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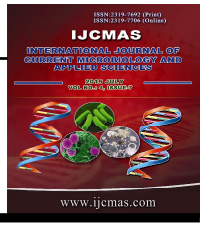

Original Research Article

Antimicrobial and Antioxidant Activity of Leaf Extracts of Two Indigenous Angiosperm Species of Tripura

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Keywords

Antimicrobial, Antioxidant, Leaf extract, *Schima wallichii*, *Melastoma malabathricum*, Indigenous plants

A B S T R A C T

Antimicrobial study of plant extracts are done for determining the ability of plant parts to kill or inhibit the growth of living pathogenic microorganisms by their extractable metabolic power. Antioxidant study is done for observing the ability of any sample that can scavenge the free radicals. In to-days stressful life role of antioxidants from natural sources are very important to combat them. In this study leaves of some indigenous angiosperm species were collected from Tripura and the ethanolic extracts were prepared to check their antimicrobial potential against a number of pathogenic organisms. The antibacterial study was done using the leaf extracts of *Schima wallichii*, *Phlogocanthus thyrsiflorus*, *Cissampelos pareira*, *Smilax zeylanica*, *Hydnocarpus kurzii*, and *Melastoma malabathricum*. *Schima wallichii* and *Melastoma malabathricum* showed the significant activities. Both the leaf extracts produced zones of inhibition in the range of 15mm to 20mm against the bacteria used in the antibacterial study. The MIC value of the crude extracts of *Schima wallichii* and *Melastoma malabathricum* were found to be 150µg/ml for one Gram positive and one Gram negative bacteria i.e. *Staphylococcus aureus* and *Escherichia coli* respectively. The extracts showed bacteriostatic mode of action against both *Staphylococcus aureus* and *Escherichia coli*. Further, the antifungal and the antioxidant activities were checked for these two indigenous angiosperms and the results were remarkable. In Antifungal study both the leaf extracts produced zone of inhibition ranging from 10 to 20 mm against the fungi used. In antioxidant assay IC_{50} of *Schima wallichii* and *Melastoma malabathricum* were found to be 14µg/ml and 16.5µg/ml respectively in comparison to the standard control ascorbic acid where the value was found 8.5µg/ml.

Introduction

In many developing countries bacteria has become a big threat for mankind. Due to its harmful effects many people are suffering enormously as a consequence of infections. Since the industrially made antibiotics are becoming resistant by many bacteria and are of very high cost it is not possible for the folk people to afford this. Now medicinal

plants are used by the folk people as an alternative source of medicine which is very economic to them (Jose *et al*., 2011). So in this study the potential of some indigenous angiosperm against some human pathogenic bacteria are studied and to show the path that they can be used in the treatment of various infectious diseases caused by pathogenic bacteria. Microbial Pathogens such as *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, Bacillus subtilis, Listeria monocytogenes, Pantoea ananatis* are widely distributed in nature responsible for mortality and morbidity in the population. It has been reported that there are many cases of human Salmonellosis annually with 3 million deaths worldwide. *E.coli* has been isolated from various environments and is reported to cause several deaths. *Staphylococcus aureus* causes a variety of suppurative (pus-forming) infections and more serious infections such as pneumonia, mastitis, meningitis, and urinary tract infections. *S. aureus* is a major cause of hospital acquired infection of surgical wounds and infections associated with indwelling medical devices. *E.coli, B.subtilis* are well known to causal agents of food poisoning.

Tripura a state of India (22□ 56' to 24□ 32'N latitude and 90□ 09' to 92□ 20'E longitude) encompasses the total area of 10,498 sq km. 6292 sq km of the state is covered by the forest. Due to the suitable annual rainfall and the temperature, Tripura has a great plant bio-diversity. A large number of ethnic people or tribe inhabited in this part of India (Awadh *et al*., 2004). There are several tribal people like Tripuri, Reang, Jamatia, Halam, Munda, Santhal, Chakma, Bhutia, Lepcha, and Magh. They have a vast knowledge of using plants, and plant products for curing diseases in ethnic mode (Toledo *et al*., 2009). Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which have been found in-vitro to have antimicrobial properties. The leaf extract of different plants have been used in the present work to check their antibacterial effectiveness against animal bacterial pathogens were *Phlogocanthus thyrsiflorus, Schima wallichii, Smilax zeylanica, Hydnocarpus kurzii, Cissampelos pareira, Melastoma malabathricum*.

Little account of antimicrobial properties of these plants are described below:

***Phlogocanthus thyrsiflorus* Nees.**

Due to the presence of phytochemicals like saponins, diterpene lactone, flavonoids in the leaves the plant has medicinal value. It is effective in Whooping cough, fever etc. Leaf part is beneficial in liver and spleen diseases. It has prominent free radical scavenging property so it may prove as a very good medicinal herb (Upadhyay, 2009). It has antimicrobial activity also (Singh and Singh, 2010).

***Schima wallichii* (DC.) Korth.**

Schima wallichii has anti-cancer activities, and have the ability to induce apoptotic mechanisms (Diantini et al., 2012). Several phytoconstituents are present in this plant like flavonoids, alkaloids and triterpenoids and shows antimicrobial activity against wide range of microorganisms (Saikat *et al*., 2008).

Some research on this plant reported that ethanol extract of leaves has cytotoxic activity to leukemia cancer cells. Ethyl acetate fraction of leaves has cytotoxic and pro-apoptosis activity to leukemia and breast cancer cells leukemia.

***Smilax zeylanica* L.**

Phytochemical analysis of the plant extract revealed the presence of reducing sugars, tannins, saponins, gums, steroids, alkaloids, and flavonoids. Traditionally, roots and leaves of *S. zeylancia* are used as a substitute for the official drug, Sarasparilla, in the treatment of venereal diseases; decoction is applied for rheumatism, pain in the lower extremities, sores swellings, and abscesses, and also used in the treatment of dysentery. Leaves and roots contain diosgenin (Kar and Sen, 1984).

***Hydnocarpus kurzii* (King.) Warb.**

The seed oil is known as hydnocarpus oil, leprosy oil. The active principles of the oil are hydnocarpic and chaulmoogric acids which are strongly antibacterial. It also shows the thrombolytic activity (Sikder *et al*., 2011).

***Cissampelos pareira* L.**

The phytochemical tests demonstrated the presence of phytochemicals like alkaloids, flavonoids, tannins, terpenoids in root extract (Ngoci *et al*., 2014). *Cissampelos pareira* root shows protective nature against gastric cancer.

***Melastoma malabathricum* L.**

The phytochemical constituents of the dried leaf extract contain flavonoids, triterpenes, tannins, saponins and steroids, but not alkaloids. Based on the HPLC analysis several flavonoids, namely quercetin, quercitrin and rutin were found in methanol extract. Methanol extract of *Melastoma malabathricum* leaves exerted antioxidant and liver protective activity in rats (Siti *et al*., 2013).

Materials and Methods

Collection of plant materials

A thorough survey was done on indigenous angiosperm flora of Tripura near Udaipur area. Based on the survey we have selected seven plants and collected them carefully and were taken to the Mycology-Plant Pathology laboratory Department of Botany, Visva Bharati. Voucher specimens were kept in the herbarium of Department of Botany, Tripura University. The plant members are as following: *Schima wallichii* (Theaceae), *Phlogacanthus thyrsiflorus* (Acanthaceae), *Cissampelos pareira* (Menispermaceae), *Smilax zeylanica* (Smilacaceae), *Melastoma malabathricum* (Melastomataceae), *Hydnocarpus kurzii* (Achariaceae), *Lepidagathis incurve* (Acanthaceae). Fresh leaves were washed thoroughly 2-3 times with running tap water and then with sterile water followed by shade dried, powdered and used for extraction.

Solvent extraction of dried leaf

2 grams of shade dried, powder of leaf of each plant materials were filled separately in the conical flask and extracted successively with 50 ml of ethanol. Then they were kept in the rotary shaker for 36 hours at 28^{o}C at 100 rpm. After shaking the contents of the conical flask were taken in the centrifuge tube. Then the materials were centrifuged in cooling centrifuge at 10,000rpm for 10 minutes. Supernatant from respective tube were taken and poured in the petridishes and allowed to evaporate under the aseptic condition. Petridishes were kept open in the open air for two days for evaporation of the alcohol. After the reduction of volume it was passed through Cellulose Acetate membrane filter (0.22µ pore size) and put inside laminar air flow for complete evaporation.

Each of these solvent extract was weighed and dissolved in 2 ml DMSO. Finally they were preserved at 4□ C in airtight bottles for further use.

Antibacterial effect of the leaf extract

Pathogenic bacteria such as *Escherichia coli* MTCC1667, *Staphylococcus aureus* MTCC96, *Salmonella typhimurium* MTCC98, *Pseudomonas aeruginosa* MTCC741, *Bacillus subtilis* MTCC121, *Listeria monocytogenes* MTCC657, *and Pantoea ananatis* MTCC2307 were used in the present study. These bacterial strains were treated with the leaf extracts of *Phlogocanthus thyrsiflorus*, *Schima wallichii*, *Smilax zeylanica*, *Hydnocarpus kurzii*, *Cissampelos pareira*, *Melastoma malabathricum.* The assay for antibacterial activity of each plant leaf extract was tested by disc diffusion method (Kirby *et al*., 1966). Bacterial suspensions (1.5×10^8) were inoculated on Nutrient Agar plates evenly using sterile swab. The Whatman No. 1 filter paper was used to prepare the paper disc (6mm in diameter). Then the discs impregnated with the leaf extracts were placed individually on the Nutrient Agar plates with flamed forceps. A standard commercial disc of Ciprofloxacin 10 µg/ml was used as positive control and DMSO impregnated disc was used as negative control in each case. The plates were incubated at 37˚C for 24 hours in inverted position. Zones of inhibition produced by the sensitive organisms were observed by a clear circular area around the discs and were compared with the zone of inhibition produced by the positive control (Ciprofloxacin 10 µg/ml) and the negative control (DMSO impregnated disc).

MIC Study

Since the leaf extract of *Schima wallichii* and *Melastoma malabathricum* showed a great antibacterial activity, MIC study of these two extracts was studied against one Gram positive and one Gram negative bacteria i.e. *Staphylococcus aureus* and *Escherichia coli* respectively. The MIC was determined by counting the Colony Forming Units (CFU). Different concentration of these plant extracts including 25, 50,100,150,200 µg/ml were used in this study. The ethanolic fraction of these plant materials were dissolved in DMSO and added to the Nutrient Broth in different concentration. After adding of fixed volume of bacterial culture, culture tubes were incubated at 37ºC for overnight. Then 100 µl of cultures were taken from each tube and spread on Nutrient Agar plates using glass rod spreader after proper dilution. Then all the plates were incubated at 37ºC for overnight. On the next day CFU were counted and CFU/ml was calculated. MIC value for each plant extract was also calculated.

Mode of action of the plant extracts

To determine the mode of action of the plant extracts, time killed study with the leaf extracts of *Schima wallichii* and *Melastoma malabathricum* was performed against *Staphylococcus aureus* and *Escherichia coli* respectively. For this purpose the leaf extracts were added to the actively growing liquid cultures of the bacteria at their minimum inhibitory concentration found previously. Activities of the leaf extracts in relation to time were measured by colony count method (Ray et al., 1999).

Antifungal activity of the plant extracts

The ethanolic leaf extracts of *Schima wallichii* and *Melastoma malabathricum* were prepared and their antifungal activity was tested against *Helminthosporium oryzae*

MTCC351, *Alternaria alternata* VBAV007, *Fusarium oxysporum* MTCC2480, *Colletotrichum acutatum* MTCC281, *Candida albicans* MTCC1644, *Aspergillus parasiticus* MTCC2796 procured from IMTech, Chandigarh. The assay for antifungal activity of each plant leaf extract was tested by agar well diffusion method (Fernandez-Garayzabal *et al*., 1992). Fungal suspensions were cultured in Malt Extract Broth for 1-3 days depending on their growth pattern. 100µl of these cultures were spread on Malt Extract Agar in petri dishes. Then the wells were prepared on the petri dishes with the help of the cork borer. In each well 50µl of leaf extracts were poured. Then the plates were kept in incubation at 28ºC for 1 to 3 days. The fungi which showed clear zone were considered sensitive and those not as resistant.

Antioxidant activity (DPPH free radical scavenging activity) of the plant extracts

Antioxidant activity of the plant extracts was done on the basis of the radical scavenging effect of the stable DPPH (2 2-diphenyl-2-picrilhydrazyl) radical (Braca *et al*., 2002). The solutions of the plant extracts were prepared in methanol as 0.01 gm of plant extracts were dissolved in 1 ml of methanol. 0.004% of DPPH was prepared. Different concentrations from 5 to 50 µg/ml were prepared by mixing the DPPH stock solution and respective amount of the methanolic solution of the plant extracts. Ascorbic acid was used as standard in the same concentrations. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using Spectrophotometer. Methanol with DPPH solution (0.004%) was used as blank. The optical density was recorded and % inhibition was calculated using the formula given below:

Percentage of inhibition of DPPH activity (%) = A-B/A×100

Where A = optical density of the blank and B = optical density of the sample.

Results and Discussion

Antibacterial effect of the leaf extract

All the bacteria were sensitive to the positive control and resistant to the negative control. All the bacteria were also found sensitive to the leaf extracts of *Schima wallichii* and *Melastoma malabathricum*. Both *Schima wallichii* and *Melastoma malabathricum* showed the maximum activity against *E.coli*, *Pseudomonas aeuruginosa*, *Staphylococcus aureus* and *Listeria monocytogens*. *Hydnocarpus kurzii* also exhibited good activity against *Listeria monocytogens* and moderate activity against *E.coli*. *Phlogocanthus thyrsiflorus* and *Smilax zeylanica* showed morderate potential against *Bacillus subtilis and E.coli* respectively. The measurement of inhibition zones (in mm) produced by the leaf extracts and the positive control are presented in the Table 1 and Figures 1 and 2. From the above study it was observed that leaf extracts of *Schima wallichii* and *Melastoma malabathricum* were most effective against all the selected microorganisms.

Minimum Inhibitory Concentration (MIC) of the plant extracts

The Minimum Inhibitory Concentration (MIC) of the leaf extracts against *Staphylococcus aureus* and *Escherichia coli* were determined by counting the Colony Forming Units (CFU). The MIC values of both *Schima wallichii* and *Melastoma malabathricum* against both the bacteria were found to be 150µg/ml (Table 3 and 4).

Table.1 Zone of inhibitions (in mm) as shown by ethanolic extracts (50mg/ml) of selected plant species of Tripura against bacteria

	E. coli	*Bacillus subtilis*	*Pantoea ananatis*	*Salmonella typhimurium*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Listeria monocytogens*
Phlogocanthus Thyrsiflorus	--	8	--	--	--	--	--
Schima Wallichii	20	17	17	8	19	19	18
Smilax Zeylanica	10	--	--	--	--	--	--
Hydnocarpus Kurzii	8	--	--	--	--	--	18
Cissampelos Pareira	--	--	--	--	--	--	--
Melastoma malabathricum	20	18	17	17	18	18	20
Ciprofloxacin (10µg/ml)	20	20	20	20	21	21	20

Table.3 MIC of *Schima wallichii* against *Staphylococcus aureus* and *Escherichia coli*

	CFU/ml	
	Staphylococcus aureus	*Escherichia coli*
Concentration (µg/ml)		
Control	1.2× 10□	3.3× 10^9
25	1.5× 10^8	6.0× 10^8
50	1.2× 10^8	1.5× 10^8
100	1.0× 10^8	1.2× 10^7
150	**2.0× 10^6**	**8.3× 10^5**
200	1.5× 10^4	3.1× 10^3

Table.4 MIC of *Melastoma malabathricum* against *Staphylococcus aureus* and *Escherichia coli*

	CFU/ml	
	Staphylococcus aureus	*Escherichia coli*
Concentration (µg/ml)		

Control	1.0× 10□	2.0× 10^9
25	1.8× 10^8	3.0× 10^8
50	1.5× 10^8	2.5× 10^8
100	1.2× 10^8	1.6× 10^7
150	**1.0× 10^6**	**4.2× 10^5**
200	3.0× 10^3	1.2× 10^3

Figure.1 Antibacterial effects of the leaf extracts (50mg/ml)

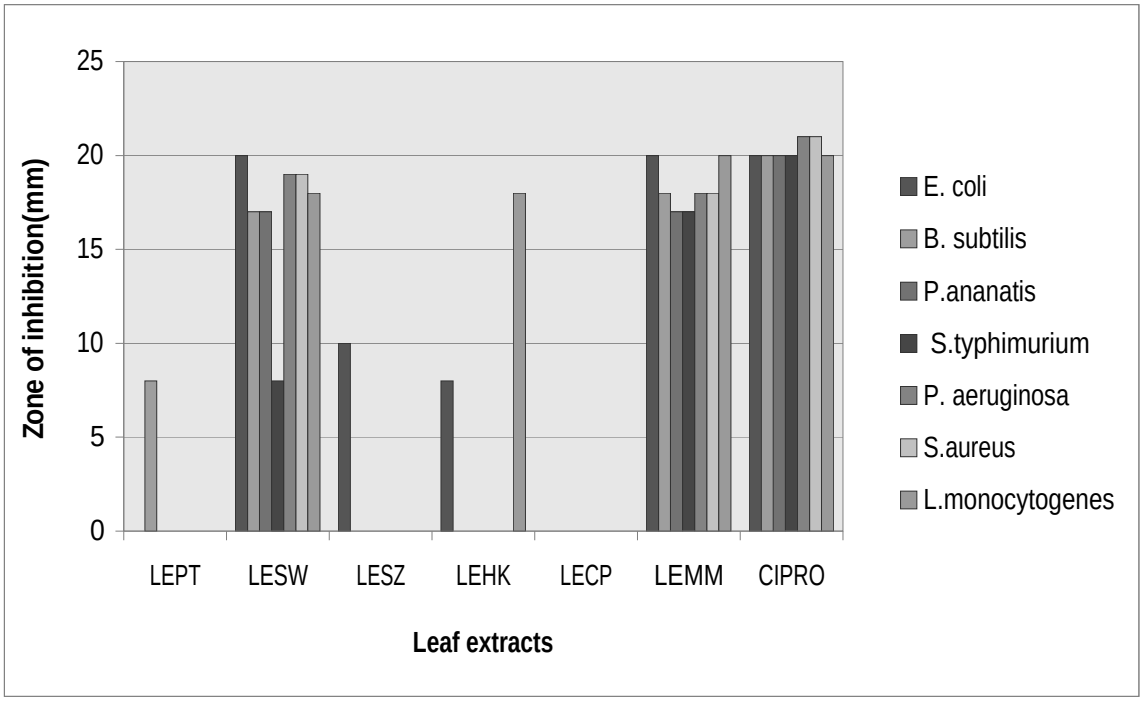


Figure.2 Antibacterial effects of the leaf extracts (25mg/ml)

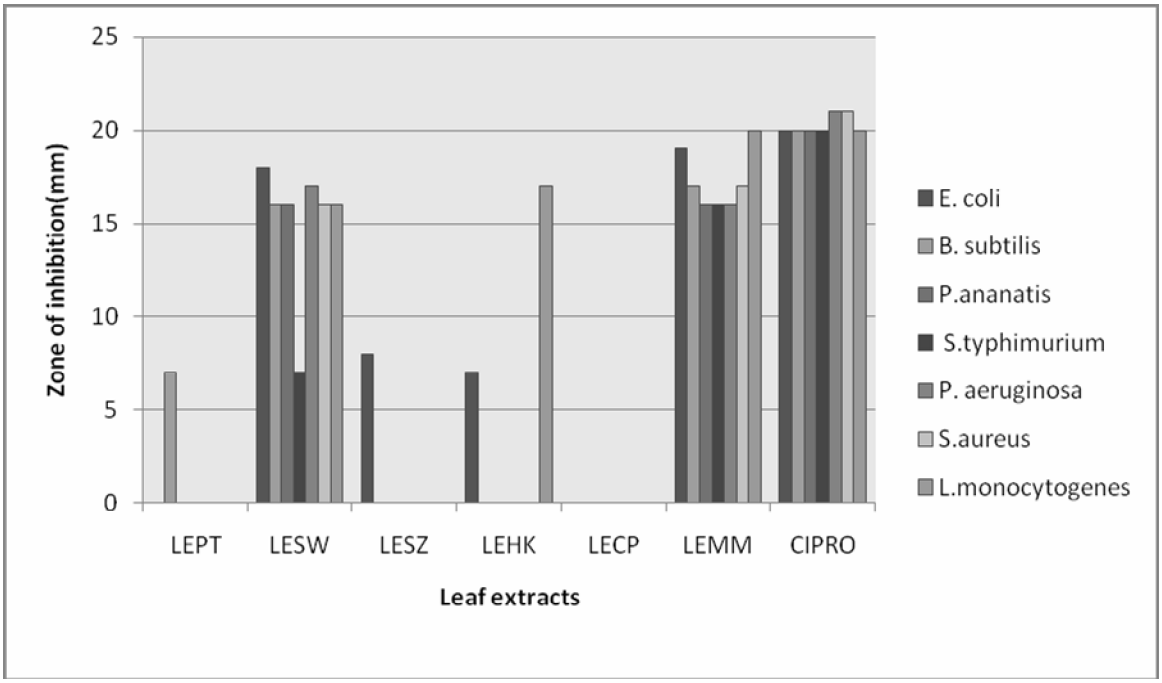


Figure.3 Mode of action of leaf extracts of *Schima wallichii* on *Staphylococcus aureus*

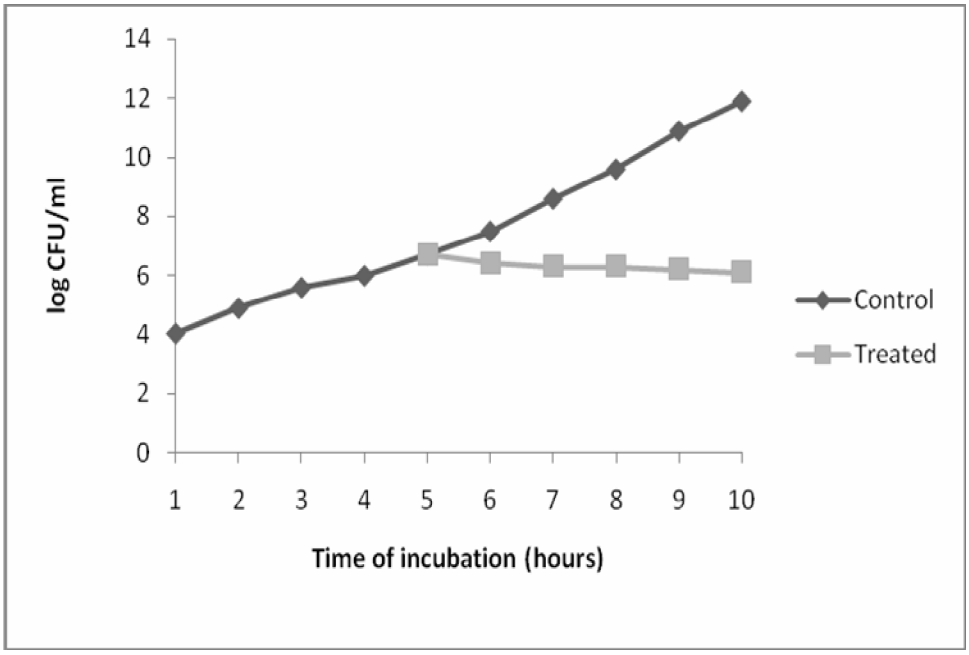


Figure.4 Mode of action of leaf extracts of *Schima wallichii* on *Escherichia coli*

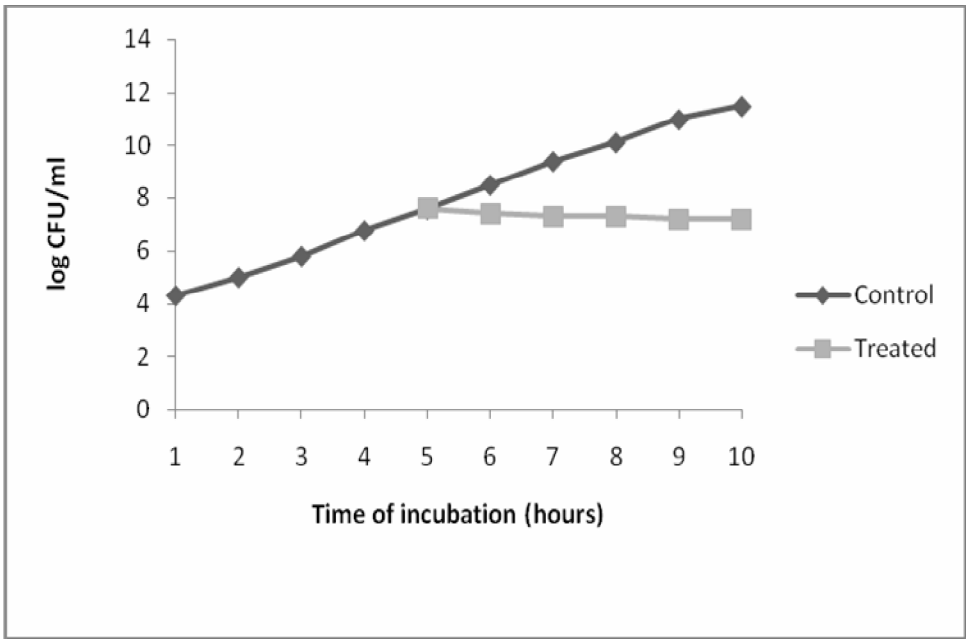


Figure.5 Mode of action of leaf extracts of *Melastoma malabathricum* on *Staphylococcus aureus*

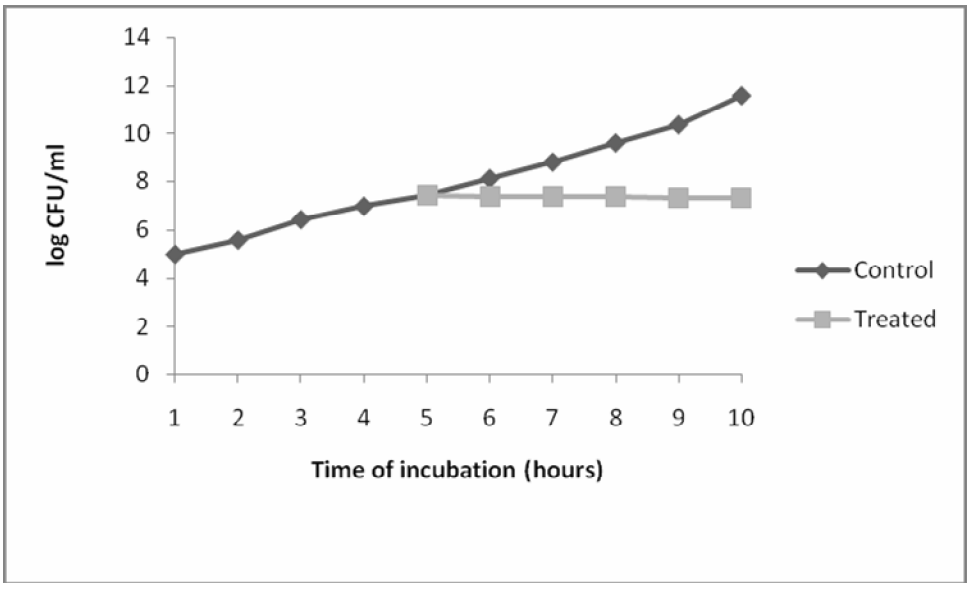


Figure.6 Mode of action of leaf extracts of *Melastoma malabathricum* on *Escherichia coli*

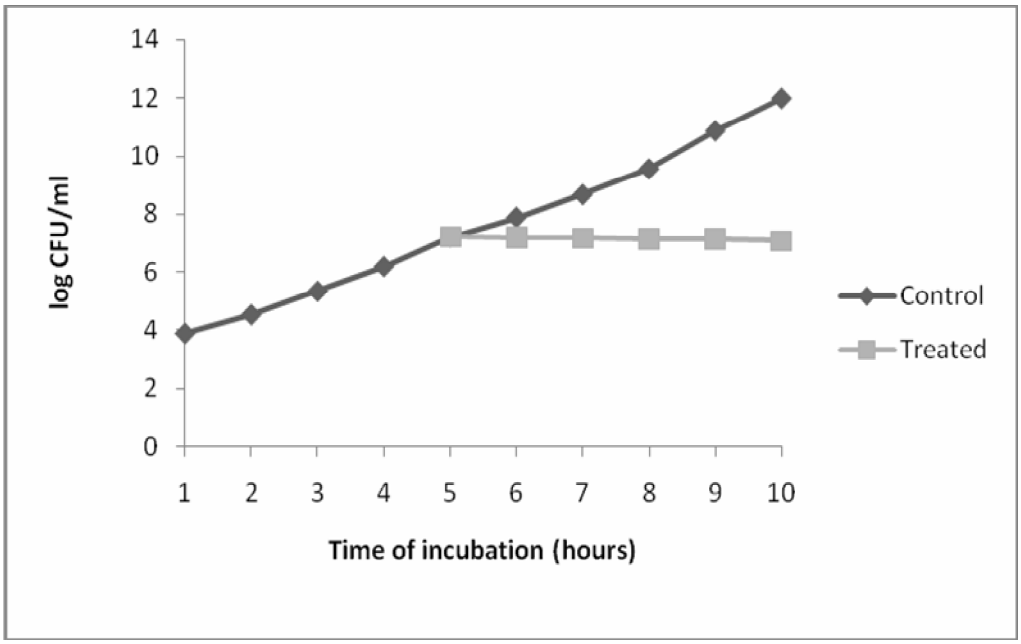


Figure.7 Antifungal activity of the leaf extracts (50mg/ml)

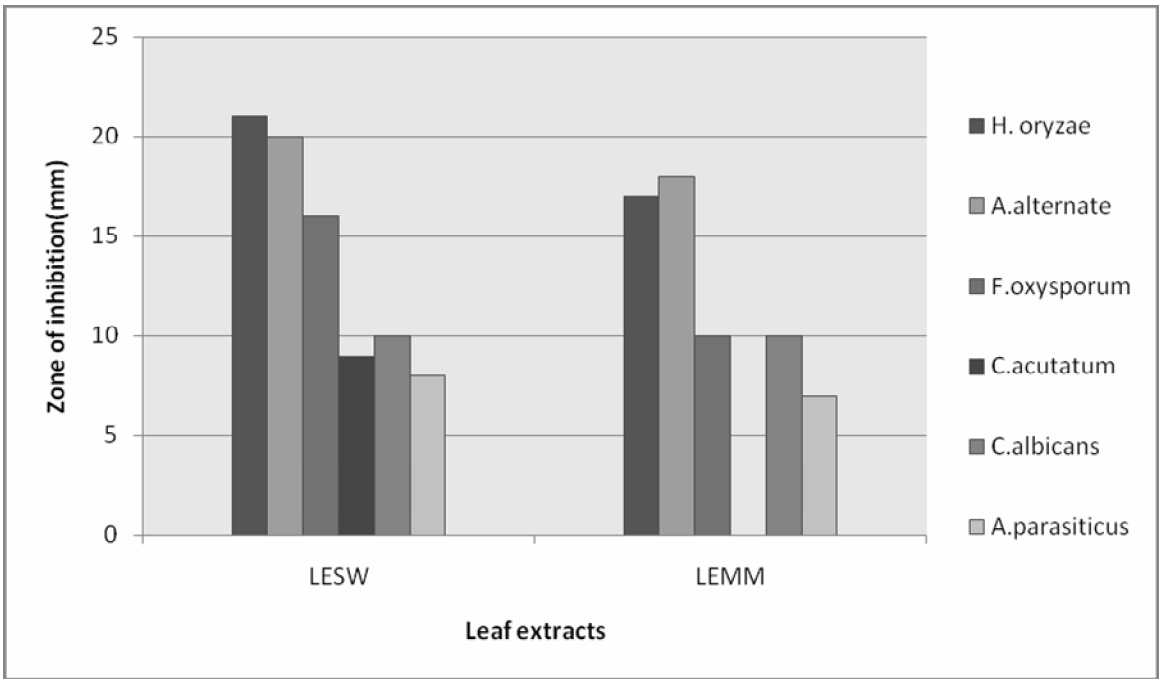


Figure.8 Antifungal activity of the leaf extracts (25mg/ml)

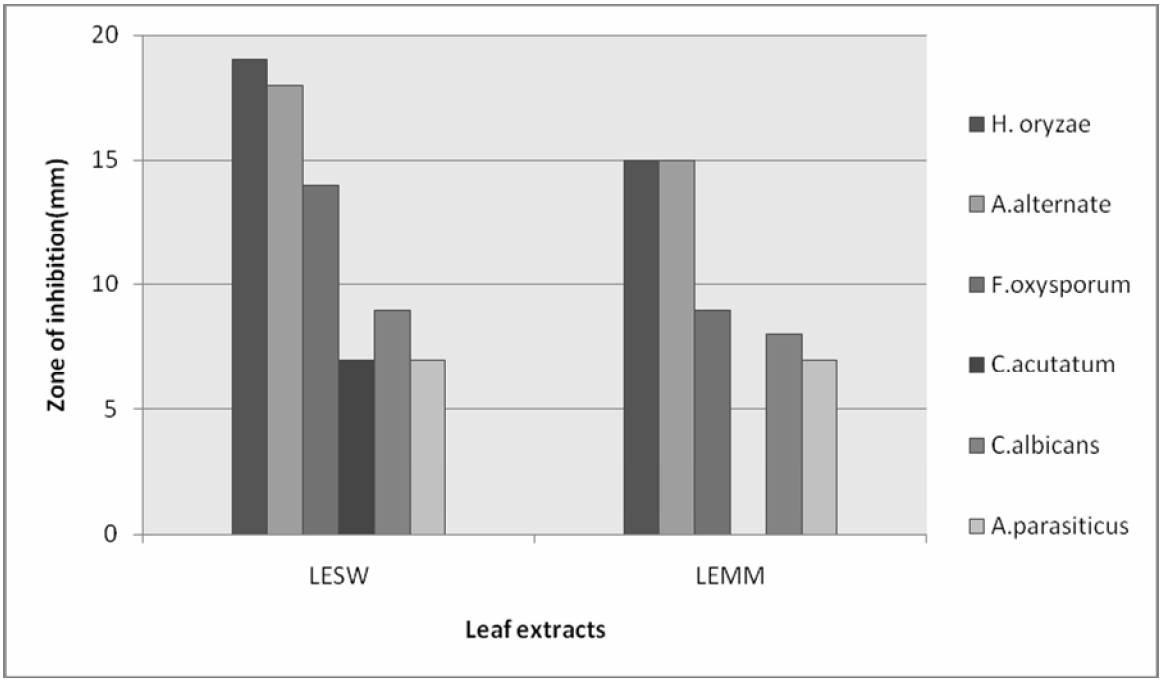
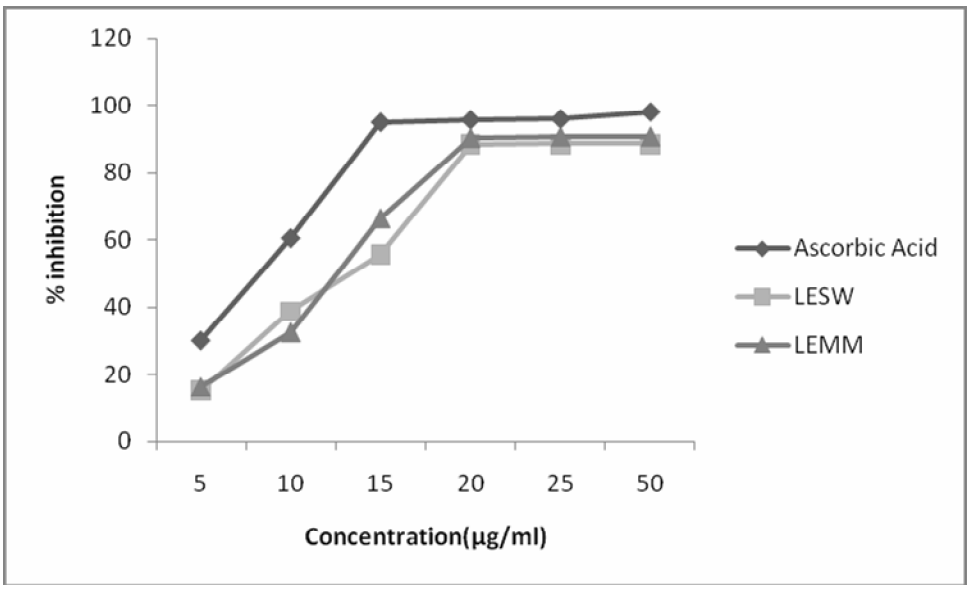


Table.3 Zone of inhibitions (in mm) as shown by ethanol leaf extracts against selected fungi

		Helminthosporim oryzae	*Alternaria alternate*	*Fusarim oxysporum*	*Colletotrichum acutatum*	*Candida albicans*	*Aspergillus parasiticus*
Schima Wallichii	25 mg/ml	19	18	14	7	9	7
	50 mg/ml	21	20	16	9	10	8
Melastoma malabathricum	25 mg/ml	15	15	9	--	8	7
	50 mg/ml	17	18	10	--	10	8

Figure.9 DPPH free radical scavenging activity of standard ascorbic acid and leaf extracts



Mode of action of the plant extracts

Mode of action of the leaf extracts were determined by counting the Colony Forming Units (CFU) of every hour. Both the leaf extracts showed bacteriostatic mode of action against *Staphylococcus aureus* and *Escherichia coli* (Figure 3, 4, 5, and 6).

Antifungal activity of the plant extracts

The inhibition zones were found on the ME plates after 2-3 days around the sensitive fungi. All the fungi were sensitive to the leaf extracts of *Schima wallichii* and *Melastoma malabathricum*. *Colletotrichum acutatum* was only resistant to *Melastoma malabathricum*. Leaf extracts of *Schima wallichii* and *Melastoma malabathricum* showed great activity against the plant pathogenic fungi *Helminthosporium oryzae* and *Alternaria alternata* and morderate activity against human pathogenic fungi *Candida albicans* and *Aspergillus parasiticus*. The measurement of inhibition zones (in mm) produced by the leaf extracts are presented in the table: 3 and figure: 7 and 8.

Antioxidant activity of plant extracts

The two crude ethanolic extracts of *Schima wallichii*, *Melastoma malabathricum* and standard Ascorbic acid tested for the in vitro antioxidant activity using the DPPH method showed antioxidant activity, with IC_{50} values of 14µg/ml, 16.5µg/ml and 8.5µg/ml respectively. The DPPH scavenging activity is represented in the figure: 9.

The leaf extracts of *Schima wallichii* and *Melastoma malabathricum* showed better antibacterial potential in comparison to other leaf extracts, and these two leaf extracts were used in antifungal as well as antioxidant study. Both the leaf extracts showed good activity against human pathogenic fungi like *Candida albicans*, *Aspergillus parasiticus* as well as plant pathogenic fungi like *Alternaria alternata*, *Helminthosporium oryzae* etc. *Candida albicans* causes oral and vaginal candidiasis, *Aspergillus parasiticus* causes aspergillosis. *Alternaria alternata* causes leaf spot, *Helminthosporium oryzae* causes brown spot of rice, *Colletotrichum acutatum* affects the fruit crops and *Fusarium oxysporum* causes the Fusarium wilt. Thus ability to kill such an enormous spectrum of pathogens belonging to both eukaryotes as well as

prokaryotes causing diseases both to plants and humans indicates a great prospect of using leaf extracts of these two indigenous angiosperm species of Tripura. Antioxidation potential at very low concentration also added value to these plant extracts towards their applicability.

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