

COMPARATIVE PHYTOCHEMICAL PROFILING AND ANTIFUNGAL POTENTIAL OF
TWO ENDEMIC VARIETIES OF *PIPER BETLE* L.

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ABSTRACT

Piper betle L. (family Piperaceae) is a well-known medicinal plant extensively referenced in Ayurvedic literature and recognized with a geographical indication (GI) tag. It exhibits a wide range of pharmacological properties, including antiseptic, analgesic, antibacterial, carminative, and stimulant activities. Numerous cultivars of *P. betle* exist, differing in their phytochemical composition; however, detailed comparative documentation of these variations remains limited. In the present study, two commonly cultivated varieties from Mysuru, namely 'Mysuru veelyadele' and 'Ambadi veelyadele', were selected to comparatively evaluate their phytochemical profiles and antifungal potential against selected phytopathogenic fungi. Preliminary proximate analysis was conducted to quantify carbohydrates, total phenolics, flavonoids, ascorbic acid, and α -tocopherol using standard protocols with both aqueous and solvent leaf extracts. Noticeable variations in phytochemical content were observed between the two varieties. Among the extracts tested, the ethyl acetate fraction exhibited significant biological activity and was therefore subjected to partial purification using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and Liquid Chromatography–Mass Spectrometry (LC-MS). Compounds separated by TLC were further analyzed by HPLC to confirm their purity. Phytochemical characterization revealed the presence of gallic acid, trans-cinnamic acid, caffeic acid, chlorogenic acid, syringic acid, protocatechuic acid, vanillin, p-coumaric acid, catechin, and epicatechin. LC-MS analysis identified a compound with a molecular mass of 290.035 and a retention time of 0.68 minutes in both varieties. Notably, four compounds detected in 'Mysuru veelyadele' were absent in 'Ambadi veelyadele', indicating distinct phytochemical variability between the cultivars.

KEYWORDS: *Piper betle*, Phytochemical analysis, Antifungal activity.

INTRODUCTION

The plant kingdom represents a vast reservoir of natural bioactive compounds that can be effectively utilized in the management of diseases affecting plants, animals, and humans.^[1,2] Consequently, there is a growing global

interest in the exploration and characterization of such phytochemicals, many of which remain largely uninvestigated. India, recognized as one of the world's richest centers of biodiversity, harbors approximately 49,000 plant species, including around 17,500 species of

higher plants. It is ranked among the 12 global mega gene centers and is also identified as one of the Vavilovian centers of origin and diversity of crop plants. Furthermore, India encompasses two of the world's 25 biodiversity hotspots, namely the Indo-Burma region and the Western Ghats–Sri Lanka region. The country accounts for nearly 12% of the world's flora, including about 5,725 endemic species of higher plants distributed across approximately 141 endemic genera and over 47 families. This diversity reflects a rich genetic reservoir comprising both native and introduced species, positioning India as both a primary and secondary center of diversity for several economically important crops, particularly those of South and Southeast Asian origin.^[3]

Despite this immense biodiversity, many plant species remain underexplored for their bioactive constituents. These naturally derived compounds are increasingly recognized as environmentally safe and biologically compatible alternatives for controlling disease-causing organisms. Some plant species are so region-specific that they are accorded Geographical Indication (GI) status, which provides legal recognition and protection of their origin and uniqueness under international frameworks such as the World Trade Organization (WTO).^[4] One such GI-recognized plant is betelvine (*Piper betle* L.), which has been selected for the present investigation.

In this study, two commonly cultivated varieties of betelvine from Mysuru, namely 'Mysuru veelyadele' (GI-tagged) and 'Ambadi yele', were selected for comparative analysis. Phytochemical profiling and antimicrobial activity of both aqueous and solvent leaf extracts were evaluated against selected phytopathogenic fungi to assess their bioactive potential. The findings of this study aim to provide insights into the antimicrobial efficacy of *P. betle* and support its potential application in the development of plant-based formulations for the management of plant diseases. The investigation primarily focused on (i) phytochemical analysis of leaf extracts of *Piper betle* L., and (ii) evaluation of its antimicrobial activity against phytopathogenic fungi.

MATERIALS AND METHODS

Isolation of phytopathogens: Six phytopathogenic fungi were isolated from infected plant samples, including tobacco, paddy, neem, and jackfruit, collected during field surveys conducted in 2015–2016. Diseased plant tissues (1–2 cm in size) were excised and surface sterilized using 70% ethanol to eliminate surface contaminants. The sterilized segments were then aseptically inoculated onto Czapek Dox Agar (CDA) medium and incubated at $25 \pm 2^\circ\text{C}$ for a period of seven days to facilitate fungal growth and isolation.

In addition, four post-harvest pathogens were isolated from infected samples of onion, garlic, orange, groundnut, and sorghum seeds.^[5] These isolates were subsequently maintained under laboratory conditions for further analysis.

Preparation of extracts

Aqueous extract: Two different varieties of *P. betle* L. namely Mysuru veelyadele and Ambadi veelyadele. were selected. The selected leaf samples (100 g) of plants were thoroughly washed, blot dried and macerated with 100 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and centrifuged at 4000 g for 30 min. The supernatant was filtered through Whatmann No.1 filter paper and sterilized at 121°C for 20 min, which served as the mother extract.^[6]

Solvent extract: Two varieties of *Piper betle* L., namely 'Mysuru veelyadele' and 'Ambadi veelyadele', were selected for the study. Fresh leaf samples (100 g) were thoroughly washed with distilled water, blot dried, and homogenized with 100 mL of sterile distilled water using a blender for 10 minutes. The resulting macerate was initially filtered through double-layered muslin cloth and subsequently centrifuged at $4000 \times g$ for 30 minutes. The supernatant obtained was further filtered through Whatman No. 1 filter paper and sterilized at 121°C for 20 minutes. The sterile filtrate thus obtained was designated as the stock (mother) extract.^[6]

Phytochemical analysis of leaf extract: Leaf extracts and solvent fractions obtained using methanol, hexane, acetone, ethyl acetate, chloroform, and water from both varieties of *Piper betle* L. were subjected to quantitative phytochemical analysis. The estimations included total phenolic content, total carbohydrates, flavonoids, α -tocopherol, and ascorbic acid, following standard protocols.^[8]

Estimation of total phenolic compounds: Total phenolic content of the extracts was determined following the method of Kujala with minor modifications, using gallic acid as the standard.^[9] Aliquots of the extracts (0–100 μL) and gallic acid (0–100 μg) were prepared in 0.5 mL of distilled water and mixed with 500 μL of 50% Folin–Ciocalteu reagent. The reaction mixtures were allowed to stand for 10 minutes, after which 1 mL of 20% sodium carbonate (Na_2CO_3) was added.

The mixtures were incubated at ambient temperature for an additional 10 minutes, and the absorbance of the resulting solution was measured at 730 nm using a spectrophotometer. Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of sample powder.

Estimation of total flavonoids content: The aluminium chloride colorimetric method is based on the formation of stable complexes between aluminium chloride and flavonoids. Specifically, aluminium chloride forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl groups of flavones and flavonols. Additionally, it forms acid-labile complexes with ortho-dihydroxyl groups present in the A- or B-ring of

flavonoids. Quercetin is commonly used as a standard for the quantification of flavonoids due to its well-characterized reactivity.

The estimation of total flavonoid content was carried out using a modified aluminium chloride colorimetric method as described by Woisky and Salatino.^[35] A standard calibration curve was prepared using quercetin. Aliquots of plant extracts (0–100 μ L) and quercetin standard solutions (0–100 μ g) were prepared in 80% ethanol. Graded concentrations of these solutions were then mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water.

The reaction mixtures were incubated at room temperature for 30 minutes, after which the absorbance was measured at 415 nm using a spectrophotometer. Appropriate controls were included in the analysis. The total flavonoid content in the extracts was calculated from the quercetin standard calibration curve and expressed as quercetin equivalents.^[10]

Estimation of α -tocopherol: Vitamin E (α -tocopherol) content was determined following the method of Kivcak and Mert^[16], which is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions by tocopherol. The resulting ferrous ions form a red-colored complex with 2,2'-dipyridyl, which can be measured spectrophotometrically.

Briefly, 1 g of each sample extract was initially mixed with 10 mL of hexane and further extracted with 200 mL of hexane under continuous stirring for 24 hours. The extract was filtered through Whatman No. 1 filter paper, and the hexane fraction was concentrated under vacuum to obtain a dry residue, which was stored at -20°C . A known quantity (10 mg) of the dried extract was subsequently dissolved in 10 mL of chloroform.

Aliquots (20–100 μ L) of the prepared extract and standard α -tocopherol solution (20–100 μ L; 10 mg dissolved in 10 mL absolute alcohol) were transferred into separate volumetric flasks, and the volume was adjusted to 3 mL with chloroform. To each flask, 1 mL of 2,2'-dipyridyl reagent (prepared in ethanol and stored at 4°C in a dark bottle) and 1 mL of ferric chloride (FeCl_3) solution (200 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 mL ethanol) were added and mixed thoroughly.

The reaction mixtures were allowed to stand for 15 minutes, after which the absorbance was measured at 520 nm against a reagent blank containing chloroform, dipyridyl reagent, and FeCl_3 solution. The concentration of α -tocopherol in the samples was calculated using a standard calibration curve.^[11]

Estimation of total carbohydrates: Total sugar content of the extracts was estimated using the Dubois method.^[9] Aliquots of the extract and glucose standards (0–100 μ g)

were prepared and the volume was adjusted to 1 mL with distilled water. To each sample, 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid (36 N H_2SO_4) were added.

The reaction resulted in the development of an orange-colored complex, the absorbance of which was measured immediately at 520 nm using a spectrophotometer. The total sugar concentration in the extracts was determined from a standard calibration curve prepared using glucose.^[12]

Estimation of ascorbic acid: Total ascorbic acid content was determined following the method of Das Gupta et al.^[13], using pure ascorbic acid as the standard (0–16 μ g). An aliquot of 50 μ L of each solvent extract was taken, and the volume was adjusted to 1 mL with 5% trichloroacetic acid (TCA).

Subsequently, 1 mL of 2,4-dinitrophenylhydrazine (DNPH) reagent was added to the mixture. The reaction mixture was incubated in a boiling water bath for 10 minutes and then allowed to stand at room temperature for 15 minutes. After incubation, 60% ice-cold sulfuric acid was added carefully to develop the color.

The absorbance of the resulting solution was measured at 540 nm using a spectrophotometer. The total ascorbic acid content of the samples was calculated using a standard calibration curve prepared with known concentrations of ascorbic acid.

Thin layer chromatography (TLC): Thin layer chromatographic (TLC) analysis was performed using silica gel 60G (Merck 7731) coated on glass plates with a thickness of 0.25 mm. Extracts obtained from hexane, chloroform, ethyl acetate, acetone, methanol, and aqueous solvents were applied onto activated analytical TLC plates (20 $\times$ 20 cm, 0.25 mm thickness). The chromatographic separation was carried out using a solvent system comprising hexane, ethyl acetate, acetone, and diethyl ether in the ratio of 50:50:50:20.

Among the various extracts, the ethyl acetate fraction exhibited significant antimicrobial activity and was found to be rich in tannins based on preliminary phytochemical analysis. Therefore, this extract was selected for further purification. The ethyl acetate extract was subjected to preparative TLC using thicker plates (20 $\times$ 20 cm, 2 mm thickness) and separated using an appropriate solvent system, primarily based on hexane.^[14]

HPLC analysis of phenolic compounds: Phenolic compounds were analyzed using High-Performance Liquid Chromatography (HPLC) (Shimadzu) equipped with a CR-4A Chromatopac data integrator, Rheodyne injector, SCL-6A system controller, LC-6A pump, and SPD-6AV UV-Visible detector. Separation was achieved using a reversed-phase C18 column with a gradient

elution system comprising methanol and 0.1% (v/v) acetic acid in HPLC-grade water as the mobile phase. The flow rate was maintained at 0.9 mL min^{-1} , and detection was carried out at 278 nm.

Liquid Chromatography–Mass Spectrometry (LC–MS) analysis was performed using an Agilent 1100 SL Trap model equipped with a thermostated column compartment, diode array detector, and autosampler. The mobile phase composition and gradient conditions were the same as those used for HPLC analysis. The flow rate was set at 0.5 mL min^{-1} , and the sample injection volume was $10 \text{ }\mu\text{L}$. UV–Visible spectra were recorded over a wavelength range of 200–700 nm, and chromatograms were monitored at 280 and 340 nm.

Mass spectrometric analysis was conducted in negative ion mode using an electrospray ionization (ESI) source. The operating parameters were as follows: nebulizer gas (N_2) pressure at 40 psi, drying gas flow rate at 12 L min^{-1} , and drying gas temperature at 350°C . Full-scan mass spectra were acquired over a mass-to-charge (m/z) range of 50–800.^[15]

Antifungal activity assay

Aqueous extract: Antifungal activity of the plant extracts was evaluated using the poisoned food technique.^[16] Czapek Dox Agar (CDA) medium was amended with different concentrations (10%, 25%, 50%, 75%, and 100%) of aqueous extracts from the test plants, namely ‘Mysuru veelyadele’ and ‘Ambadi veelyadele’. Approximately 15 mL of the prepared medium was poured into sterile Petri plates and allowed to solidify. A 5 mm diameter mycelial disc obtained from seven-day-old cultures of phytopathogenic fungi—*Rhizoctonia solani*, *Fusarium oxysporum*, *Rhizopus artocarp*, *Phomopsis azadirachtae*, *Sclerotium rolfsii*, and *Pyricularia oryzae*—as well as post-harvest pathogens including *Aspergillus niger*, *Fusarium moniliforme*, *Penicillium* sp., and *Aspergillus flavus* were aseptically placed at the center of each plate. The inoculated plates were incubated at $25 \pm 2^\circ\text{C}$ for seven days.

Following the incubation period, radial mycelial growth (mm) was measured and recorded for each treatment. Each experiment was conducted in triplicate. Plates containing CDA medium without plant extract served as the control. Antifungal activity was expressed as percentage inhibition of mycelial growth, calculated using the formula:

$$\% \text{ inhibition} = [(dc - dt) / dc] \times 100$$

Where dc represents the average radial growth in the control and dt represents the average radial growth in the treatment.

Solvent extract: Dried solvent extracts of ‘Mysuru veelyadele’ and ‘Ambadi veelyadele’ were used to evaluate antifungal activity. Briefly, 1 g of each dried extract was dissolved separately in 10 mL of the

respective solvents, namely hexane, chloroform, ethyl acetate, acetone, and methanol.

Antifungal activity was assessed using the poisoned food technique.^[17] Czapek Dox Agar (CDA) medium was amended with different concentrations of each solvent extract (100, 200, 300, 400, 500, and 1000 ppm). CDA medium supplemented with 100 μL of the respective solvent without extract served as the control. Approximately 15 mL of the amended medium was poured into sterile Petri plates (90 mm diameter) and allowed to solidify. A 5 mm diameter mycelial disc, taken from the actively growing margin of a seven-day-old fungal culture, was aseptically placed at the center of each plate containing both control and extract-amended media. The plates were incubated at $25 \pm 2^\circ\text{C}$ for seven days. Each treatment was performed in triplicate.

Following incubation, the diameter of fungal colonies and growth characteristics were recorded. Antifungal activity was expressed as the percentage inhibition of mycelial growth relative to the control and was calculated using the method described by Srivastava and Singh.^[18]

RESULTS

Isolation of phytopathogens: Six phytopathogenic fungi, namely *Rhizoctonia solani*, *Fusarium oxysporum*, *Rhizopus artocarp*, *Phomopsis azadirachtae*, *Sclerotium rolfsii*, and *Pyricularia oryzae*, were isolated from infected plant samples. In addition, four post-harvest pathogens—*Aspergillus niger*, *Fusarium moniliforme*, *Penicillium* sp., and *Aspergillus flavus*—were isolated from infected groundnut seeds, onion and garlic, sorghum seeds, orange, and lemon. All the isolated fungal cultures were maintained at 4°C for further studies.

Phytochemical analysis revealed that both ‘Mysuru veelyadele’ and ‘Ambadi veelyadele’ contained high levels of vitamin E, particularly in the hexane extracts. Comparative analysis indicated that ‘Mysuru veelyadele’ exhibited higher concentrations of flavonoids, total carbohydrates, phenolics, ascorbic acid, and α -tocopherol compared to ‘Ambadi veelyadele’. Overall, both varieties were found to be especially rich in flavonoids relative to other phytochemical constituents (Table 1 and Table 2).

Table 1: Proximate Composition of various extracts in Ambadi veelyadele.

Extracts of <i>Piper betle</i> L. (Ambadi veelyadele)	Total carbohydrates (mg/gm)	Total phenolics (mg/gm)	Flavonoids (mg/gm)	Ascorbic acid (mg/gm)	α – tocopherol (mg/gm)
Hexane	-	-	-	-	6.7±0.02
Chloroform	-	-	-	-	8.0±.001
Ethyl acetate	2.7± 0.1	2.1±0.04	4.0±0.01	0.32±0.01	-
Acetone	3.3±0.02	5.1±0.03	6.0±0.03	0.96± 0.01	-
Methanol	4.0±0.04	5.5±0.05	8.0±0.02	0.62±0.03	-
Aqueous	6.0±0.00	2.0±0.05	4.0±0.01	0.32±0.01	-
TOTAL	16	14.7	22	14.7	

- Values are the mean of three replicates
- $\pm$ standard error.
- The means followed by the same letter (S) are not significantly different at $P < 0.05$ when subjected to Tukey's HSD.

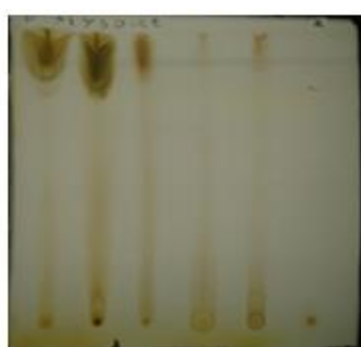
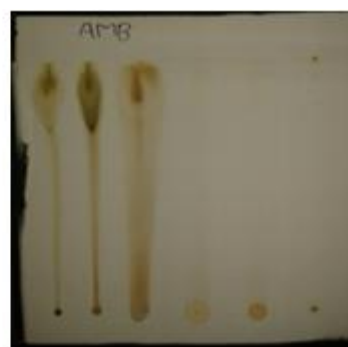
Table 2: Proximate Composition of various extracts in Mysuru veelyadele.

Extracts of <i>Piper betle</i> L. (Mysuru veelyadele)	Total carbohydrates (mg/gm)	Total phenolics (mg/gm)	Flavonoids (mg/gm)	Ascorbic acid (mg/gm)	α -tocopherol (mg/gm)
Hexane	-	-	-	-	0.02± 8.0
Chloroform	-	-	-	-	0.01± 7.7
Ethyl acetate	3.0± 0.01	4.1±0.04	10.1±0.01	0.32±0.01	-
Acetone	8.7±0.02	8.5±0.03	10.0±0.03	0.64±0.01	-
Methanol	4.0±0.04	7.5±0.05	8.0±0.02	1.36±0.02	-
Aqueous	2.0±0.01	2.0±0.05	6.0± 0.01	0.32± 0.01	-
TOTAL	17.7	22.1	32	2.64	15.7

- Values are the mean of three replicates
- $\pm$ standard error.
- The means followed by the same letter (S) are not significantly different at $P < 0.05$ when subjected to Tukey's HSD.

Thin layer chromatography (TLC): Since the ethyl acetate extract exhibited significant antimicrobial activity and was found to be rich in tannins based on preliminary phytochemical analysis, it was selected for further purification. The extract was subjected to preparative Thin Layer Chromatography (TLC) using plates of 20 × 20 cm size and 2 mm thickness. Separation was carried out using a solvent system comprising hexane, ethyl acetate, acetone, and diethyl ether in the ratio of 50:50:50:20. Upon development, three distinct bands were observed and designated as upper, middle, and

lower fractions. Each band was carefully scraped off and transferred into separate beakers. The compounds were eluted using acetone and filtered to obtain clear extracts. These filtrates were subsequently subjected to analytical TLC to assess purity. Analytical TLC revealed a single spot for each fraction, indicating the presence of relatively pure compounds. These purified fractions were further characterized using High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC–MS) for detailed analysis (Figure 1,2 and 3).

**Mysuru veelyadele****Ambadi veelyadele****Figure 1: TLC profile of Mysuru veelyadele and Ambadi veelyadele.**

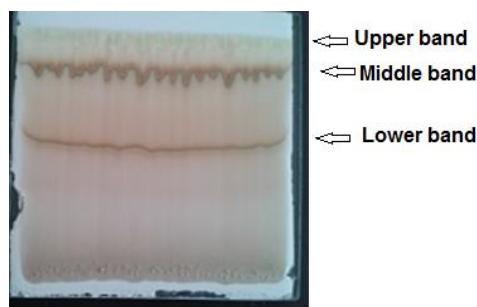


Figure 2: Preparatory TLC of Mysuru veelyadele.



Figure 3: Preparatory TLC of Ambadi veelyadele.

High performance liquid chromatography: The phenolic standards used for the analysis included p-coumaric acid, caffeic acid, syringic acid, chlorogenic

acid, protocatechuic acid, gallic acid, vanillin, trans-cinnamic acid, catechin, and epicatechin.

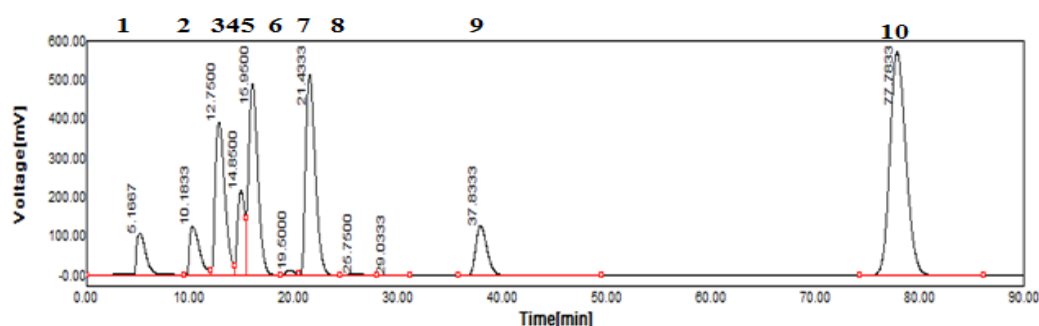


Figure 4: HPLC Chromatogram of standard phenolics.

HPLC of Ambadi veelyadele: HPLC analysis of the fractions enabled the identification of phenolic compounds by comparison with standard references. The lower and middle fractions predominantly showed the presence of gallic acid. In contrast, the upper fraction

exhibited a more complex profile, revealing the presence of multiple phenolic compounds, including gallic acid, trans-cinnamic acid, chlorogenic acid, caffeic acid, syringic acid, and p-coumaric acid (Figure 4).

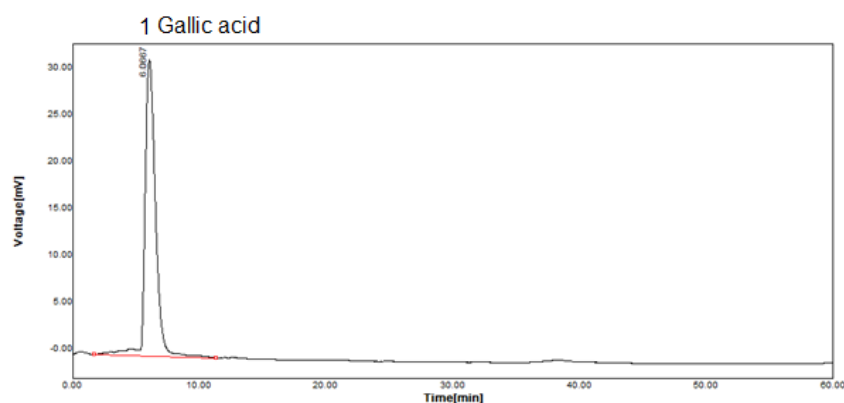


Figure 5: HPLC Profile of Ambadi veelyadele upper layer.

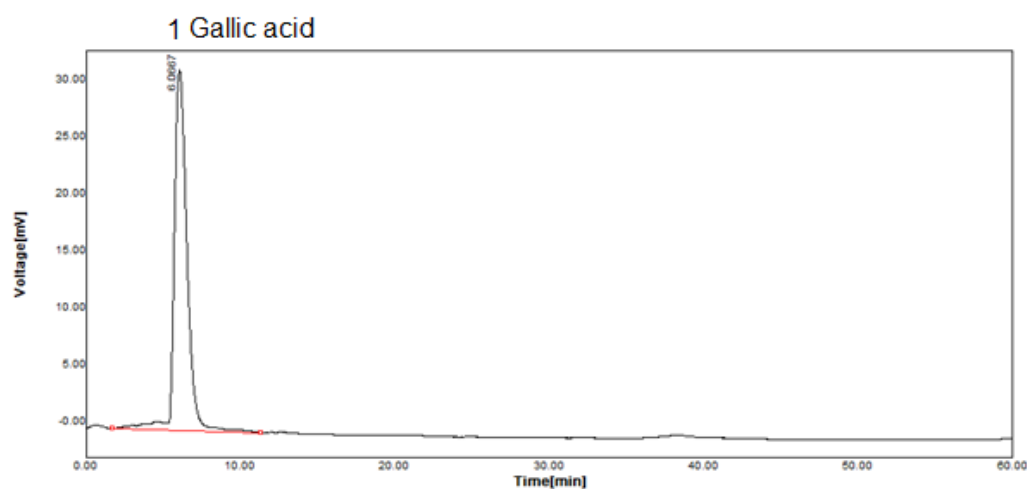


Figure 6: HPLC Profile of Ambadi veelyadele middle layer.

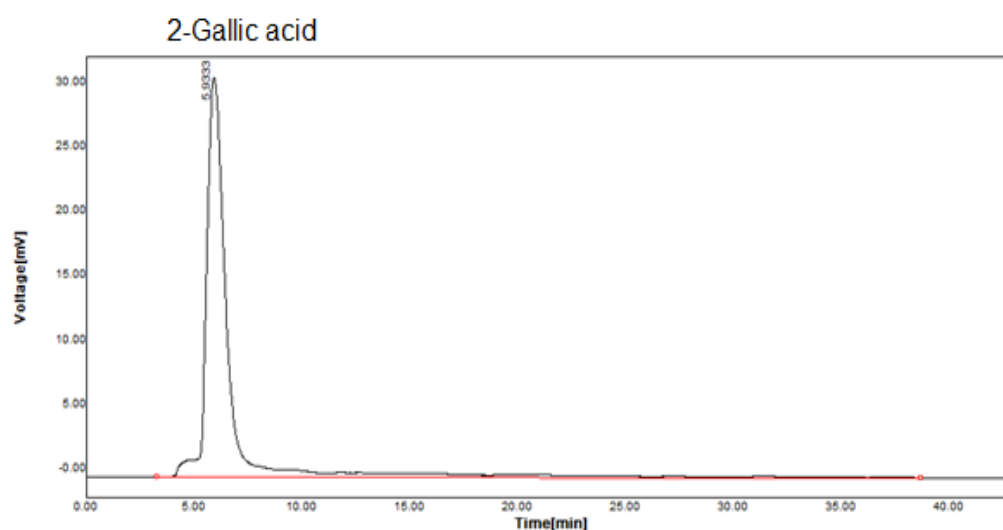


Figure 7: HPLC Profile of Ambadi veelyadele lower layer.

HPLC of Mysuru veelyadele: From the HPLC profile of Mysuru veelyadele following compounds were identified by comparing with the standards. In HPLC profile of upper band which shows the presence of 1-

Gallic acid. HPLC profile of middle and lower band showed the presence of 1-Chlorogenic acid and 1-Transcinnamic acid (Figure 5-10).

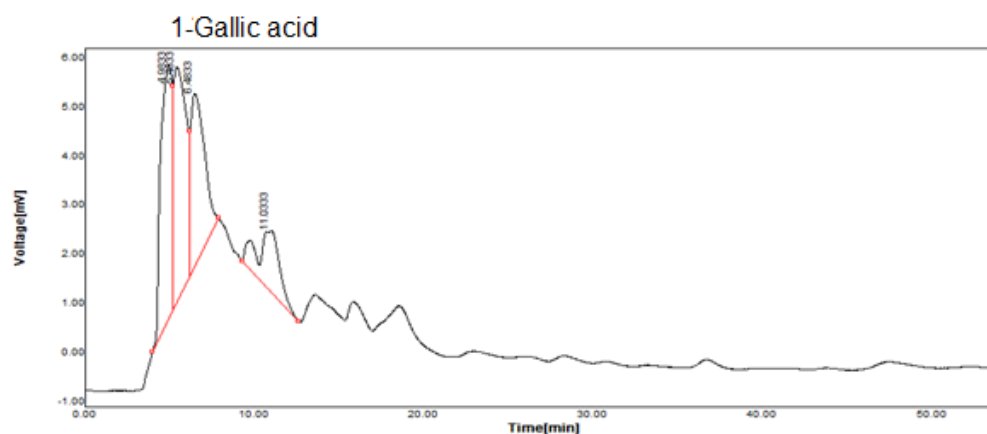


Figure 8: HPLC Chromatogram of Mysuru veelyadele.

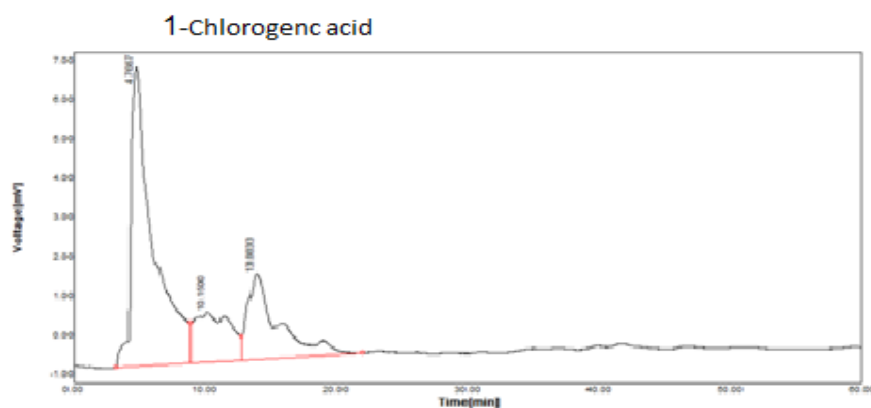


Figure 9: HPLC Profile of Mysuru veelyadele middle layer.

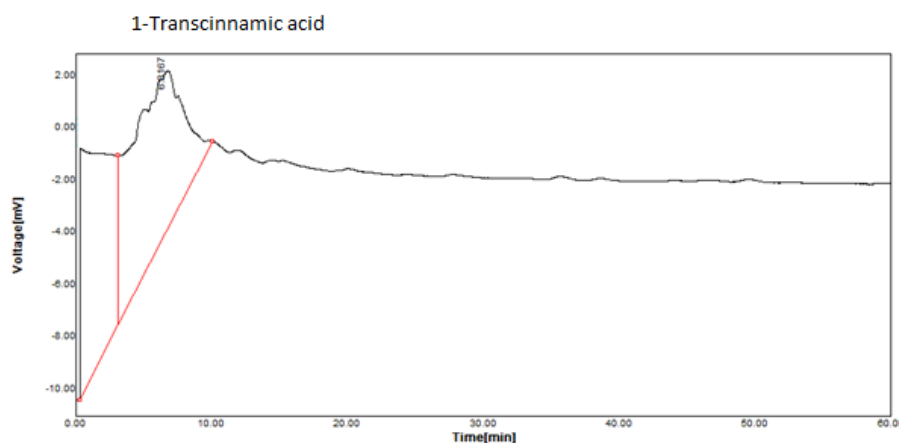


Figure 10: HPLC Profile of Mysuru veelyadele Lower layer.

LCMS: In addition to HPLC, LCMS was also carried out for Methanol extract to characterise the molecules which is present in both the varieties

In the Ambadi veelyadele (Negative mode)

1. Retention time 1.485 molecular mass 371.061
2. Retention time 0.68 molecular mass 290.035

In the Mysuru veelyadele (Negative mode)

1. Retention time 3.141 molecular mass 445.16
2. Retention time 3.534 molecular mass 593.265

3. Retention time 1.536 molecular mass 593.201
4. Retention time 1.436 molecular mass 341.048
5. Retention time 0.68 molecular mass 290.032

These are the abundant molecules present in these samples. One molecule having a molecular mass of 290.035 and retention time 0.68 is common in both Mysuru and ambadi veelyadele. Further characterisation is in progress (Figure 11-12).

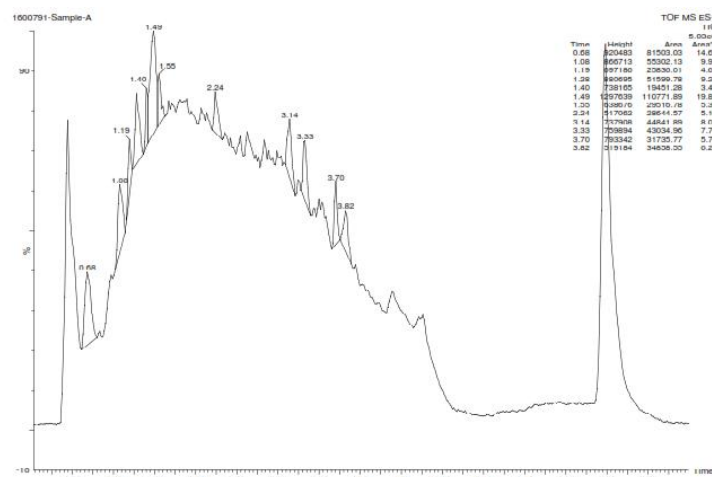


Figure 11: LC MS Profile of methanolic extract of Ambadi veelyadele.

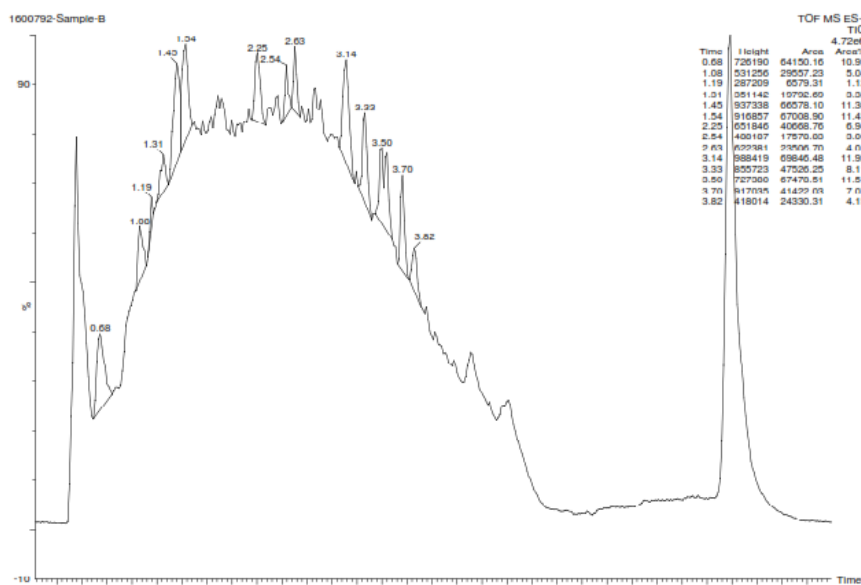


Figure 12: LC MS Profile of methanolic extract of Mysuru veelyadele.

Antifungal activity on phytopathogens

Aqueous extract: Among the two varieties evaluated, the aqueous extract of 'Mysuru veelyadele' exhibited varying degrees of antifungal activity, with notable percentage inhibition observed against *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Phomopsis azadirachtae* at concentrations of 25%, 50%, and 75%.

In comparison, 'Ambadi veelyadele' demonstrated significant antifungal activity against *R. solani* at 25% concentration, while higher concentrations (75% and 100%) were required to achieve notable inhibition against *S. rolfsii* and *P. azadirachtae*.

Other tested phytopathogens exhibited moderate growth even at the highest concentration (100%) of aqueous extracts in both varieties, indicating that complete inhibition was not achieved under the tested conditions.

Table 3: Percentage inhibition (PI) of growth of plant pathogens by aqueous extract of Mysuru veelyadele and Ambadi veelyadele.

Table 5. Percentage inhibition (PI) of growth of plant pathogens by aqueous extract of Mysuru veelyadele and Ambadi veelyadele.											
Sl. No.	Organism	Mysuru veelyadele					Ambadi veelyadele				
		Percentage of mycelia inhibition									
		Concentration range									
		10%	25%	50%	75%	100%	10%	25%	50%	75%	100%
1	<i>Fusarium oxysporum</i>	39±0.01 ^c	44±0.01 ^c	53±0.01 ^c	61±0.04 ^c	61±0.01 ^c	27±0.01 ^b	48±0.01 ^c	50±0.01 ^c	56±0.01 ^c	61±0.04 ^c
2	<i>Rhizopus artocarp</i>	00 ±0.01 ^a	00 ±0.02 ^a	0.0±0.03 ^a	00 ±0.04 ^a	00±0.01 ^a	00±0.01 ^a	00 ±0.04 ^a	00±0.04 ^a	00 ±0.04 ^a	00 ±0.03 ^a
3.	<i>Phomopsis azadirachtae</i>	39 ±0.01 ^c	44±0.02 ^c	47±0.01 ^c	100±0.01 ^d	100±0.03 ^d	56 ±0.01 ^c	47±0.01 ^c	58±0.01 ^c	100 ±0.01 ^d	100 ±0.01 ^d
4	<i>Pyricularia oryzae</i>	33 ±0.01 ^c	39±0.02 ^c	44±0.01 ^c	72±0.01 ^{cd}	72±0.01 ^{cd}	39±0.01 ^c	44±0.01 ^c	62±0.01 ^c	67±0.01 ^c	78±0.01 ^{cd}
5	<i>Rhizoctonia solani</i>	11±0.01 ^b	100±0.01 ^d	100 ±0.01 ^d	100 ±0.04 ^d	100±0.03 ^d	33±0.01 ^b	100±0.03 ^d	100 ±0.01 ^d	100 ±0.04 ^d	100±0.01 ^d
6	<i>Sclerotium rolfsii</i>	0.0±0.01 ^a	11±0.04 ^b	100±0.04 ^d	100±0.01 ^d	100±0.03 ^d	00 ±0.03 ^a	00±0.01 ^a	11±0.03 ^b	61±0.03 ^c	100 ±0.01 ^d
Post harvest pathogens											
7	<i>Fusarium moniliforme</i>	28±0.02 ^b	41±0.01 ^c	44±0.01 ^c	50 ±0.04 ^c	67±0.04 ^c	28±0.01 ^b	44±0.01 ^c	56±0.01 ^c	62 ±0.01 ^c	77±0.01 ^{cd}
8.	<i>Penicillium sp.</i>	00 ±0.02 ^a	39±0.01 ^c	50±0.01 ^c	50±0.04 ^c	67±0.01 ^c	00 ±0.03 ^a	33±0.01 ^c	44±0.01 ^c	50±0.04 ^c	64±0.04 ^c
9.	<i>Aspergillus niger</i>	11± 0.02 ^b	16±0.01 ^b	28±0.01 ^b	28±0.01 ^b	22±0.01 ^b	00 ±0.03 ^a	00±0.01 ^a	13±0.01 ^b	28 ±0.01 ^b	22±0.01 ^b
10.	<i>Aspergillus flavus</i>	00± 0.02 ^a	27±0.03 ^b	20±0.04 ^b	27±0.01 ^b	44±0.03 ^c	00±0.01 ^a	11 ±0.03 ^b	14±0.01 ^b	17 ±0.01 ^b	20±0.01 ^b

- Values are the mean of three replicates
- ± standard error.
- The means followed by the same letter (S) are not significantly different at P<0.05 when subjected to Tukey's HSD.

Solvent extract: Among the solvent extracts evaluated, hexane and ethyl acetate extracts of ‘Mysuru veelyadele’ exhibited significant antifungal activity against *Phomopsis azadirachtae*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Sclerotium rolfsii* at a concentration of 100 ppm. In contrast, extracts of ‘Ambadi veelyadele’ were effective only against *R. solani* and *S. rolfsii* at the same concentration.

The ethyl acetate extract of ‘Mysuru veelyadele’ demonstrated broad-spectrum antifungal activity, effectively inhibiting *P. azadirachtae*, *P. oryzae*, *R. solani*, *S. rolfsii*, *Fusarium oxysporum*, and *Rhizopus artocarp*i at 100 ppm. Among post-harvest pathogens, growth inhibition was observed in *Aspergillus niger* and *A. flavus* at 200 ppm, *Fusarium moniliforme* at 400 ppm, and *Penicillium* sp. at 500 ppm.

Hexane extracts of ‘Mysuru veelyadele’ showed inhibition of *F. oxysporum* at 500 ppm, while post-harvest pathogens such as *A. niger*, *A. flavus*, and *F. moniliforme* were inhibited at concentrations ranging from 200 to 500 ppm, and *Penicillium* sp. at 400 ppm.

Methanol and chloroform extracts of ‘Mysuru veelyadele’ exhibited significant antifungal activity against *P. azadirachtae*, *P. oryzae*, and *R. solani* at 1000 ppm, whereas post-harvest pathogens showed only moderate inhibition at this concentration. No inhibitory

activity was observed with acetone extracts of either variety.

In ‘Ambadi veelyadele’, the hexane extract showed significant inhibition of *R. solani* and *S. rolfsii* at 100 ppm, *P. azadirachtae* and *P. oryzae* at 200 ppm, and *F. oxysporum* at 500 ppm, while *R. artocarp*i remained unaffected. Among post-harvest pathogens, *A. niger* was inhibited at 100 ppm, *Penicillium* sp. and *F. moniliforme* at 500 ppm, and *A. flavus* at 1000 ppm.

The ethyl acetate extract of ‘Ambadi veelyadele’ exhibited notable inhibition of *P. azadirachtae*, *P. oryzae*, *R. solani*, *S. rolfsii*, and *R. artocarp*i at 300 ppm, and *F. oxysporum* at 400 ppm. Post-harvest pathogens such as *Penicillium* sp. and *F. moniliforme* were inhibited at 400 ppm, while *A. niger* and *A. flavus* were inhibited at 200 ppm.

Methanol extracts of ‘Ambadi veelyadele’ showed limited activity, with inhibition observed only against *P. azadirachtae* at 1000 ppm, while other phytopathogens exhibited moderate growth. Chloroform extracts were effective against *P. azadirachtae*, *P. oryzae*, and *R. solani* at 1000 ppm but showed no significant activity against other pathogens. Acetone extracts of both varieties showed no inhibitory activity against any of the tested plant pathogens (Table 4 and 5).

Table 4: Percentage inhibition (PI) of growth of plant pathogens by hexane extract of Mysuru veelyadele and Ambadi veelyadele.

Sl. No.	Organism	Mysuru veelyadele						Ambadi veelyadele					
		Percentage of mycelia inhibition											
		Concentration range											
		100ppm	200ppm	300ppm	400ppm	500ppm	1000ppm	100ppm	200ppm	300ppm	400ppm	500ppm	1000ppm
1	<i>Fusarium oxysporum</i>	23 ±0.01 ^b	33 ±0.01 ^b	45.6 ±0.01 ^b	88 ±0.02 ^{cd}	100 ±0.02 ^d	100 ±0.02 ^d	00 ±0.02 ^a	25 ±0.01 ^b	38 ±0.02 ^b	80 ±0.01 ^{cd}	100 ±0.01 ^d	100 ±0.01 ^d
2	<i>Rhizopus artocarp</i>	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.01 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.02 ^a	00 ±0.01 ^a
3	<i>Phomopsis azadirachtae</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	75 ±0.02	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
4	<i>Pyricularia oryzae</i>	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.01 ^d	68 ±0.01 ^c	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.01 ^d
5	<i>Rhizoctonia solani</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d
6	<i>Sclerotium rolfsii</i>	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d
Postharvest pathogens													
7	<i>Fusarium moniliforme</i>	00 ±0.01 ^a	30 ±0.01 ^b	58.17 ±0.01 ^c	80 ±0.01 ^{cd}	100 ±0.02 ^d	100 ±0.01 ^d	15 ±0.01 ^b	22 ±0.01 ^b	45 ±0.01 ^b	78 ±0.02 ^c	100 ±0.02 ^d	100 ±0.01 ^d
8	<i>Penicillium sp.</i>	43 ±0.02 ^b	74.14 ±0.01 ^c	92 ±0.01 ^{cd}	100 ±0.02 ^d	100 ±0.01 ^d	100 ±0.01 ^d	40 ±0.01 ^b	72 ±0.01 ^c	75 ±0.01 ^c	78 ±0.02 ^c	100 ±0.02 ^d	100 ±0.01 ^d
9	<i>Aspergillus niger</i>	88 ±0.02 ^{cd}	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.02 ^d	100 ^d	22 ±0.01 ^b	44 ±0.01 ^b	94 ±0.01 ^{cd}	100 ±0.02 ^d	100 ±0.02 ^d	100 ±0.02 ^d
10	<i>Aspergillus flavus</i>	86 ±0.02 ^{cd}	86 ±0.02 ^{cd}	89 ±0.02 ^{cd}	90 ±0.02 ^{cd}	100 ±0.02 ^d	100 ±0.01 ^d	33 ±0.01 ^b	36 ±0.01 ^b	44 ±0.01 ^b	68 ±0.02 ^c	82 ±0.02 ^{cd}	100 ±0.02 ^d

- Values are the mean of three replicates
- ± standard error.
- The means followed by the same letter (S) are not significantly different at P<0.05 when subjected to Tukey's HSD.

Table 5: Percentage inhibition (PI) of growth of plant pathogens by ethyl acetate extract of Mysuru veelyadele and Ambadi veelyadele.

Sl. No.	Organisms	Mysuru veelyadele						Ambadi veelyadele					
		Percentage of mycelia inhibition											
		Concentration range											
		100ppm	200ppm	300ppm	400ppm	500ppm	1000ppm	100ppm	200ppm	300ppm	400ppm	500ppm	1000ppm
1	<i>Fusarium oxysporum</i>	100 ±0.0 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.0 ^d	100 ±0.01 ^d	4 ±0.01 ^b	15 ±0.01 ^b	15 ±0.01 ^b	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
2	<i>Phomopsis azadirachtae</i>	100 ±0.01 ^d	10 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	8 ±0.01 ^b	25 ±0.0 ^b	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
3	<i>Rhizopus artocarp</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	00 ±0.01 ^a	00 ±0.01 ^a	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
4	<i>Pyricularia oryzae</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	3 ±0.01 ^b	7 ±0.0 ^a	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
5	<i>Rhizoctonia solani</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	50 ±0.01 ^c	52 ±0.01 ^c	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.0 ^d
6	<i>Sclerotium rolfsii</i>	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	25 ±0.01 ^b	38 ±0.01 ^c	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
Post harvest pathogen													
7	<i>Fusarium moniliforme</i>	18 ±0.01 ^b	26 ±0.01 ^b	48 ±0.01 ^c	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	25 ±0.0 ^b	25 ±0.01 ^b	26 ±0.01 ^b	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
8	<i>Penicillium sp.</i>	31 ±0.01 ^c	41 ±0.01 ^c	82 ±0.01 ^{cd}	90 ±0.01 ^{cd}	100 ±0.01 ^d	100 ±0.01 ^d	22 ±0.01 ^b	34 ±0.01 ^c	44 ±0.01 ^c	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
9	<i>Aspergillus niger</i>	00 ±0.01 ^a	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	22 ±0.01 ^b	44 ±0.01 ^c	78 ±0.01 ^{cd}	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d
10	<i>Aspergillus flavus</i>	00 ±0.01 ^a	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	100 ±0.01 ^d	33 ±0.01 ^b	36 ±0.01 ^c	48 ±0.01 ^c	78 ±0.01 ^{cd}	100 ±0.01 ^d	100 ±0.01 ^d

- Values are the mean of three replicates
- ± standard error.
- The means followed by the same letter (S) are not significantly different at P<0.05 when subjected to Tukey's HSD

DISCUSSION

Biological control has gained considerable importance in modern agriculture as a sustainable alternative to minimize the adverse effects associated with the excessive use of chemical pesticides.^[21] In this context, the present study investigated the phytochemical composition and in vitro antifungal potential of aqueous and solvent extracts of two varieties of *Piper betle* L., namely 'Mysuru veelyadele' and 'Ambadi veelyadele', against selected plant pathogens.

Preliminary phytochemical screening revealed the presence of carbohydrates, proteins, flavonoids, tannins, saponins, terpenoids, steroids, and alkaloids in the aqueous extracts of both varieties. However, carbohydrates, proteins, flavonoids, tannins, and saponins were absent in hexane and chloroform extracts, while these constituents were present in ethyl acetate, acetone, and methanol extracts. In contrast, steroids and terpenoids were detected in hexane and chloroform extracts but were absent in ethyl acetate, acetone, and methanol extracts. These findings are consistent with earlier reports indicating variability in phytochemical composition depending on the solvent used for extraction.^[22,23] Similar observations have been reported by Prakash et al.^[24], who identified tannins, flavonoids, alkaloids, terpenoids, glycosides, and carbohydrates in *P. betle*, while saponins, resins, and proteins were absent. Arani et al.^[25] also reported the presence of tannins, flavonoids, carbohydrates, and proteins in *P. betle* extracts.

Quantitative analysis indicated that both varieties contained high levels of vitamin E, particularly in hexane extracts. Comparative evaluation revealed that 'Mysuru veelyadele' possessed higher concentrations of flavonoids, total carbohydrates, phenolics, ascorbic acid, and α -tocopherol compared to 'Ambadi veelyadele'. Overall, flavonoids were found to be the predominant phytochemical constituents in both varieties, corroborating previous studies on *P. betle*.^[26]

Purification and characterization of bioactive compounds were carried out using preparative and analytical Thin Layer Chromatography (TLC), followed by High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC–MS). HPLC analysis of the ethyl acetate extracts revealed the presence of six phenolic compounds—gallic acid, trans-cinnamic acid, chlorogenic acid, caffeic acid, syringic acid, and p-coumaric acid—in 'Ambadi veelyadele'. In contrast, 'Mysuru veelyadele' showed the presence of three major phenolic compounds, namely gallic acid, chlorogenic acid, and trans-cinnamic acid, with gallic acid being predominant in both varieties. Previous studies have reported hydroxychavicol as a major bioactive compound in *P. betle*.^[27,28] LC–MS analysis further confirmed the presence of several □ □ □ □ □ molecules in both varieties, including a common compound with a molecular mass of 290.035 and a

retention time of 0.68 minutes. Further structural characterization of these compounds is in progress.

The antifungal activity studies demonstrated that the aqueous extract of 'Mysuru veelyadele' exhibited varying degrees of inhibition against *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Phomopsis azadirachtae*, whereas 'Ambadi veelyadele' showed significant activity primarily against *R. solani*. Other phytopathogens exhibited moderate growth in both varieties. Similar antifungal effects of *P. betle* against *R. solani* have been reported by Seema et al.^[29], while limited activity against *Colletotrichum falcatum* has also been documented.^[30]

Among solvent extracts, hexane and ethyl acetate fractions of 'Mysuru veelyadele' showed pronounced antifungal activity against *P. azadirachtae*, *Pyricularia oryzae*, *R. solani*, and *S. rolfsii*. Ethyl acetate extract, in particular, exhibited broad-spectrum activity and effectively inhibited several phytopathogens, including *Fusarium moniliforme* at relatively low concentrations. These findings are in agreement with previous reports demonstrating the antifungal efficacy of ethyl acetate extracts of *P. betle*.^[31,33] In contrast, 'Ambadi veelyadele' extracts were comparatively less effective, showing activity against fewer pathogens. Methanol extracts showed limited activity, while acetone extracts exhibited no antifungal effect in either variety.

The observed antifungal activity of *P. betle* extracts may be attributed to the presence of phenolic compounds, particularly hydroxychavicol and related constituents, which are known to possess strong antimicrobial properties.^[34] The higher efficacy of ethyl acetate extracts may be due to their ability to solubilize and concentrate these phenolic compounds.

Overall, the results of this study confirm that both aqueous and solvent extracts of *P. betle* L. possess significant antifungal properties against a range of phytopathogenic and post-harvest fungi. These findings highlight the potential application of *P. betle* as a natural source of bioactive compounds for the development of eco-friendly biopesticides. Further investigations are required to isolate, identify, and characterize the specific bioactive molecules responsible for antifungal activity and to evaluate their potential for large-scale agricultural applications.^[35]

CONCLUSION

Piper betle L. (Piperaceae) is a tropical, shade-loving, perennial evergreen climber of significant medicinal importance. Its therapeutic use has been well documented in classical Ayurvedic texts such as the *Charaka Samhita*, *Sushruta Samhita*, and *Ashtanga Hridaya*. The plant is known to possess diverse pharmacological properties, including antiseptic, analgesic, antibacterial, carminative, and stimulant activities. It is also recognized with a Geographical Indication (GI) tag. Despite the existence of numerous

cultivars, detailed comparative studies on their phytochemical variability remain limited.

In the present investigation, two commonly cultivated varieties from Mysuru, namely 'Mysuru veelyadele' and 'Ambadi veelyadele', were selected for comparative analysis of their phytochemical composition. Preliminary quantitative analyses of carbohydrates, phenolics, flavonoids, ascorbic acid, and α -tocopherol were carried out using aqueous and various solvent extracts following standard protocols. The results indicated significant variation in the concentration of phytochemicals between the two varieties.

Among the extracts, the ethyl acetate fraction exhibited notable biological activity and was therefore subjected to partial purification using Thin Layer Chromatography (TLC), followed by characterization through High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC–MS). Compounds separated via TLC were further analyzed by HPLC to confirm their purity. The analysis revealed the presence of key phenolic compounds, including gallic acid, trans-cinnamic acid, caffeic acid, chlorogenic acid, syringic acid, and p-coumaric acid. LC–MS analysis identified a compound with a molecular mass of 290.035 and a retention time of 0.68 minutes, which was common to both varieties. Notably, four compounds detected in 'Mysuru veelyadele' were absent in 'Ambadi veelyadele'. Further studies aimed at detailed characterization of these bioactive compounds are currently in progress.

The *in vitro* antifungal activity of aqueous and solvent leaf extracts of both varieties was evaluated against phytopathogenic fungi, including *Fusarium oxysporum*, *Rhizopus artocarp*i, *Phomopsis azadirachtae*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Sclerotium rolfsii*, as well as post-harvest pathogens such as *Fusarium moniliforme*, *Penicillium digitatum*, *Aspergillus niger*, and *Aspergillus flavus*, using the poisoned food technique. The aqueous extracts of both varieties showed significant inhibitory activity against *R. solani*, *P. azadirachtae*, and *S. rolfsii*, while exhibiting comparatively lower activity against other pathogens.

Among the solvent extracts, hexane and ethyl acetate fractions of 'Mysuru veelyadele' demonstrated significant antifungal activity against *P. azadirachtae*, *P. oryzae*, *R. solani*, and *S. rolfsii* at 100 ppm. In contrast, 'Ambadi veelyadele' showed effectiveness primarily against *R. solani* and *S. rolfsii* at the same concentration. Most of the remaining pathogens were inhibited at higher concentrations (up to 500 ppm), except *R. artocarp*i, which showed resistance. Other solvent extracts exhibited varying degrees of antifungal activity against the tested pathogens.

Overall, the study confirms the presence of diverse bioactive compounds in both varieties of *P. betle* L. and

demonstrates their significant antifungal potential against phytopathogenic fungi. These findings highlight the potential of *P. betle* as a source of natural antimicrobial agents for the development of eco-friendly, cost-effective plant disease management strategies. The results provide a scientific basis for further characterization of bioactive compounds and support the use of betel leaf as a valuable herbal resource in sustainable agriculture.

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