# JSS MAHAVIDYAPEETHA

**JSS COLLEGE OF ARTS, COMMERCE AND SCIENCE**
(An Autonomous College of University of Mysore; Re-accredited by Naac with 'A' Grade)
**B.N.ROAD, MYSURU-25**

**UGC-MINOR RESEARCH PROJECT**

# "Elucidation of Phytochemical and Antimicrobial characteristics of Betel vine (*Piper betle* L.)"

**Submitted to**

**UNIVERSITY GRANTS COMMISSION**
**South Western Regional Office**
**Prasana Kumar Block, Palace Road**
**Bangalore - 560 009**
**Karnataka**


**Submitted by**
**Dr. M. Seema**
Department of Microbiology(UG)
JSS College of Arts,Commerce and Science
(An Autonomous College of University of Mysore)
Ooty Road, Mysore-570 025
Re-accredited by Naac with 'A' Grade
Phone:0821-2548236,Fax:0821-2548238,Email-jssautonomous@gmail.com

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Recognised by UGC as "College with Potential for Excellence"

Ph: 0821-2548236 & 2548380. FAX: 0821-2548238 E-mail: jssautonomous@gmail.com

Website: www.jsscacs.edu.in

## DECLARATION

I hereby declare that the Minor research project (MRP(S)-1499-MRP/14-15/KAMY013/UGC-SWRO) entitled "**Elucidation of Phytochemical and Antimicrobial characteristics of Betel vine (*Piper betle* L.)"** is the result of bonafide work carried out by me.

I further declare that the results are not submitted for the award of any other degree or fellowship.

**PRINCIPAL INVESTIGATOR**

JSS MAHAVIDYAPEETHA

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## CERTIFICATE

I hereby certify that the Minor Research Project (MRP(S)-1499-MRP/14-15/KAMY013/UGC-SWRO) entitled **"Elucidation of Phytochemical and Antimicrobial characteristics of Betel vine (*Piper betle* L.)"** carried out by Dr. M. Seema, Assistant Professor, Department of Microbiology, JSS College, B.N.Road, Mysuru-25. The project report has been kept in the college library and the executive summary of the study has been posted on the website of the college.

**PRINCIPAL**

**ACKNOWLEDGMENTS**

*I immensely thank JSS MahavidyaPeetha for permitting and encouraging me to take up UGC -MRP. I am grateful to Prof. B.V. Sambashivaiah, Cheif executive and Prof. M. Mahadevappa, Principal, JSS College, B.N. Road, Mysuru-25 for extending necessary facilities for my research work.*

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Dr. M. SEEMA

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# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| @ | at the rate of |
| cm | centimeter |
| Diam. | Diameter |
| et al. | et  alia |
| e.g. | For example |
| Fig. | Figure |
| g | acceleration due to gravity |
| g | gram (s) |
| g m-2 | gram per meter square |
| hrs. | hours |
| i.e., | id est, that is. |
| kg | kilogram |
| µg | microgram |
| µm | micrometer |
| mg | milligram |
| mm | millimeter |
| ml | milliliter |
| min | minute |
| M | molar |
| No. | number |
| pH | hydrogen ion,  minus log concentration |
| % | percentage |
| rpm | rotation per minute |
| sp | species (plural) |
| viz. | namely |
| w/v | weight/volume |

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# GENERAL INTRODUCTION

The plant world is a rich store house of natural chemicals that could be exploited for use of controlling many diseases of plants, animals and humans (Satish *et al.,* 2008). Thus there is an intense search for these molecules in many plants throughout the world and they are still largely unexplored. Our country is very rich in biological diversity, harbouring around 49000 species of plants, including about 17500 species of higher plants. The Indian gene centre holds a prominent position among the 12 mega-gene centres of the world. It is also one of the Vavilovian centres of origin and diversity of crop plants. Two out of the 25 global hotspots of biodiversity, namely the Indo-Burma and Western Ghats / Sri Lanka, are located here. India possesses about 12 percent of world flora with 5725 endemic species of higher plants belonging to about 141 endemic genera and over 47 families. Presently, the Indian diversity is composed of rich genetic wealth of native as well as introduced types, that is India is a primary as well as a secondary centre of diversity for several crops, and has also rich regional diversity for several South/Southeast Asian crops (Anonymous, 2007).

Many of these plants are yet to be exploited for the bioactive compounds and these compounds are emerging as safer and compatible ones to control disease causing organisms (Kumbhar *et al*., 2000). Some of these plants are endemic to such an extent that they are identified with geographical indication tag (GI). The concept of geographical indication is fast developing globally. GIs is very essential and imperative in the current global scenario to seek legal protection in WTO countries (Prajapati, 2010**)**. One of such plants having Geographical indication tag is Betelvine (*Piper betel*). This plant species is selected for the proposed project.

During the present investigation, 2 varieties of betelvine commonly cultivated in Mysore *viz.*, *Mysuru veelyadele* and *Ambadi yele* were used. Among these, the former one is provided with GI (Prajapati, 2010**)**. Phytochemical analysis and Antimicrobial activity of two varieties of betelvine aqueous leaf extracts and solvent extracts against plant pathogens was carried out to explore the antimicrobial potency. This can be exploited for further commercial use to control the plant diseases.

The present investigations were carried out on the following areas:

1. Phytochemical analysis of Leaf extracts of Piper betle L.
2. Antimicrobial activity of Piper betle L. on Phytopathogenic fungi

## MATERIALS AND METHODS

**ISOLATION OF PHYTOPATHOGENS**: Six plant pathogens were isolated from infected disease plants namely, Tobacco, Paddy, Neem and Jack fruit. Infected plants were collected during the field survey in 2015-16. The infected parts of the plants of 1-2cm surface sterilized using 70% ethyl alcohol. These surface sterilized plant parts were plated on Czapeck Dox Agar (CDA) and incubated at 25±2º C for seven days.

Four post harvest pathogens were isolated from onion, garlic, orange, ground nut and sorghum seeds.

## PREPARATION OF EXTRACTS

### AQUEOUS EXTRACT

Two different varieties of Piper 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 (Preethi mixer grinder, India) 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.

### SOLVENT EXTRACT

Thoroughly washed mature leaves of both the varieties were shade dried and then powdered with the help of a blender. Thirty grams of the powder was filled in the thimble and extracted successively with hexane, ethyl acetate, chloroform, acetone and methanol using a soxhlet extractor for 48 hrs. All extracts were concentrated using rotary flash evaporator and preserved at 5°C in airtight brown

bottle until further use.All extracts were subjected to antifungal activity against the test fungi.

## PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACT

The leaf extract and solvent extracts obtained from methanol, hexane, acetone, ethyl acetate, chloroform and aqueous extract of both the varieties of Piper betle L. were subjected to preliminary qualitative tests for the presence of carbohydrates, proteins, steroids, flavonoids, tannins ,saponins, alkaloids, terpionoids, steroids as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

***Test for Carbohydrates*:**

a. **Molisch Test:** To 2 ml extract few drops of α-naphthal (20% in ethyl alcohol) were added. Then 1 ml of conc. $H_2SO_4$ was added along the side of the test tube. Reddish violet ring at the junction of the two layers indicates the presence of carbohydrates (Telrandhe *et al*., 2010).
b. **Fehling's Test :** 10 ml of Fehling solution (copper sulphate in alkaline condition) was added to the concentrated extracts and heated on a steam bath. Brick-red precipitate indicates the presence of carbohydrate.

***Test for Proteins:***

*a.* **Biuret Test:** To 3 ml of extract was added 4% NaOH and few drops of 1% $CuSO_4$ solution. Violet or pink colour indicates the presence of proteins. (Telrandhe *et al*., 2010).
*b.* **Ninhydrin Test:** To 1 ml of extract 1% Ninhydrin reagent was added and heated on a steam bath. Violet colour indicates the presence of proteins.

***Test for Alkaloids:***

To 2 ml of extract 2 ml Conc. HCl and few drops of Mayer's reagent was added. A green or white precipitate indicates the presence of alkaloids (Culki, 1994).

***Test for Tannins:***

About 0.5 ml of the leaves extract was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

***Test for Saponin:***

About 2ml of the leaf extract was boiled in 20 ml of distilled water in a water bath and then filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

***Test for Flavonoids :***

To determine the presence of flavonoids in the plant sample (Sofowara, 1993; Harbrone, 1973), A 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of the plant extract followed by addition of con. $H_2SO_4$. A yellow colouration in extract indicates the presence of flavonoids. The yellow colouration disappeared on standing.

***Test for steroids:***

Two ml of acetic anhydride was added to 0.5 g of extract with 2 m $H_2SO_4$. The colour changed from violet to blue or green in samples indicating the presence of steroids.

***Test for terpenoids (Salkowski test) :***

Five ml of the extract was mixed in 2 ml of chloroform and con.$H_2SO_4$ (3 ml ) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

Estimation of Total Phenolics, Carbohydrate , Flavanoids , α-Tocopherols and ascorbic acid was carried out

**Estimation of total phenolic compounds**

A total phenolic content of the extracts was determined according to the method of Kujala(kujala TS et al., 2000) with minor modifications, using gallic acid as standard. The extracts ranging from 0-100 µl and 0-100µg of gallic acid were dissolved in 0.5 ml of water and were mixed with 500µl of 50% Folin-Ciocalteau reagent. The mixtures were then allowed to stand for 10min followed by the addition of 1ml of 20% $Na_2CO_3$. After 10min of incubation at ambient temperature, the absorbance of the supernatant was measured at 730nm. The total phenolics content was expressed as gallic acid equivalents (GAE) in milligrams per gram powder.

**Estimation of total flavonoids content**

The basic principle of Aluminium chloride colorimetric method is that Aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition it also it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. Quercetin is reported to be suitable of various concentrations were used to build up the calibration curve.

The aluminium chloride colorimetric method was modified from the procedure reported by Woisky and Saltino (Woisky R, Saltino A, 1998). Standard calibration curve was prepared using quercetin. The differents extracts ranging from 0-100 µl and 0-100 µg of quercetin were dissolved in 100 ml of 80% ethanol and then graded concentrations of the above solution were mixed with 1.5ml of 95% ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1 M potassium acetate and 2.8ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415nm. Proper controls were also done. The amount of flavonoids was calculated in the different extracts of the plant using the standard calibration curve for quercetin.

**Estimation of α- tocopherol**

Vitamin E was determined according to the method of Kivcak and Mert (Kivcak B, Mert T, 2001). It is based on the reduction of ferric iron to ferrous iron by tocopherol, which forms a red colour with 2, 2 dipyridyl. 1g of each extract was taken

in 10ml of hexane and transferred to conical flask which was further extracted with 200ml of hexane with stirring for 24 hrs. The contents of the flasks were later filtered through a whtmann No.1 filter paper . The hexane extract was further distilled in vacuum to get dry extract and stored at -20ºC. 10 mg of dry hexane extract was dissolved in 10ml of chloroform.

Aliquots of hexane extract solution (20-100µl) and α- tocopherol solution (20-100µl) (10mg of α- tocopherol (sigma) was dissolved in 10ml of absolute alcohol) was transferred to separate volumetric flask and the volume was made up to 3ml with chloroform. Then 1ml of 2, 2' dipyridyl dissolved in 25ml of absolute ethanol and stored in dark bottle at 4C) and 1ml of $FeCl_3$ solution (200mg of $FeCl_3.6H_2O$ in 100ml ethanol and stored in brown bottle and kept in refrigerator until used) was added and mixed well. After 15min, read against the blank at 520nm. A blank was run, using 3ml of chloroform, 1ml of 2,2' dipyridyl reagent and 1ml of $FeCl_3$ solution. The amount of α- tocopherol was calculated using the calibration curve for α-tocopherol.

**Estimation of total carbohydrates**

The total sugar concentrations of the extracts was estimated by Dubois method (Dubois M et al., 1956). Different aliquots of extract along with the glucose (0-100µg) where made up to 1ml with distilled water. To this 1ml of 5% phenol and 5ml of concentrated 36N $H_2SO_4$ were added. Orange colour developed was read at 520nm immediately. The total sugar concentration was calculated according to this standard glucose calibration curve.

**Estimation of ascorbic acid**

The total ascorbic content was determined by Das guptha et al., (Das guptha GC and Guha BC, 1941) method, taking pure ascorbic acid as standard (0-16µgm). 50µl of different solvent extract were taken and the volume was made up to 1ml using 5% TCA. This was followed by the addition of 1ml DNPH (Di Nitro Phenyl Hydrazine). The reaction mixture was then incubated in boiling water both for 10min and allowed to stand for 15min at room temperature. 60% ice cold sulphuric acid was then added and the absorbance was read at 540nm. The total ascorbic acid was calculated using a standard ascorbic acid calibration curve.

## THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatographic (TLC) analyses were made on 0.25mm thick silica gel 60G (Merk,7731) prepared on glass plates. The extracts obtained from hexane , chloroform , ethyl acetate , acetone, methanol and aqueous extracts were loaded on activated analytical TLC 0.25mm (20cm x 20cm) and separated using solvent system hexane , ethyl acetate, acetone, di ethyl ether ( 50:50:50:20).

As the ethyl acetate extract was showing better antimicrobial activity and was rich in tannins as found out by chemical analysis, we thought of purifying the compound from the same. The ethyl acetate extract was loaded on to the preparative TLC (20X20cm, 2mm thickness) and separated using the solvent system hexane.

## HPLC ANALYSIS OF PHENOLIC COMPOUNDS

Phenolic compounds was analysed using HPLC (Shimadzu) consisting of an CR- 4A chromatopack data integrator, rheodyne injector, a SCL- 6A system controller, LC 6A pump and a SPD 6AV UV visible spectroscopic detector. A reversed- phase high- performance liquid chromatographic method has been developed and validated for estimation. A C18 column was used with a gradient elution of methanol and 0.1% (v/v) acetic acid in HPLC- grade water as mobile phase at a flow rate of 0.9 ml per minute. UV detection was performed at 278 nm.

## LC-MS

LC-MS analysis was performed by 1100 SL trap model with thermo stated column compartment, diode array and standard auto sampler. Mass analysis was carried out with 0.5 mL/min flow rate and negative ion mode. The mobile phase and the solvent gradient were the same with that which was used in HPLC. The sample injection volume was 10 µl. The UVVIS spectra were recorded in the range of 200–700nm and chromatograms were acquired at 280 and 340nm. All of the analyses used the ion-spray source in negative mode with the following settings: nebulizer gas (N2) 40.0 psi, drying gas 12 L/min and drying gas temperature 350 °C. Full scan data was acquired by scanning from m/z 50 to 800.

## ANTIFUNGAL ACTIVITY ASSAY

### Aqueous extract

Antifungal activity of the plant extract was carried out by poison food technique (Nene and Thaplyal, 1979). Czapek Dox Agar medium (CDA) with 10, 25, 50, 75, 100% concentration of aqueous extracts of test plants namely, *Mysuru veelyadele* and *Ambadi veelyadele* were prepared. About 15 ml of the medium was poured into each petriplate and allowed to solidify. 5 mm disc of seven-day-old culture of *Rhizoctonia solani, Fusarium oxysporum, Rhizopus artocarpi, Phomoposis azardirachta ,Sclerotium rolfsii* and *Pyricularia oryzae* and Four postharvest pathogens *Aspergillus niger, Fusarium moniliforme Penicillium* sp. and *Aspergillus flavus* were placed at the center of the petriplate and incubated at 25±2°C for seven days. After incubation period, radial colony growth (mm) were measured and recorded in each treatment. For each treatment three replicates were maintained. CDA medium without the aqueous extract served as control. The fungal toxicity of the extracts in terms of % inhibition of mycelial growth was calculated using the following formula:

% inhibition = dc – dt /dc x 100, where dc = average increase in mycelial growth in control, dt = average increase in mycelial growth in treatment.

### Solvent extract

One gram of each of the dried evaporated solvent extract of Mysuru veelyadele and Ambadi veelyadele was separately dissolved in 10 ml of respective solvents i.e., hexane, chloroform, ethyl acetate, acetone and methanol. Antifungal activity of the solvent extract was carried out by poisoned food technique (Nene and Thaplyal, 1987). The Czapek Dox Agar (CDA) containing 100, 200, 300, 400, 500 and 1000 ppm concentration of each solvent extract was prepared. The CDA medium amended with 100 µl of the solvent without any extract served as control. The solvent extract amended medium was poured into sterile 90 mm diameter petriplates (15 ml per plate).The mycelia disc (5 mm) obtained from the margin of seven-day-old culture was inoculated at the centre of the petriplate to both control and solvent extract amended CDA medium. The petriplates were incubated at 25 ±

2°C for seven days. The experiment was replicated three times. The diameter of the fungal colonies and growth characteristics in each petridish were recorded. The antifungal activity was expressed as percentage of mycelial growth inhibition with respect to control was computed using Srivatsava and Singh (2001) method.

## RESULTS

### Isolation of phytopathogens

Six phytopathogens were isolated from the disease plants namely, *Rhizoctonia solani, Fusarium oxysporum, Rhizopus artocarpi, Phomoposis azardirachtae ,Sclerotium rolfsii* and *Pyricularia oryzae* and Four postharvest pathogens were isolated from ground nut seeds, onion & garlic, Sorghum seeds, orange and lemon *viz, Aspergillus niger* ,*Fusarium moniliforme* ,*Penicillium* sp. and *Aspergillus flavus.* All these cultures were maintained at 4°C.

**Table 1a: Phytopathogens isolated from diseased plants**

| **Sl. No.** | **Disease** | **Host** | **Pathogen isolated** |
|---|---|---|---|
| 1 | Sore shin | Tobacco | *Rhizoctonia solani* |
| 2 | wilt | Tobacco | *Fusarium oxysporum* |
| 3 | Rhizopus rot | Jack fruit | *Rhizopus artocarpi* |
| 4 | Damping off | Tobacco | *Sclerotium rolfsii* |
| 4 | Die back of neem | Neem | *Phomoposis azardirachtae* |
| 5 | Blast of paddy | Paddy | *Pyricularia oryzae* |

**Table 1b: Post harvest pathogens**

| Sl.no. | Plant material | organism |
|---|---|---|
| 1 | Sorgum seeds | *Aspergillus niger* and *Fusarium moniliforme* |
| 2 | Groundnut seeds | *Aspergillus flavus* |
| 3 | Onion and Garlic | *Aspergillus niger* |
| 4 | Orange and lemon | *Penicillium* sp. |

**Fig.1: Phytopathogen isolated from diseased plants:**

**Fig.1a: Blast of Paddy**

Infected paddy field *Pyricularia oryzae*

**Fig.1b: Sore shin disease of tobacco**

Healthy tobacco nursery bed

Sore shin Infected nursery beds

Nursery bed showing baldness

Sore shin infected tobacco seedlings

Pure culture of *Rhizoctonia solani* (Sore shin pathogen)

**Fig.1c: Tobacco wilt**

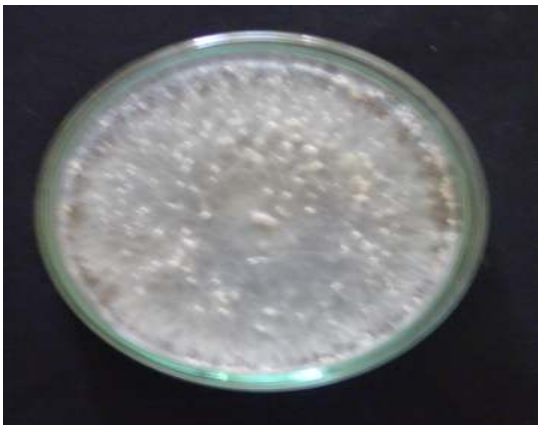

*Fusarium* wilt of tobacco

*Fusarium oxysporum*

**Fig.1d: Damping off of tobacco**

Healthy nursery

Infected seedlings



*Sclerotium rolfsii*

**Fig.1e: Die back of neem**

Infected neem tree

*Phomoposis azardirachtae*

**Fig.1f: Rhizopus rot**



Infected Jack fruit *Rhizopus artocarpi*

**Fig.2: Post harvest pathogens**

**Fig.2a: Sorghum seeds**



Infected sorghum *Fusarium moniliforme*

**Fig.2b: Ground nut seeds**



Infected groundnut seeds *Aspergillus flavus*

**Fig.2c: Onion and garlic**

Infected onion and garlic

*Aspergillus niger*

**Fig.2d: orange and Lemon**

Orange and lemon

*Penicillium* sp.

**QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN MYSORE AND AMBADI VEELYADELE**

Qualitative analysis revealed that carbohydrate, proteins, flavanoids, alkaloids, tannins, Terpinoids, saponins and steroids were present in aqueous extract. Whereas, carbohydrate, proteins, flavanoids, tannins and saponins were absent in Hexane and chloroform extract but were present in other solvent extracts. Terpenoids and steroids were absent in ethyl acetate, acetone and methanol extract but present in hexane and chloroform.

**Table 2: Qualitative analysis of phyto-constituents of aqueous and solvent extracts of both the varieties of Piper betle L.**

| Phytochemicals | Aqueous extract | Hexane | Chloroform | Ethyl acetate | acetone | methanol | Observation |
|---|---|---|---|---|---|---|---|
| Carbohydrate | + | - | - | + | + | + | Brick-red precipitate |
| Proteins | + | - | - | + | + | + | Violet colour |
| Flavanoids | + | - | - | + | + | + | Yellow colouration |
| Alkaloids | + | + | + | + | + | + | green precipitate |
| Tannins | + | - | - | + | + | + | Blue black colouration |
| Terpenoids | + | + | + | - | - | - | Reddish brown colouration |
| Saponins | + | - | - | + | + | + | Stable persistant froth |
| Steroids | + | + | + | - | - | - | Blue/ Green colouration |

Phytochemical analysis also revealed that both Mysore and Ambadi veelyadele are having high amount of vitamin E in hexane extract, Mysore veelyadele is rich in flavonoids, total carbohydrates, phenolics, ascorbic acid and alpha tocopherol when compared to Ambadi veelyadele. Both varieties are rich in flavonoids, compared to other contents.

**Table 3: Proximate Composition of various extracts in soxhlet**

**Table 3a: Ambadi veelyadele**

| **Extracts of Piper betle L.** Ambadi | 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±0.01 | 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 ± 0.02 | - |
| 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 | |

**Table 3b** :Mysuru veelyadele

| **Extracts of Piper betle L.** Mysore | Total carbohydrates (mg/gm) | Total phenolics (mg/gm) | Flavonoids (mg/gm) | Ascorbic acid (mg/gm) | α – tocopherol (mg/gm) |
|---|---|---|---|---|---|
| Hexane | -- | - | - | - | 8.0 ±0.02 |
| Chloroform | - | - | - | - | 7.7±0.01 |
| Ethyl acetate | 3.0 ± 0.01 | 4.1 ±0.04 | 10.0 ±0.01 | 0.32±0.02 | - |
| 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 |

## THIN LAYER CHROMATOGRAPHY (TLC)

As the ethyl acetate extract was showing better antimicrobial activity and was rich in tannins as found out by chemical analysis, we thought of purifying the compound from the same. The ethyl acetate extract was loaded on to the preparative TLC(20X20cm,2mm thickness) and separated using the solvent system hexane , Ethyl acetate, acetone , diethyl ether (50:50:50:20) ethyl acetate extract are loaded to the preparatory TLC separated using solvent system three bands were obtained ,named as upper band, middle band and lower band ,these 3 bands were scraped into the beaker acetone were added and filtered , the filtrate was again

loaded on to the analytical TLC to check the purity of molecule, single spot was obtained in all the three different bands and they were subjected further HPLC and LC MS

Mysuru veelyadele Ambadi veelyadele

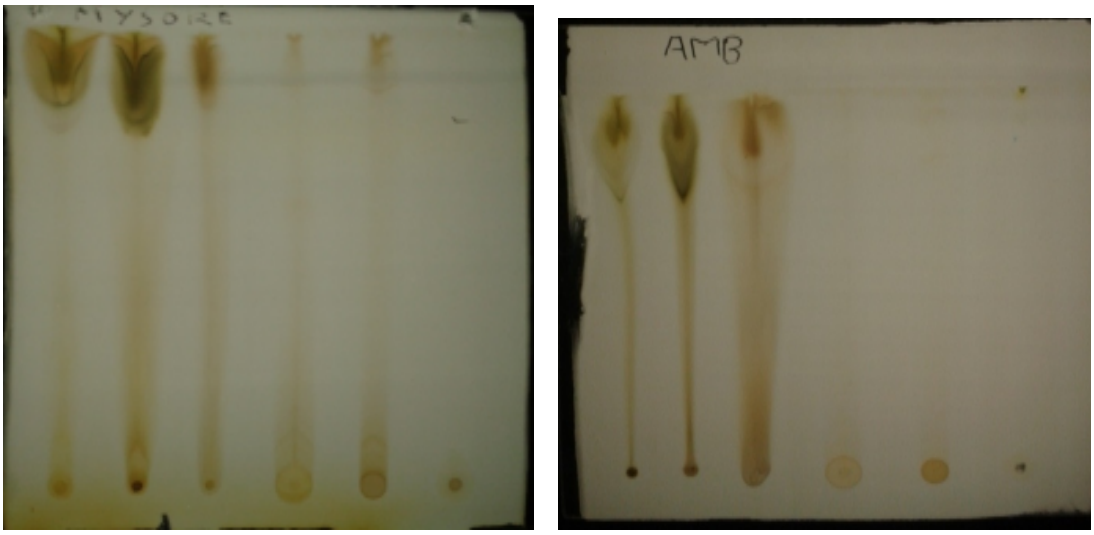



**Fig 3: TLC profile of Mysuru veelyadele and Ambadi veelyadele**

Mysuru veelyadele Ambadi veelyadele

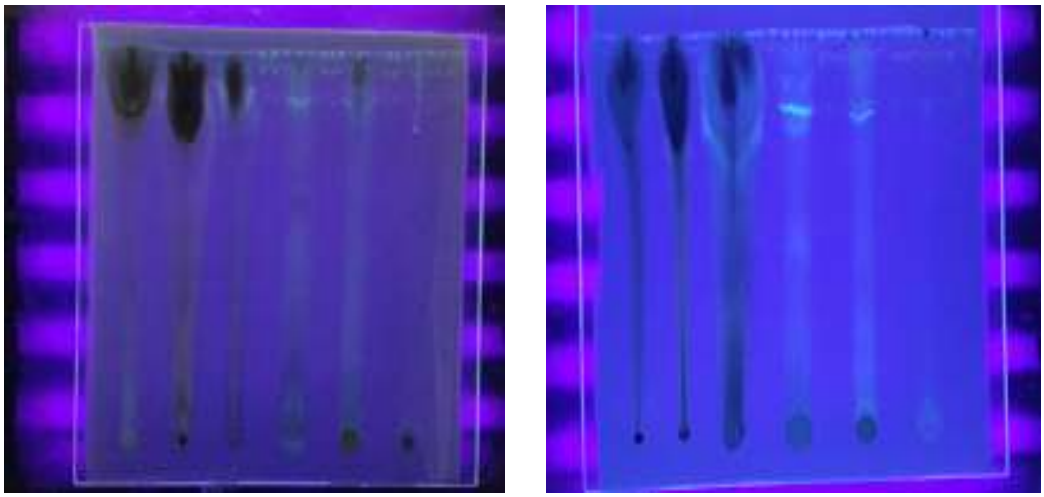


**Fig.4:TLC profile of Mysuru veelyadele and Ambadi veelyadele under UV**

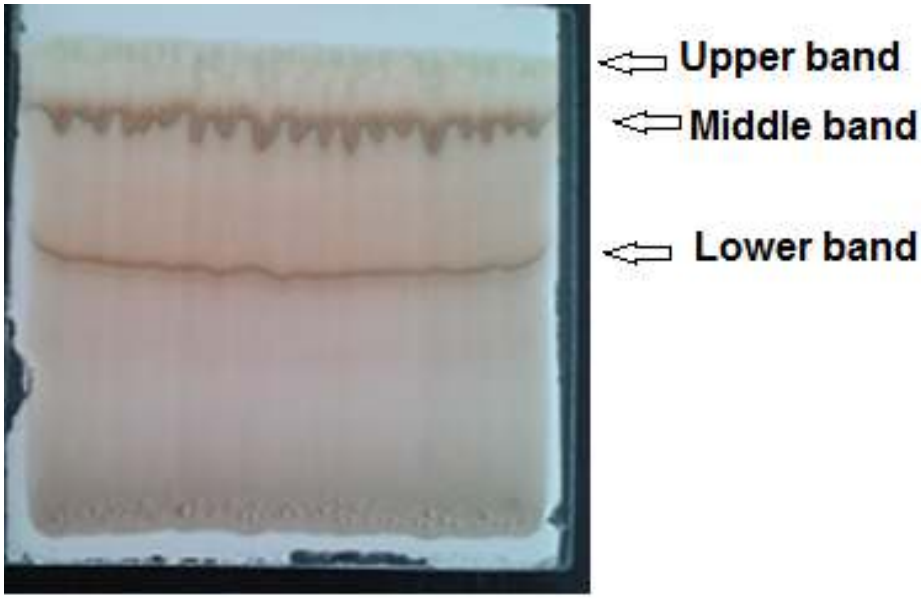



**Fig.5a: Preparatory TLC of Mysuru veelyadele**

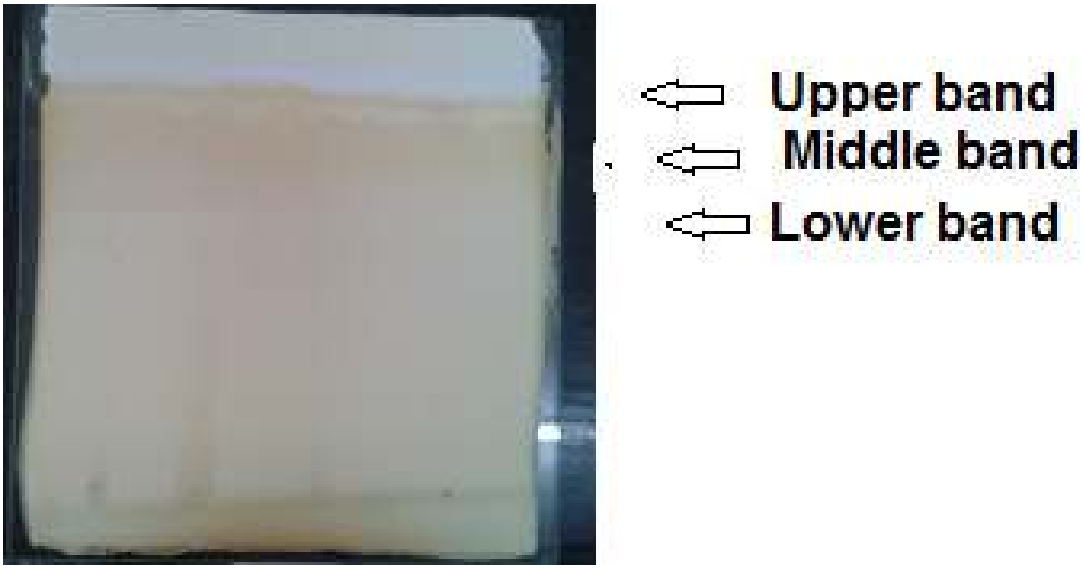



**Fig.5b: Preparatory TLC of Ambadi veelyadele**

## HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The phenolic standards used were Coumaric acid, Caffeic acid, Synergic acid, Chlorogenic acid, Procalactin, Gallic acid, Vanillin, Transcinamic acid, Calcihin and Epicatichin

**Fig.6:** HPLC Chromatogram of standard phenolics

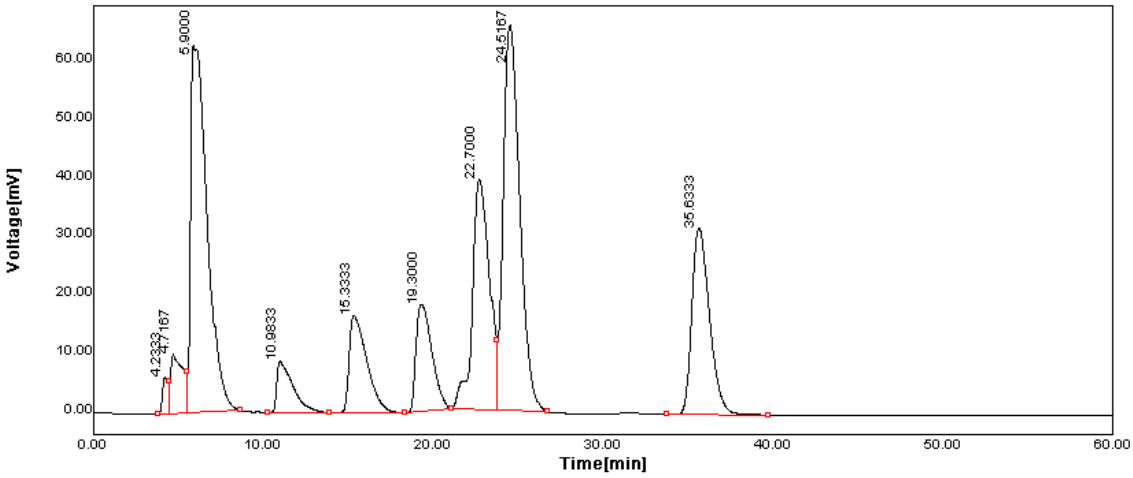



**Fig.6a:** Gallic acid

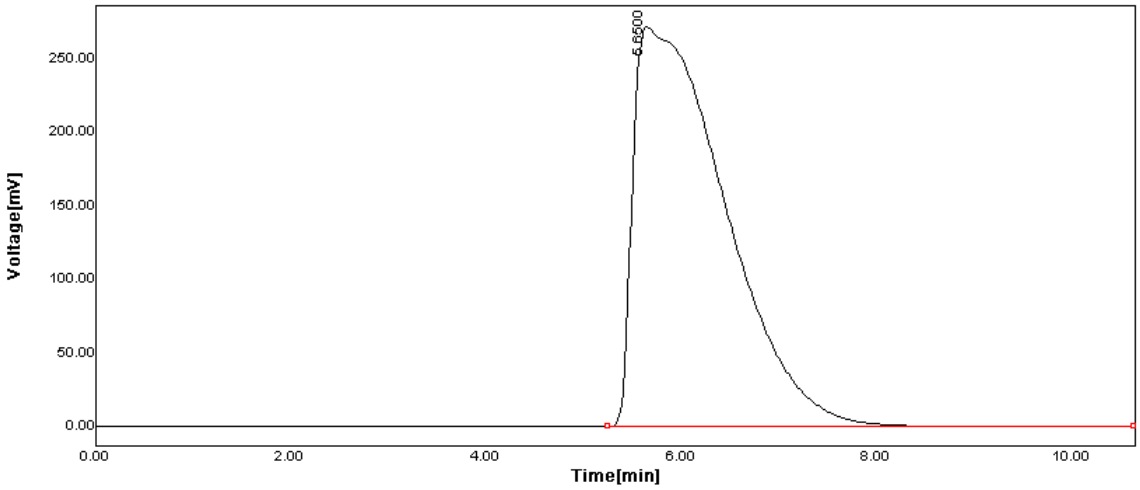


**Fig.6b:** Transcinnamic acid

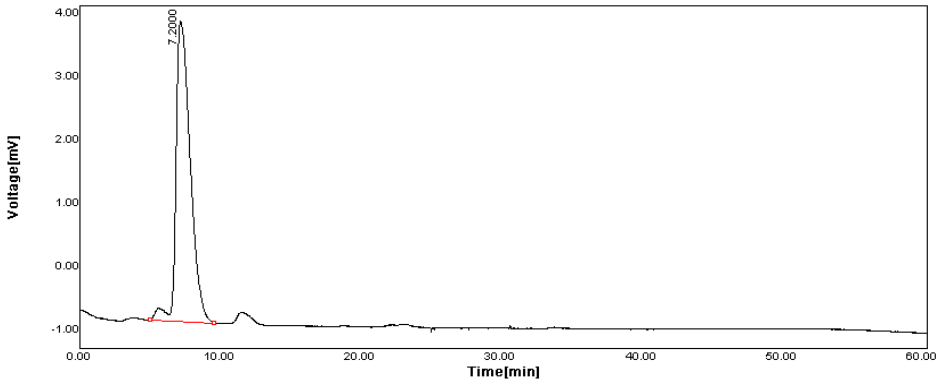



**Fig.6c:** Caffeic acid

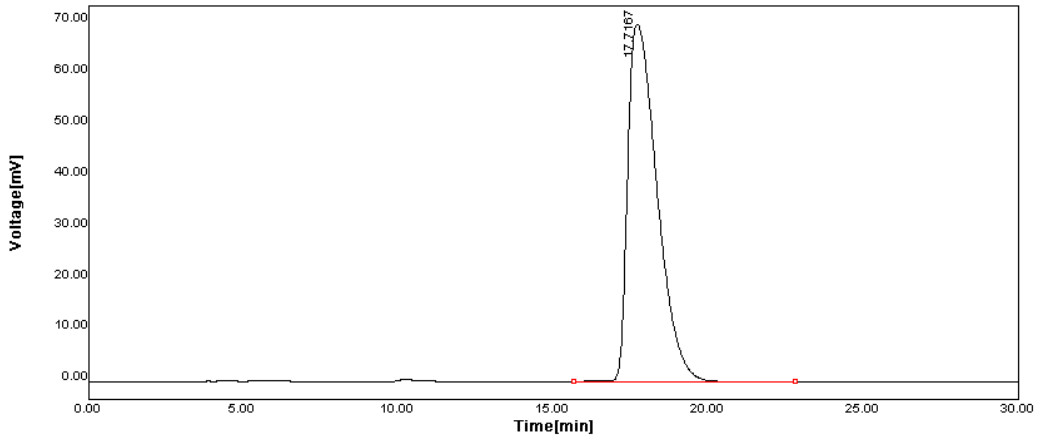



**Fig.6d:** Chlorogenc acid

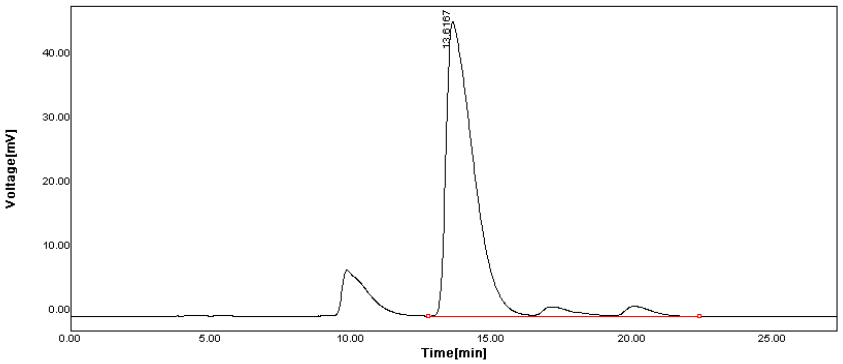


**Fig.6e:** Synergic acid

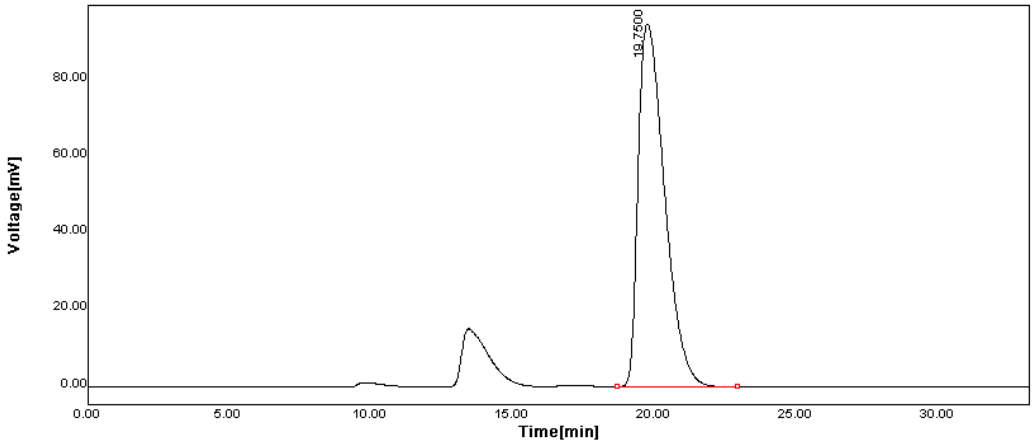



**Fig.6f:** Protocatechoic acid

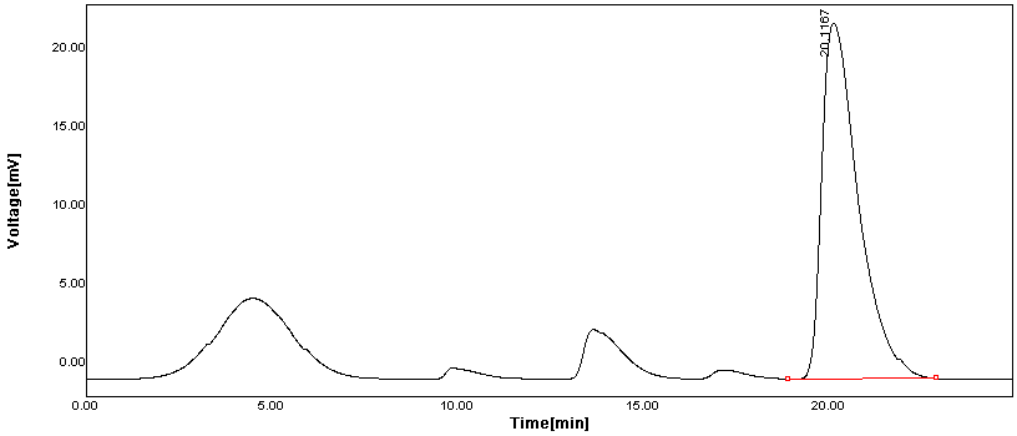



**Fig.6g:**
Vanillin

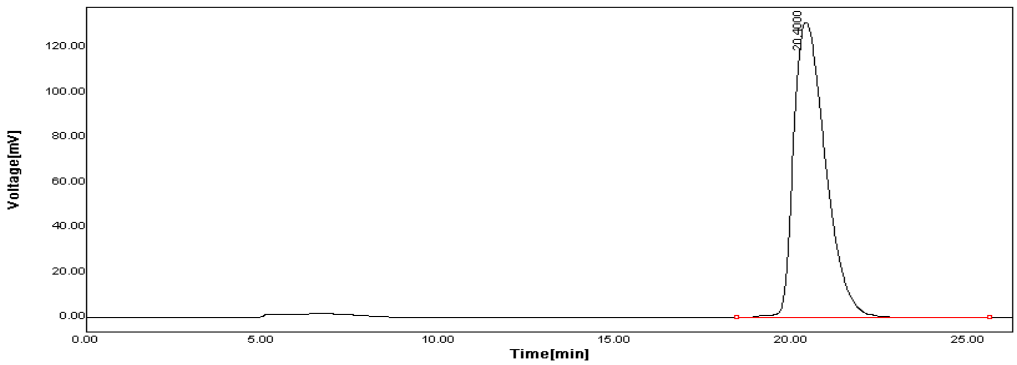


**Fig.6h:** Coumaric acid

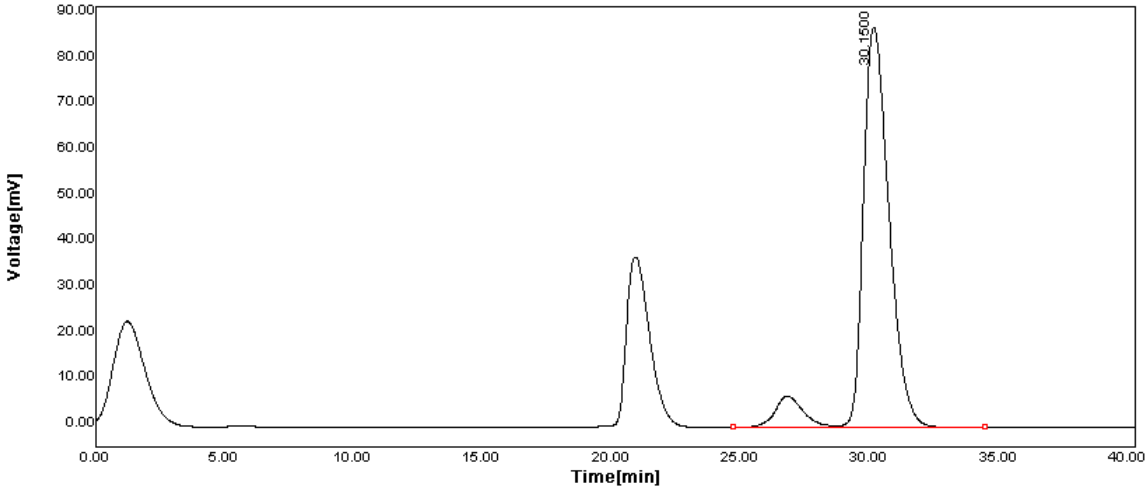
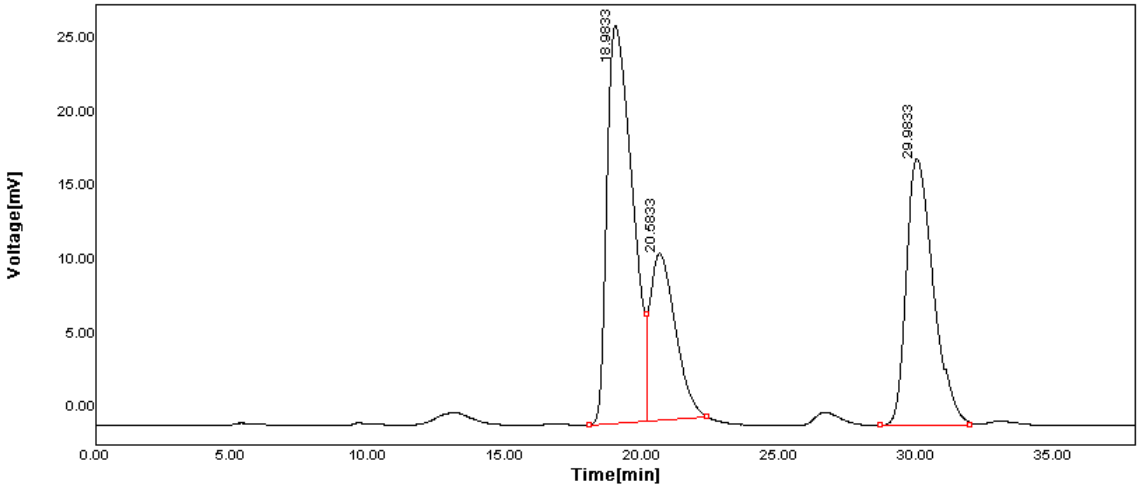



**Fig.6i:** Epicatachin



**HPLC of Ambadi veelyadele**

From the HPLC profile, following compounds were identified by comparing with the standards. In the lower and middle layer, showed the presence of Gallic acid. In the upper layer the HPLC profile of shows the presence of 1-Gallic acid, 2-Transcinnamic acid, 3- Chlorogenc acid,4- Caffeic acid, 5- Synergic acid, 6-Coumaric acid

**Fig.7: HPLC Chromatogram of *Ambadi veelyadele***

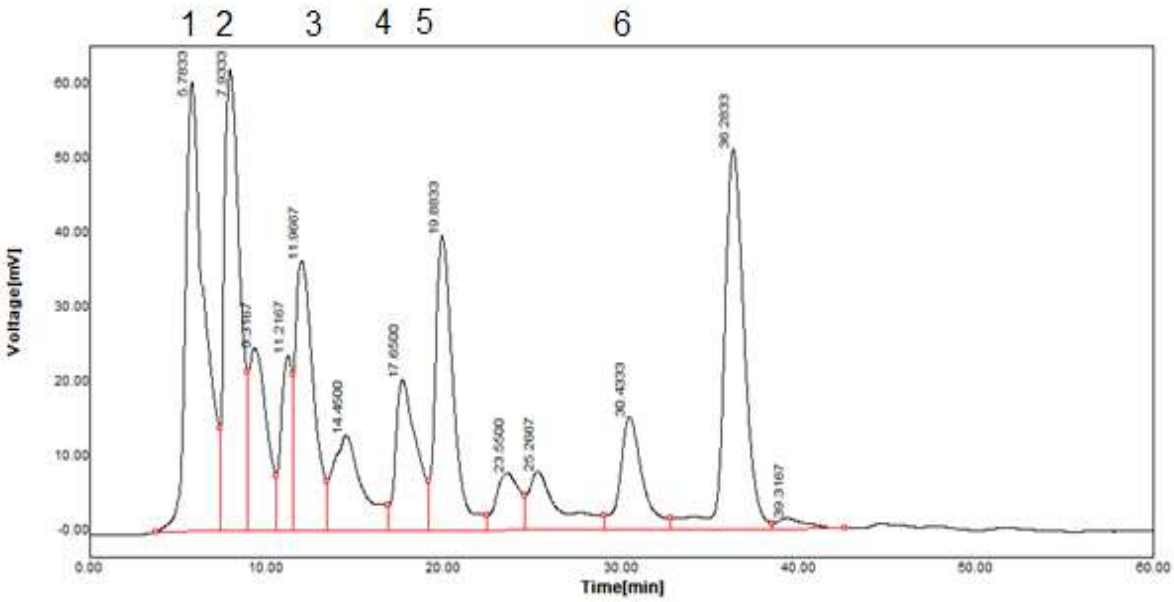



**Fig.7a: HPLC Chromatogram of Ambadi veelyadele (upper layer).**

1-Gallic acid, 2-Transcinnamic acid, 3- Chlorogenc acid,4- Caffeic acid, 5- Synergic acid and 6- Coumaric acid.

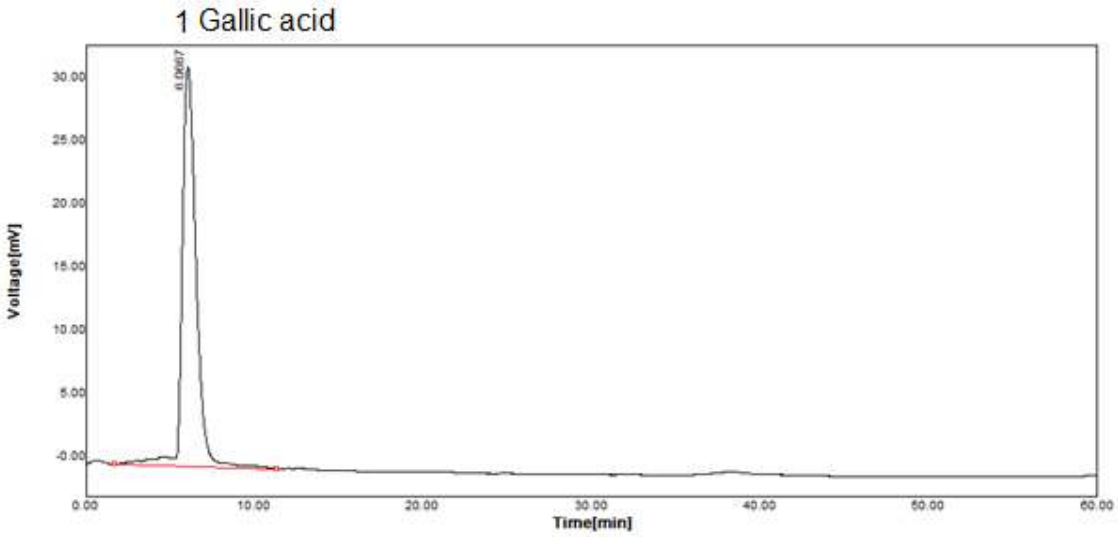



**Fig.7b: HPLC Chromatogram of ambadi veelyadele (middle layer)**

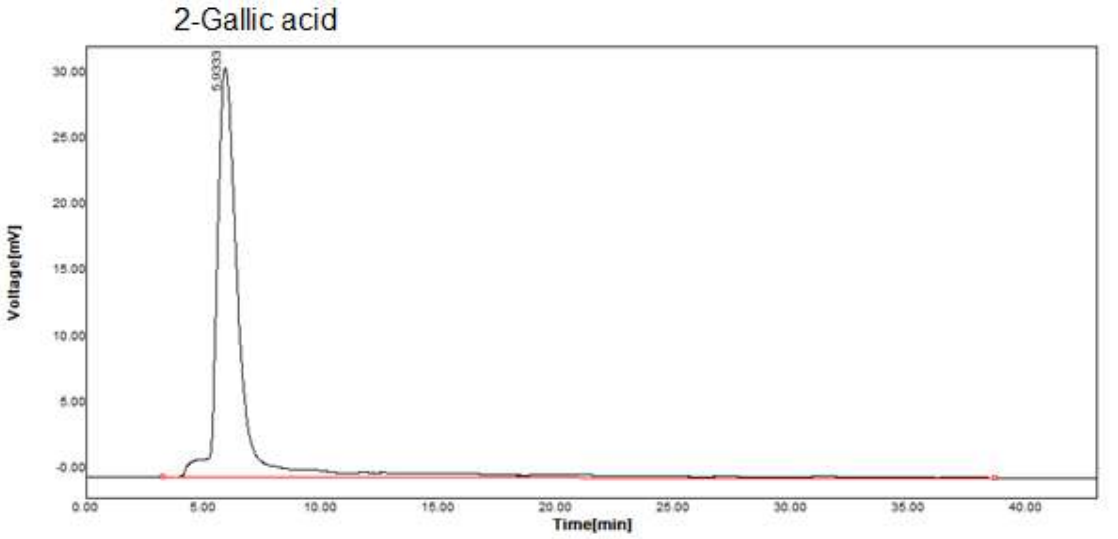



**Fig.7c: HPLC Chromatogram of ambadi veelyadele(lower layer)**

**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-Chlorogenc acid and 1- Transcinnamic acid

**Fig.8: HPLC Chromatogram of Mysuru veelyadele**

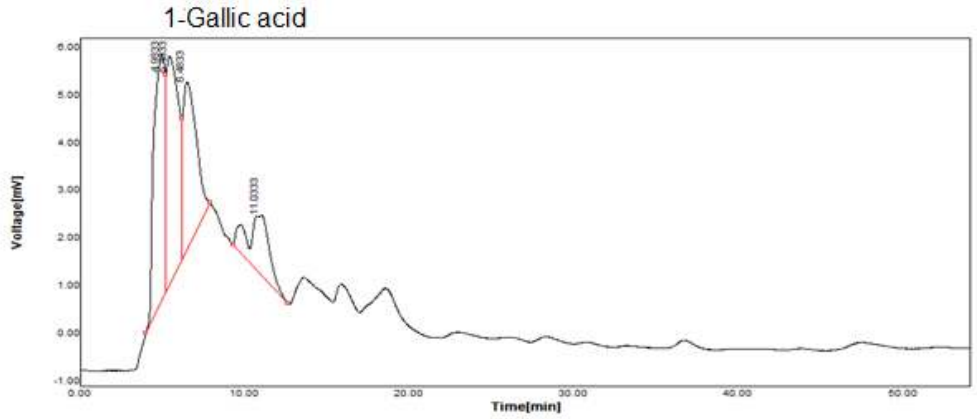



**Fig.8a: HPLC Chromatogram of Mysuru veelyadele (upper layer)**

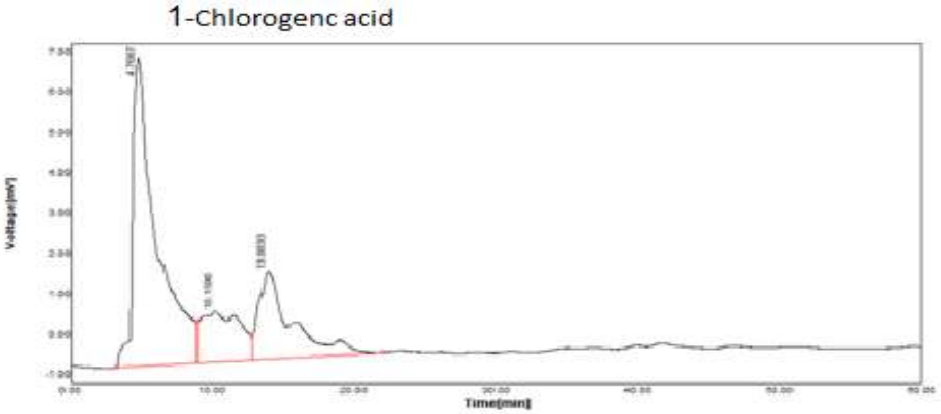



**Fig.8b: HPLC Chromatogram of Mysuru veelyadele ( middle layer)**

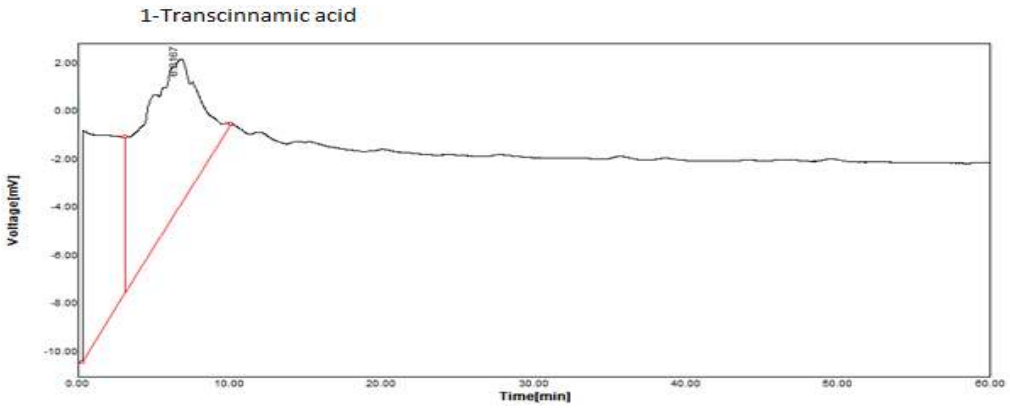



**Fig.8c:HPLC Chromatogram of Mysuru veelyadele ( Lower layer)**

**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.

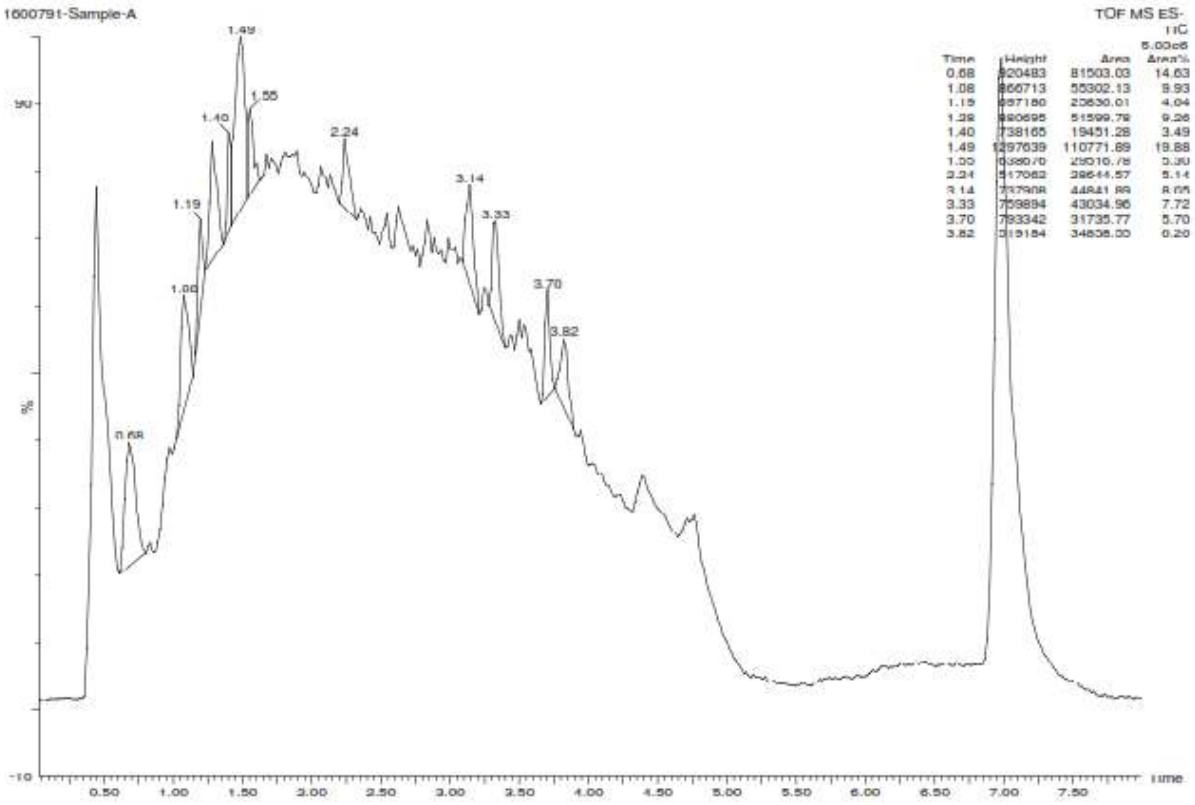



**Fig.9: LC MS Profile of methanolic extract of Ambadi veelyadele**

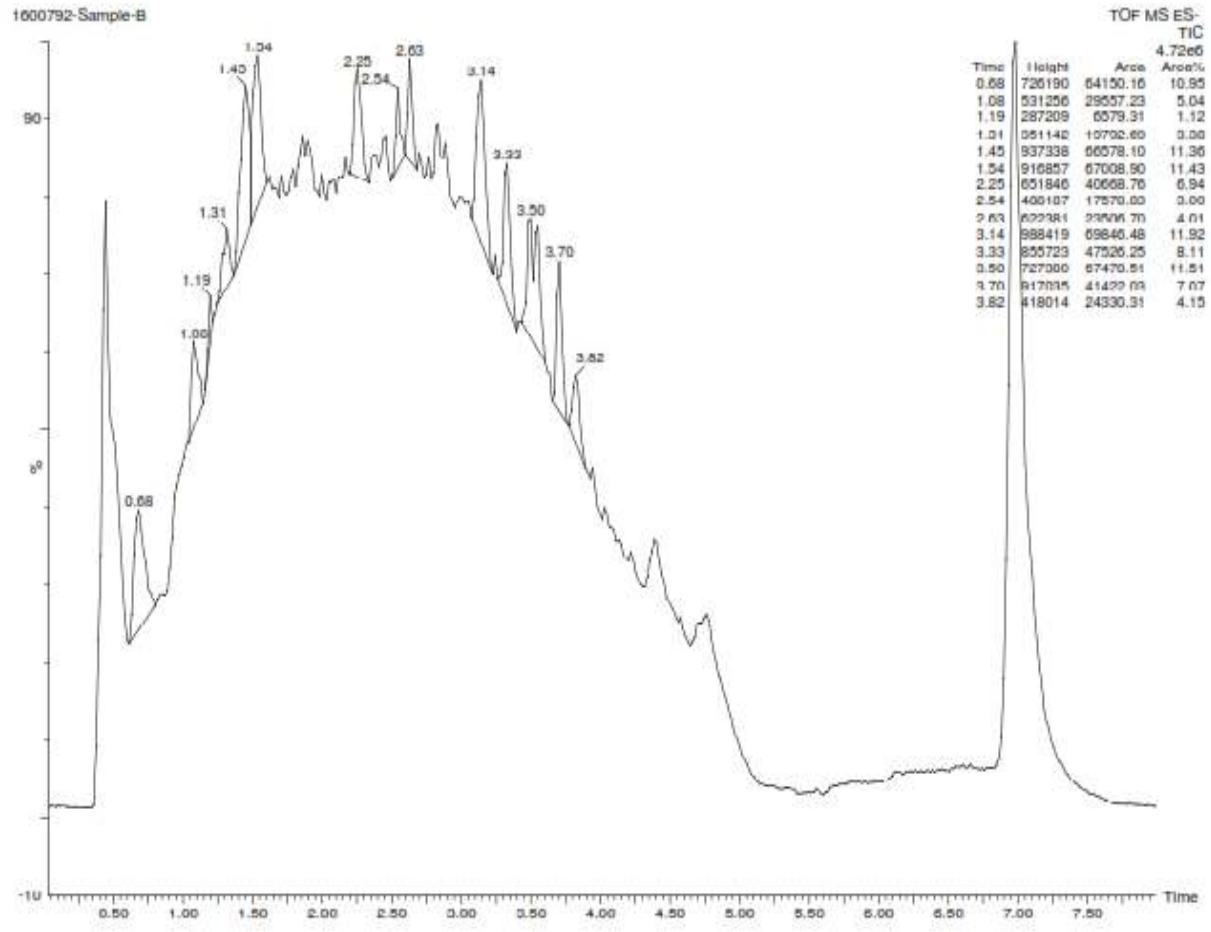


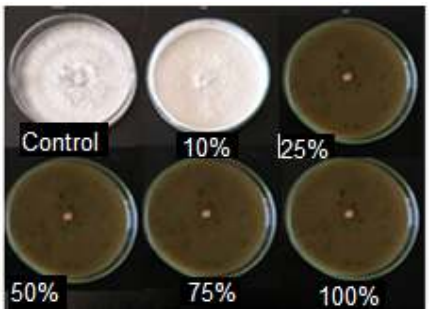

**Fig.10: LC MS Profile of methanolic extract of Ambadi veelyadele**

## ANTIFUNGAL ACTIVITY ON PHYTOPATHOGENS

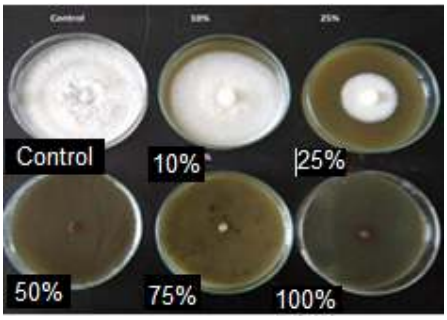
### AQUEOUS EXTRACT

Among the two varieties screened, aqueous extract of Mysuru veelyadele have recorded varied percentage of inhibition against *Rhizoctonia solani, Sclerotium rolfsii* and *Phomopsis azadirachtae* at 25%, 50% and 75%. Ambadi veelyadele recorded significant antifungal activity against *Rhizoctonia solani* at 25%, whereas *Sclerotium rolfsii* and *Phomopsis azadirachtae* at 75% and 100%. Other phytopathoogens showed moderate growth at 100% concentration in both the varieties, but did not completely inhibit the growth even at 100% concentration.

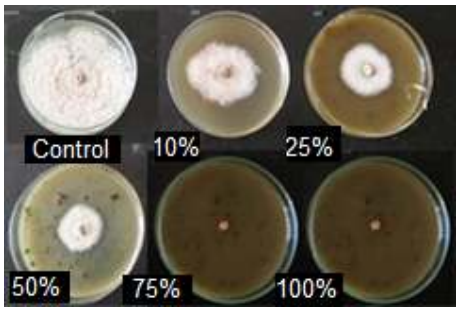
**Fig.11a: Effect of aqueous extract of Mysuru veelyadele on *Rhizoctonia solani* , *Sclerotium rolfsii* and *Phomopsis azadirachtae***




*Rhizoctonia solani*

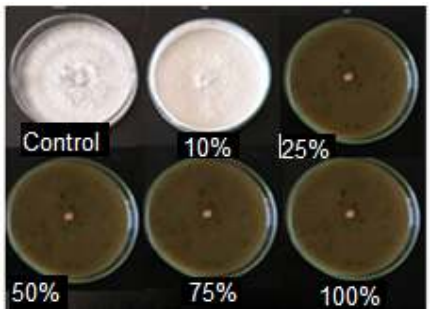



*Sclerotium rolfsii*

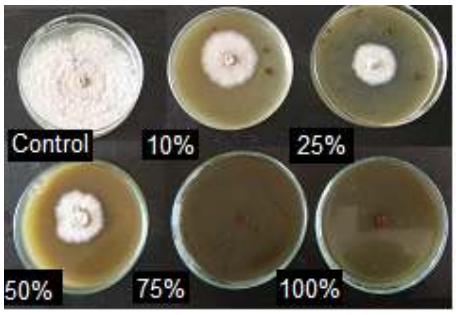



*Phomopsis azadirachtae*

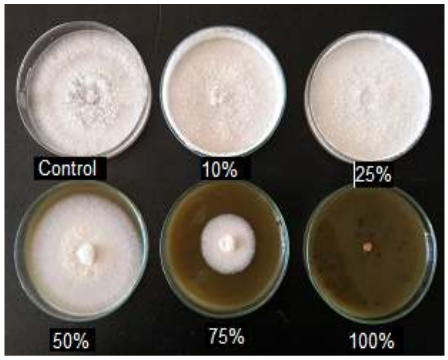
**Fig.11b: Effect of aqueous extract of Ambadi veelyadele on *Rhizoctonia solani* *Sclerotium rolfsii* and *Phomopsis azadirachtae***




*Rhizoctonia solani*




*Phomopsis azadirachtae*




*Sclerotium rolfsii*

**Fig.12a:Percentage inhibition of aqueous extract of Mysuru veelyadele**

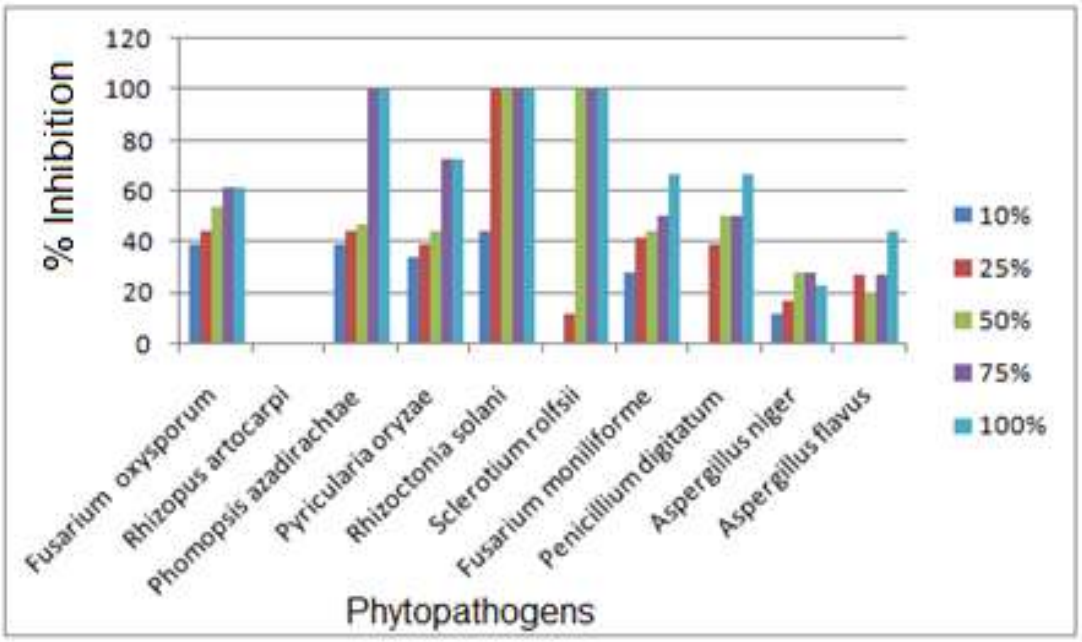



**Fig.12b: Percentage inhibition of aqueous extract of Ambadi veelyadele**

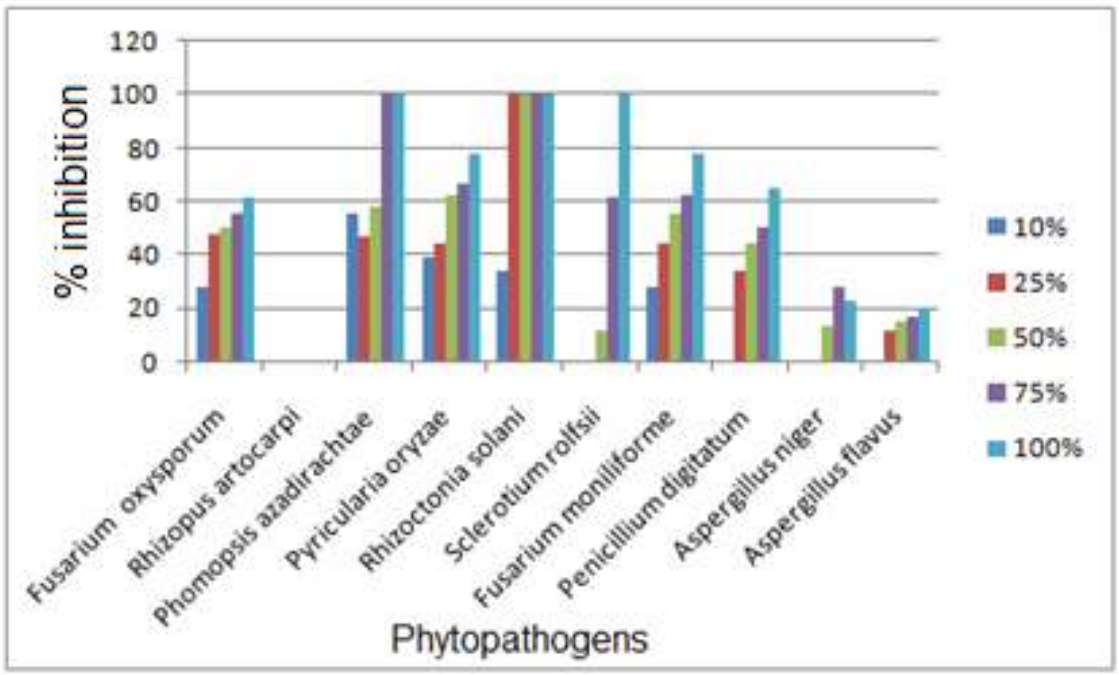


**Table 4: Percentage inhibition (PI) of growth of plant pathogens by aqueous extract of Mysuru veelyadele and Ambadi veelyadele**

| Sl. No. | Organism | Mysuru veelyadele | | | | | | Ambadi veelyadele | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Percentage of mycelia inhibition | | | | | | | | | | |
| | | Concentration range | | | | | | | | | | |
| | | 10% | 25% | 50% | 75% | 100% | | 10% | 25% | 50% | 75% | 100 % |
| 1 | *Fusarium oxysporum* | 39 $^{c}$ | 44$^{c}$ | 53$^{c}$ | 61 $^{c}$ | 61 $^{c}$ | | 27$^{b}$ | 48$^{c}$ | 50$^{c}$ | 56$^{c}$ | 61 $^{c}$ |
| 2 | *Rhizopus artocarpi* | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ | | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ | 00 $^{a}$ |
| 3. | *Phomopsis azadirachtae* | 39 $^{c}$ | 44 $^{c}$ | 47 $^{c}$ | 100$^{d}$ | 100 $^{d}$ | | 56 $^{c}$ | 47$^{c}$ | 58$^{c}$ | 100 $^{d}$ | 100 $^{d}$ |
| 4 | *Pyricularia oryzae* | 33 $^{c}$ | 39$^{c}$ | 44$^{c}$ | 72 $^{cd}$ | 72 $^{cd}$ | | 39 $^{c}$ | 44$^{c}$ | 62$^{c}$ | 67$^{c}$ | 78$^{cd}$ |
| 5 | *Rhizoctonia solani* | 11$^{b}$ | 100$^{d}$ | 100 $^{d}$ | 100 $^{d}$ | 100 $^{d}$ | | 33$^{b}$ | 100 $^{d}$ | 100 $^{d}$ | 100 $^{d}$ | 100 $^{d}$ |
| 6 | *Sclerotium rolfii* | 00 $^{a}$ | 11 $^{b}$ | 100 $^{d}$ | 100 $^{d}$ | 100 $^{d}$ | | 00 $^{a}$ | 00 $^{a}$ | 11$^{b}$ | 61$^{C}$ | 100 $^{d}$ |
| Post harvest pathogens | | | | | | | | | | | | |
| 7 | *Fusarium moniliforme* | 28 $^{b}$ | 41$^{c}$ | 44$^{c}$ | 50 $^{c}$ | 67 $^{c}$ | | 28 $^{b}$ | 44 $^{c}$ | 56 $^{c}$ | 62 $^{c}$ | 77$^{cd}$ |
| 8. | *Penicillium sp.* | 00 $^{a}$ | 39$^{c}$ | 50$^{c}$ | 50$^{c}$ | 67 $^{c}$ | | 00 $^{a}$ | 33 $^{c}$ | 44$^{c}$ | 50 $^{c}$ | 64 $^{c}$ |
| 9. | *Aspergillus niger* | 11 $^{b}$ | 16$^{b}$ | 28$^{b}$ | 28$^{b}$ | 22 $^{b}$ | | 00 $^{a}$ | 00 $^{a}$ | 13$^{b}$ | 28 $^{b}$ | 22 $^{b}$ |
| 10. | *Aspergillus flavus* | 00 $^{a}$ | 27$^{b}$ | 20$^{b}$ | 27$^{b}$ | 44 $^{c}$ | | 00 $^{a}$ | 11 $^{b}$ | 14 $^{b}$ | 17 $^{b}$ | 20 $^{b}$ |

**Figures having the same letters are not significantly different according to Ducan's multiple range test (P<0.05).**

## SOLVENT EXTRACT

Among the solvent extracts tested, hexane and ethyl acetate extract of Mysuru veelyadele has shown significant activity against *Phomopsis azadirachtae, Pyricularia oryzae, Rhizoctonia solani, Sclerotium rolfsii* at 100 ppm and *Ambadi veelyadele* was effective on only two phytopathogens *viz., Rhizoctonia solani* and *Sclerotium rolfsii* at 100ppm.

The concentration of 100ppm of Hexane and ethyl acetate extract of Mysuru veelyadele was effective in inhibition of mycelia growth of *P. azadirachtae, P. oryzae, R. solani* and *S. rolfsii* at 100 ppm.

Ethyl acetate extract of Mysuru veelyadele has shown significant activity against *P. azadirachtae, P. oryzae, R. solani, S. rolfsii, F. oxysporum* and *R. artocari* at 100 ppm. Post harvest pathogens namely, *F. moniliforme* showed growth

inhibition at 400ppm*, penicillium sp. at* 500ppm and *A. niger and A.flavus* at 200ppm.

In hexane extract *F.oxysporum* was inhibited at 500ppm. Post harvest pathogens namely, *A. niger* at 200ppm*, Penicillium sp.at 400ppm, A. flavus,* and *F.moniliforme* was inhibited at 200ppm, 300ppm and 500ppm.

Methanol and Chloroform extracts showed significant growth inhibition of *Phomopsis azadirachtae, Pyricularia oryzae* and *Rhizoctonia solani* at 1000 ppm. Post harvest pathogens showed moderate growth at this concentration.

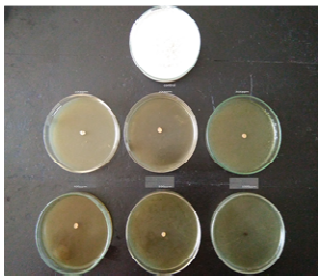
Total absence of inhibitory activities by Acetone extract of both Mysuru veelyadele and Ambadi veelyadele on plant pathogens were observed

*Rhizopus artocarpi* was inhibited by ethyl acetate extract at 100ppm but not in hexane extract.

**Fig.13: Effect of Ethyl acetate extract of Mysuru veelyadele on Phytopathogens**

*Phomopsis azadirachtae*

*Pyricularia oryzae*

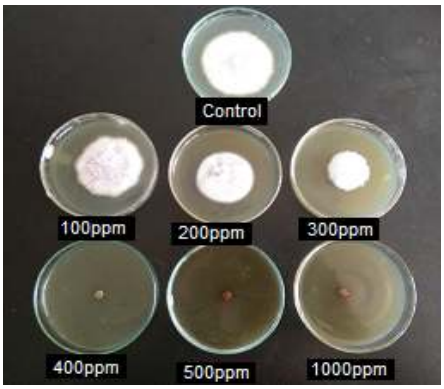


*Rhizoctonia solani*

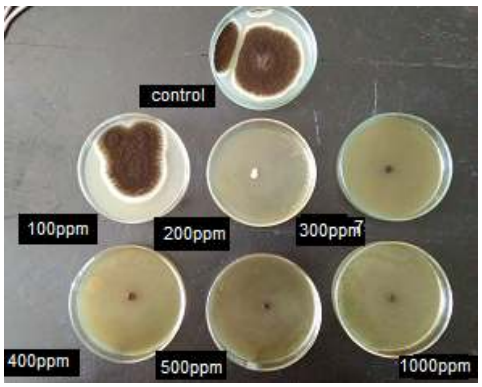
*Sclerotium rolfsii*


*Rhizopus artocari*

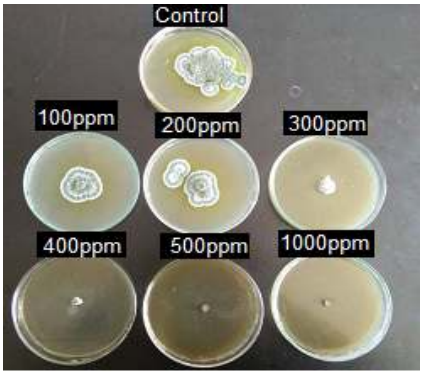
*Fusarium oxysporum*



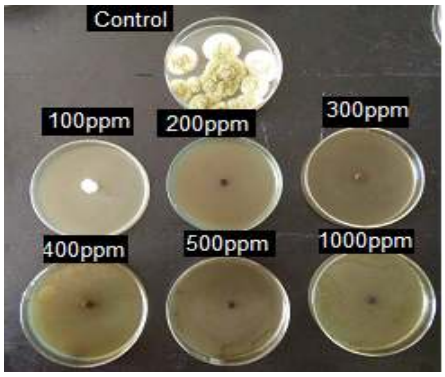

*Fusarium moniliforme*




*Aspergillus niger*




*Penicillium digitatum*




*Aspergillus flavus*

Ambadi veelyadele extract was effective on only two phytopathogens *viz.*, *Rhizoctonia solani* and *Sclerotium rolfsii* at 100ppm.

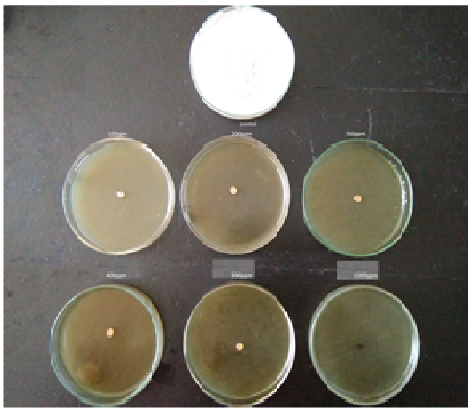
The concentration of 100ppm of hexane extract of Ambadi veelyadele showed significant inhibition of mycelia growth of *R. Solani, P. oryzae* and *S. rolfsii* at 100ppm. *P. azadirachtae* at 200ppm and *F. oxysporum* at 500ppm. No growth inhibition was seen in *R. artocarpi*. Among post harvest pathogens, *A. niger* at 100ppm, *P. digitatum* and *F. moniliforme* showed inhibiton at 500ppm and *A. flavus* at 1000ppm. Whereas ethyl acetate extract showed significant inhibition of *P.azadirachtae, P. oryzae, R. solani, S. rolfsii* and *R. artocarpi* at 300ppm and *F.oxysporum* at 400ppm. Whereas Post harvest pathogen namely, *Penicillium sp.*and *F. moniliforme* showed inhibition at 400ppm *A. niger* and *A. flavus* at 200ppm.

In methanol extract, only *Phomopsis azadirachtae* showed inhibition at 1000ppm. where as other phytopathogens showed moderate growth at different concentration. *Rhizopus artocarpi* was ineffective against methanol.

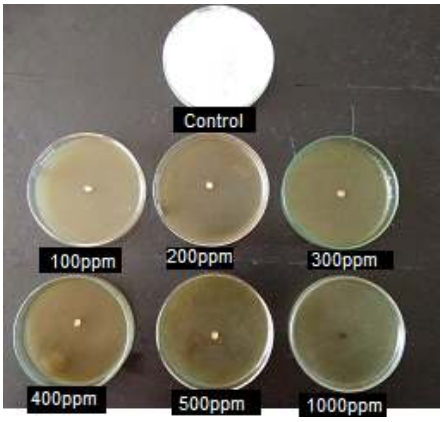
Chloroform extract was effective against *Phomopsis azadirachtae, Pyricularia oryzae* and *Rhizoctonia solani* at 1000ppm but not against other pathogens

Total absence of inhibitory activities by Acetone extract of both Mysuru veelyadele and Ambadi veelyadele on plant pathogens were observed.

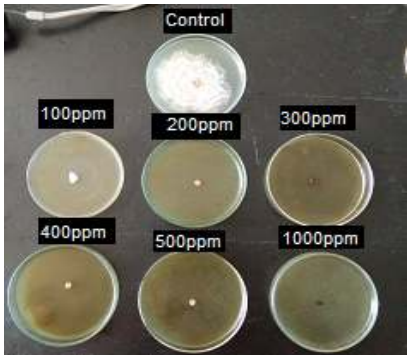
**Fig.14: Effect of Hexane extract of Mysuru veelyadele on Phytopathogens**



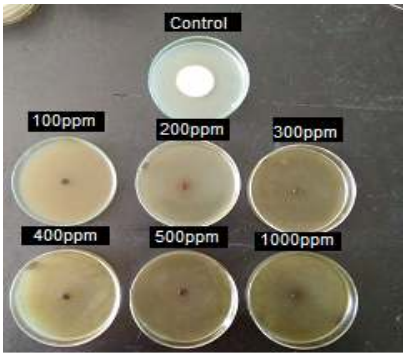
*Rhizoctonia solani*



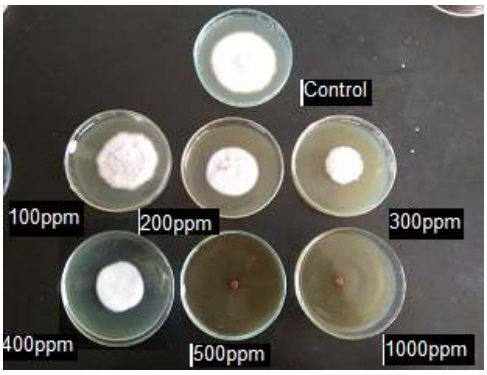

*Sclerotium rolfsii*




*Phomopsis azadirachtae*

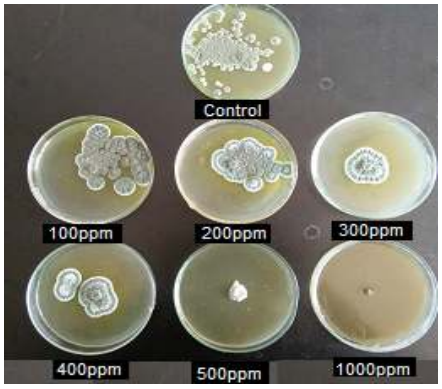



*Pyricularia oryzae*

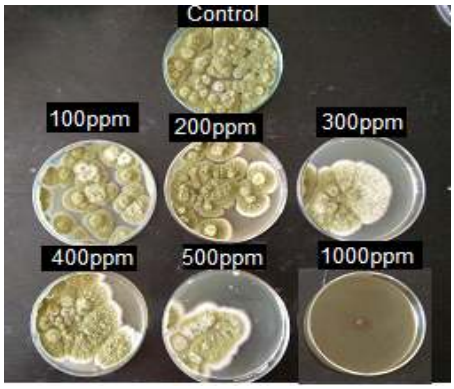



*Fusarium moniliforme*

*Aspergillus niger*




*Penicillium digitatum*




*Aspergillus flavus*

## DISCUSSION

Biological control have attained importance in modern agriculture to curb the hazards of intensive use of chemicals for pest and disease control (Baker and Cook,1079). Phytochemical analysis and *in vitro* antifungal studies were carried out to explore the fungicidal efficacy of two varieties of Piper betle L. *viz.,* Mysuru and Ambadi veelyadele aqueous and solvent extracts against plant pathogens.

In the current investigation both the varieties showed the presence of carbohydrates, proteins, flavanoids, tannins, saponins, terpenoids, steroids and alkaloids in aqueous extract. However carbohydrate, proteins, flavonoids ,tannins and saponins were absent in hexane and chloroform extract but was present in ethyl acetate, acetone and methanol. Steroids and terpinoids were absent in ethyl acetate, acetone and methanol but present in chloroform and hexane extracts. Similar findings were reported by Chakraborty and Shah, 2011 have reported that several extracts of P. betle leaves using methanol, petroleum ether, aqueous and ethyl acetate produced different results in which all the tested solvents, except for water extract had indicated the presence of flavonoids, tannins, sterols and phenol, but lack of alkaloids. Jayalakshmi *et al*.,2011 have reported the presence of carbohydrate, proteins, flavonoids and tannins in methanolic extract. Prakash *et.al.,* 2014 have also reported the presence of tannins, flavonoids and terpenoids in methanol extracts. Similarly Arani *et al.,* 2011 also reported the presence of tannins, flavanoids, carbohydrates and proteins.

Both mysore and ambadi varieties are having high amount of vitamin E in hexane extract, Mysore variety is rich in flavonoids, total carbohydrates, phenolics, ascorbic acid and alpha tocopherol when compared to Ambadi variety. Both varieties are rich in flavonoids, compared to other contents. Similar findings have been reported in Piper betle L. by Dwivedi and Tripathi 2014; Divyalashmi and Aruna Sharmili 2017.

In preparatory and analytical TLC purity of the molecule was tested and the band obtained were subjected further HPLC and LC MS. HPLC was carried out for ethyl acetate extract to characterize the phenolic profile of the two varieties of Piper betle L.. During the present investigation six phenolic compounds, comprising of Gallic acid, Transcinnamic acid, Chlorogenc acid, Caffeic acid, Synergic acid and Coumaric acid was present in Ambadi veelyadele. However, in Mysuru veelyadele three phenolic compounds, namely gallic acid, Chlorogenic acid and Transcinnamic acid were present. Gallic acid was the major compound in both the extract. Ferreres *et.al*., 2014 have reported hydroxychavicol as the major compound in Piper betle L.. Similarly, Hydroxychavicol and chavibetol were detected in ethanol extracts and aquous extracts the higher percentage of hydroxychavicol has been reported by Nurul *et al.,* 2013. In LC MS study revealed the presence of abundant molecules in both Mysuru and Ambadi veelyadele These molecules should we further characterised. One molecule having a molecular mass of 290.035 and retention time 0.68 was common in both varieties.

Among the two varieties studied, aqueous extract of Mysuru veelyadele have recorded varied percentage of inhibition against *Rhizoctonia solani, Sclerotium rolfsii* and *Phomopsis azadirachtae* and Ambadi veelyadele recorded significant antifungal activity against *Rhizoctonia solani* . Other phytopathogens showed moderate growth in both the varieties. Seema *et al*., 2011, has reported the antifungal activity of Piper betle L. on sore shin pathogen, *Rhizoctonia solani*. The effect of these extracts was ineffective against *R. artocarpi.* Prince and prabakaran, 2011 have reported that aqueous extract of Piper betle L. was not effective against *Colletotrichum falcatum* which is a soil borne fungus.

Among the solvent extracts tested, hexane and ethyl acetate extract of Mysuru veelyadele has shown significant antifungal activity against *Phomopsis azadirachtae, Pyricularia oryzae, Rhizoctonia solani, Sclerotium rolfsii.* Seema *et al*.., 2011 have also reported that ethyl acetate extract of Piper betle L. possess inhibitory effect against *Rhizoctonia solani* causing sore shin of tobacco. *Ambadi veelyadele* was effective on only two phytopathogens viz., *Rhizoctonia solani* and *Sclerotium rolfsii*. Mrinoy *et al*., 2017 has reported that methanol extract of Piper betle L. have shown

significant antifungal activity on *Rhizoctonia solani*. The hexane extract have shown significant activity against *Aspergillus niger Penicillium digitatum* ,*Aspergillus flavus* and *Fusarium moniliforme* . Ethyl acetate extract, completely inhibited the growth of *Fusarium moniliforme* at very low concentration. Similar results have been reported on Piper betle L. leaves have significant potential in the integrated pest management of common contaminants in storage grains and soil borne pathogens by Seetha lakshmi and Naidu, 2013. *Rhizopus artocarpi* was inhibited by ethyl acetate extract at 100ppm but not in hexane extract. Shitut *et al*., have reported that ethyl acetate extract of Piper betle L. have shown significant activity against human pathogenic bacteria and phytopathogenic fungi.

Previous research literature on antifungal properties of Piper betle L. have shown that they have varying degrees of growth inhibitory effect on soil borne pathogens and post harvest pathogens due to their chemical compositon. Several workers have identified the chemical compounds of these plants and showed that those fractions are very efficient in suppressing the growth of fungi. The fungicidal property of Piper betle L. might be due to the presence of hydrochavicol in the extracts as reported by Ali *et al*.,2010; Nalina and Rahim,2007. The higher activity of ethyl acetate extract could be because of the fractions containing phenolic compounds. The results confirmed that these aqueous plant extracts and solvent extracts have antifungal properties on plant pathogenic fungi. Further research work is warranted to identify the bioactive compounds having antifungal activity in these two varieties. This study is an experimental evaluation and offers a possibility to make use of Piper betle L. in biopesicidal drug development to control the post harvest pathogens and soil borne pathogens.

## SUMMARY AND CONCLUSION

Piper betle L. (Piperaceae) is a tropical shade-loving perennial evergreen climber. It is an important medicinal plant and its use is mentioned in our vedic literature such as, Charaka Samhita, Sushruta Samhita and Astanga Hridayam. It is known to have medicinal properties such as antiseptic, analgesic, antibacterial, carminative and stimulant etc. This plant is having geographical indication tag. There are many varieties and difference among these is not well documented. Hence, two varieties of betel vine commonly cultivated in Mysuru viz., 'Mysuru veelyadele' and 'Ambadi veelyadele' were considered for the present study for comparing the chemical components. Preliminary quantitative phytochemical analysis for Carbohydrates, Phenolics, Flavonoids, Ascorbic acid and α-tocopherol were made by following standard procedures using aqueous and solvent leaf extracts. Variation

in the quantity of phytochemicals was observed in both the varieties. Significant biological activity was showed by ethyl acetate extract and hence this was partially purified using TLC, HPLC and LCMS. The separated molecules from TLC were subjected to HPLC for the confirmation of purity. Studies revealed the presence of Gallic acid, Transcinnamic acid, Caffeic acid, Chlorogenic acid, Synergic acid and Coumaric acid. In LCMS a molecule having molecular mass of 290.035 and retention time of 0.68 was found in both the varieties. The four compounds identified in Mysuru veelyadele were absent in Ambadi veelyadele. Further work on characterization of active principles is in progress.

The *in vitro* antimicrobial activity of aqueous and solvent leaf extracts of two different varieties of *Piper betle* L. namely Mysuru veelyadele and Ambadi veelyadele leaf extracts were studied against phytopathogenic fungi such as, *Fusarium oxysporum, Rhizopus artocarpi, Phomopsis azadirachtae, Pyricularia oryzae, Rhizoctonia solani, Sclerotium rolfsii* and post harvest pathogens *Fusarium moniliforme, Penicillium digitatum, Aspergillus niger* and *Aspergillus flavus* by poisoned food technique. Aqueous extract of both the varieties showed significant inhibition of *Rhizoctonia solani, Phomopsis azadirachtae* and *Sclerotium rolfsii* when compared to other pathogens. Among the solvent extracts tested, hexane and ethyl acetate extract of *Mysuru veelyadele* has shown significant activity against *Phomopsis azadirachtae, Pyricularia oryzae, Rhizoctonia solani, Sclerotium rolfsii* at

100 ppm and *Ambadi veelyadele* was effective on only two phytopathogens viz., *Rhizoctonia solani* and *Sclerotium rolfsii* at 100ppm. The remaining pathogens listed above were inhibited at 500ppm except *Rhizopus artocarpi*. Other solvent extracts of these two plant varieties have shown a range of activity against most of the phytopathogens tested.

This study has revealed the presence of many bioactive molecules in both the varieties of Piper betle L. It has further confirmed that the plant extracts could be used for control of phytopathogenic fungi and shows that Piper betle L. could be exploited for new potent antimicrobial agent against plant pathogen. This study forms a basis for the characterization of the phytochemicals of the two varieties. The presence of various bioactive compounds justifies the usage of betel leaf as an herbal choice for controlling various plant diseases. Aqueous and solvent extracts

are found effective, it will help in the formulation of ecofriendly control measure, which is cheap and can be recommended to the farmers.

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