern Thailand). The observed differences in phenolic composition may be attributed to environmental factors, geographical variations, and the timing of sample collection, all of which significantly influence secondary metabolite production. For example, a study on green tea (*Camellia sinensis*) has demonstrated that the growing season highly influences its antioxidant properties and the levels of total phenolics and flavonoids [12].

Our findings align with previous research on other plant species. For instance, studies on bilberry (*Vaccinium myrtillus*) have also revealed that the leaves contain a richer profile of polyphenolic compounds compared to the stems. These compounds, which include flavonoids and phenolic acids, contribute to the plant's antioxidant properties, with the leaves showing the highest concentrations [13].

The difference in the content of phenolic compounds across different plant parts has been reported [14, 15]. Leaves, as the primary site of photosynthesis and secondary metabolite production, often accumulate higher concentrations of phenolic compounds [16]. For instance, phenolics like vanillic acid demonstrate potent antioxidant and antimicrobial properties, highlighting the superior bioactivity potential of ethanolic leaf extracts. These compounds are vital to plant defense, providing protection against UV radiation, herbivory, and microbial attacks [17]. Conversely, stem phenolics, such as 4-hydroxybenzoic acid, are integral to structural integrity. Their role in lignin biosynthesis strengthens the stem, providing resistance to mechanical stress and pathogens, further emphasizing the functional specialization of phenolic distribution in plants [18].

Our study confirms that the type of solvent significantly influences the solubility of phenolic compounds during the extraction process. Solvent polarity plays a critical role in enhancing phenolic solubility. The findings of this study align with the report by Wulandari et al. [19], which revealed that the highest total phenolic content was observed in ethanol extracts of the leaf, bark, and branch of *L. garciae*, ranging from 90–100 µg gallic acid equivalents/mg extract (µg GAE/mg). In contrast, extraction using hexane yielded a lower total phenolic content, ranging between 30–40 µg GAE/mg.

Antibacterial activity against *Xoo*

The antibacterial activity of *L. petiolata* extracts (100 mg crude extract/mL) was assessed using the agar well diffusion method, and the results revealed variation between extracts (Table 2). Ethanolic leaf extracts demonstrated the highest antibacterial activity, with a clear zone diameter of 22.45 ± 0.49 mm and an antimicrobial index of 36.42 ± 0.82. This performance was significantly superior to that of the ethanolic stem extracts (19.75 ± 0.35 mm clear zone diameter), suggesting that the phenolic-rich leaf extracts are more effective in inhibiting the growth of *Xoo* (Figure 3). This is in agreement with the findings of Hai et al. [20], who demonstrated that 70% ethanol extracts from *Clerodendrum fragrans*, *Excoecaria cochinchinensis*, and *Caesalpinia sappan* L. exhibited significant antibacterial effects against *Xoo*, with inhibition zones of 28.50 mm, 21.00 mm, and 25.70 mm, respectively.

Table 2 Efficiency of crude extracts from *L. petiolata* in inhibiting the growth of *Xoo*.

Test	Leaf		Stem	
	Ethanol	**Hexane**	**Ethanol**	**Hexane**
Clear zone diameter (mm)	22.45 ± 0.49 ^{a}	14.38 ± 0.88 ^{bc}	19.75 ± 0.35 ^{ab}	11.50 ± 4.95 ^{c}
Antimicrobial index	36.42 ± 0.82 ^{a}	22.96 ± 1.48 ^{bc}	31.92 ± 0.59 ^{ab}	18.17 ± 8.25 ^{c}

* Extract concentration: 100 mg/mL

** Data are the means of three replicates ± standard deviation. Different letters in the same row indicate significant differences (Turkey's test $p \leq 0.05$).

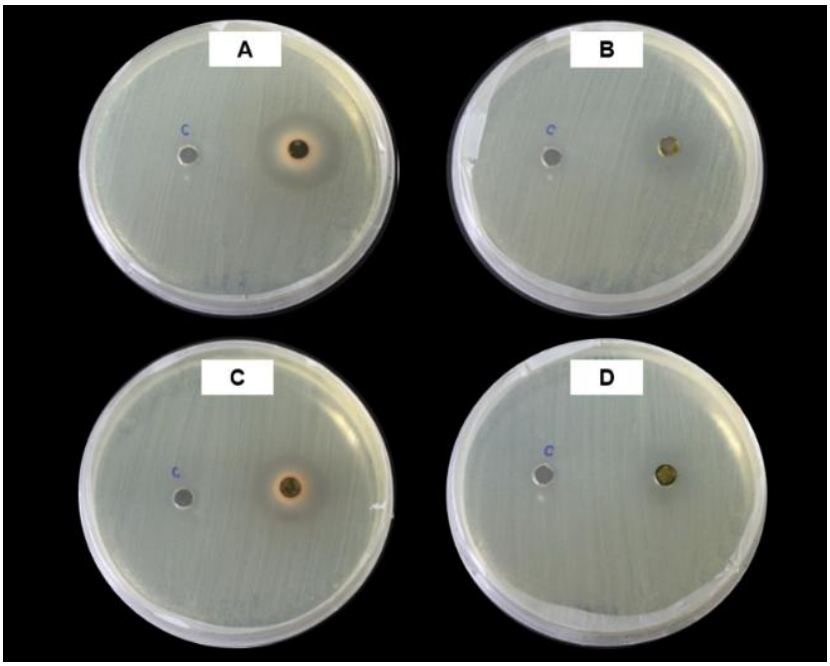

Figure 3. Antibacterial efficiency of crude extracts from *L. petiolata* against *Xoo*; crude extracts from leaves extracted with (A) ethanol and (B) hexane: crude extracts from stems extracted with (C) ethanol and (D) hexane.

In this study, hexane extracts exhibited measurable antibacterial activity despite their lower phenolic content. Hexane leaf extracts showed a clear zone diameter of 14.38 ± 0.88 mm, while the stem extracts demonstrated lower activity with a zone of 11.50 ± 4.95 mm. These findings suggest the presence of non-polar bioactive compounds, such as terpenoids and sterols, which are likely extracted by hexane and contribute to the observed antibacterial effects [21].

The MIC and MBC assays provided further insights into the potency of the extracts (Table 3). Ethanolic leaf extracts exhibited the lowest MIC (937.5 µg/mL) and MBC (1,250 µg/mL), underscoring their strong bacteriostatic and bactericidal effects. Ethanolic stem extracts required higher concentrations for similar effects, with an MIC of 2,500 µg/mL and MBC of 3,750 µg/mL. Hexane extracts consistently exhibited weaker antibacterial activity, with the highest MIC (15,000 µg/mL) and MBC (20,000 µg/mL) observed for the stem-derived hexane extract.

Table 3 Average minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude extracts from *L. petiolata* against *Xoo*.

Test	Leaf		Stem	
	Ethanol	**Hexane**	**Ethanol**	**Hexane**
MIC (µg/mL)	937.5	2,500	2,500	15,000
MBC (µg/mL)	1,250	2,500	3,750	20,000

The phenolic composition of *L. petiolata* extracts, particularly those obtained using ethanol, was found to be rich in compounds such as vanillic acid, caffeic acid, syringic acid, and 4-hydroxybenzoic acid. This profile aligns with reports on other members of the *Litsea* genus, such as *L. cubeba* [22, 23] and *L. glutinosa* [24, 25], both of which have demonstrated strong antimicrobial activities linked to their phenolic content. However, *L. petiolata* stands out due to its distinct phenolic profile and higher concentration of specific compounds like vanillic acid, which might explain its notable antibacterial activity against *Xoo*.

Vanillic acid exerts its antibacterial effects by disrupting bacterial enzymatic systems, impairing the function of vital enzymes and interfering with the overall metabolic activity of the bacteria [26]. Similarly, 4-hydroxybenzoic acid damages bacterial cell membranes, compromising their integrity and leading to leakage of cellular contents, ultimately causing bacterial cell death [27]. The antibacterial mechanisms of other phenolic compounds, such as quercetin, syringic acid, and caffeic acid, typically involve their ability to disrupt bacterial cell structures and inhibit key metabolic processes, such as protein synthesis and DNA replication, thereby preventing bacterial growth and proliferation [28].

The findings from our study suggest that the presence of antibacterial activity in hexane extracts highlights the potential role of non-polar compounds in *L. petiolata*. These compounds may act synergistically with residual phenolic compounds to amplify the antimicrobial effect [29]. The ability of hexane extracts to inhibit bacterial growth suggests that *L. petiolata* contains a broad spectrum of bioactive compounds, both polar and non-polar, that contribute to its antibacterial potential [30]. Ethanolic extracts, being rich in polar

phenolics, demonstrated the strongest antibacterial effects, while hexane extracts provided insights into the contributions of non-polar bioactive compounds. The complementary nature of these extracts highlights the potential of *L. petiolata* as a natural antibacterial agent.

The study faces two primary limitations. Firstly, the scope of sample collection is constrained to a single geographical location and a specific season, potentially failing to represent the variability in phenolic profiles of *L. petiolata* across different regions and climates. Environmental factors, such as soil composition, temperature, and sunlight exposure, are known to influence phytochemical composition, and their variability is not captured in this study. Secondly, unidentified peaks in the HPLC chromatograms highlight challenges associated with the complexity and diversity of phytochemicals in plant extracts. The lack of available reference standards for many compounds restricts comprehensive characterization. To address this, the study recommends using advanced analytical methods, such as HPLC coupled with mass spectrometry (e.g., HPLC-MS or HPLC-MS/MS), to aid in structural elucidation and improve the identification of unknown compounds. This approach would enhance the reliability and scope of phytochemical profiling.

Conclusions

This study demonstrates the potential of *L. petiolata* extracts, particularly ethanolic extracts, as effective antibacterial agents against *Xoo*. The interplay of phenolic and non-phenolic compounds highlights the importance of solvent selection in maximizing bioactivity. The complementary activity of non-phenolic compounds in hexane extracts underscores the need for further research into the full spectrum of bioactive compounds in these extracts. Furthermore, to advance these promising *in vitro* results toward practical application, greenhouse trials are necessary to evaluate the efficacy of *L. petiolata* extracts against *Xoo* under conditions that more closely mimic natural environments. Such studies will help establish optimal application strategies, effective dosage ranges, and assess potential phytotoxicity on host plants. Overall, *L. petiolata* has the potential to serve as a valuable natural resource for the management of plant pathogenic bacteria, providing an environmentally friendly alternative to conventional synthetic antimicrobial agents.

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