

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.810.058>

Antifungal Activity of Important Botanicals against Plant Pathogens

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ABSTRACT

Keywords

Medicinal plants,
Sequential
extraction,
Antifungal activity,
Agar-well diffusion

Article Info

Accepted:
07 September 2019
Available Online:
10 October 2019

Medicinal plants are store house of remedies to cure all ailments of mankind. The use of plants as medicine is widespread throughout the world. The secondary metabolites present in the plants showed various biological activities and act in plant defence mechanism. Various pathogenic organisms such as bacteria, fungi, viruses attack plants at various stages of their development and thereby reduce their yield and productivity. Among all the pathogenic organisms, phytopathogenic fungi cause severe losses in plants and crop-production. However, these fungal diseases can be managed by the use of synthetic fungicides but due to the overzealous and indiscriminate use of these synthetic fungicides, has created different types of environmental and toxicological problems. Therefore, natural products symbolize safety in contrast to the synthetics as they have eco-friendly approach and are cheap. In the present investigation, the methanol and aqueous extracts of leaves as well as rhizome of three different medicinal plants viz. *Azadirachta indica*, *Lantana camara* and *Curcuma longa* were used against five fungal phytopathogens viz. *Curvularia lunata*, *Bipolaris specifera*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Alternaria alternata* by agar well diffusion method. Methanolic extracts of plant parts showed maximum antifungal activity against the phytopathogens than the aqueous extract. *Curcuma longa* rhizome extract showed antifungal activity against all the plant pathogens and maximum zone of inhibition was showed at conc. 200µl/ml against *Rhizoctonia solani*, *Bipolaris specifera*, *Curvularia lunata* and *Macrophomina phaseolina* (11mm, 7.66mm, 8mm and 7mm respectively), followed by leaf extract of *Curcuma longa* and *Azadirachta indica*. The minimum activity was showed by *Lantana camara*. Phytochemical screening of plant extracts revealed that the maximum phytoconstituents (Saponin, steroid, alkaloid, Flavonoid, carbohydrate, tannin, anthocyanin) is present in methanolic extracts than the aqueous extract. Hence, it was concluded that methanolic extract possess sufficient antifungal activity under controlled conditions to warrant a further investigation under field conditions.

Introduction

The state of Jammu and Kashmir possess a significant portion of Himalayas (Western

Himalayas) with areas of high altitude, cold deserts and immense plant diversity including a Plethora of medicinal and aromatic plants (Hanson *et al.*, 2009) are known to have

medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times (Mahalingam *et al.*, 2011). These plants are reported to have pharmacological properties due to the presence of various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes which possess antimicrobial properties. Various insects, bacteria, viruses, fungi and pests attack plants at various stages of their development (Tapwal *et al.*, 2011), thereby reducing their productivity and leading to huge loss. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related aspect, nutritional value, organoleptic characteristics, and limited shelf life (Agrios, 2004). The harvest losses due to fungal diseases in all over world may reach upto 12% or even higher for developing countries (Hadizadeh *et al.*, 2009). Generally, phytopathogenic fungi are managed by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Harris *et al.*, 2001). The use of biodegradable material like fresh plant extract from different parts has gained importance during last few decades for plant disease control (Bajwa *et al.*, 2004).

Natural products seem to be viable solution to the environmental problems caused by the synthetic pesticides (Kim *et al.*, 2003).

These plant extracts are safe, eco friendly, easily biodegradable and cheap as they produce secondary metabolites which perform defensive role in plant and protect plant from invaders. These medicinal plants needs to be investigated in terms of antimicrobial and biochemical properties for better understanding of their properties, safety and efficiency (Bajwa *et al.*, 2007). Turmeric (*Curcuma longa* L.) is a therapeutic plant that

belongs to family Zingiberaceae. It is a prompt source of bioactive compounds like antioxidants, polyphenols and flavonoids, which may be the substitute of antibiotics used in food and food products (Chainani-Wu, 2003). Chloroform and ether extracts of turmeric have proved antibacterial, antifungal, antiviral and anti-protozoan. *Azadirachta indica* belongs to the family Meliaceae, is a evergreen tree found in most tropical countries. Fungicidal properties of neem extracts are promising and significantly reduced conidial germination in several fungi (Hanif *et al.*, 2013). The antifungal effect of leaf extract of neem against *Alternaria alternata* is well documented (Bhomick and Choudhary, 1982). *Lantana camara* is a low erect, rugged hairy, evergreen shrub (Verbenaceae) native to tropical America, extracts from the *lantana camara* leaves exhibit antimicrobial, insecticidal and nematicidal activity and also contain verbascoside, which possess antimicrobial, immunosuppressive and antitumor activities (Adiquzel *et al.*, 2005). Biologically active compounds present in these plants have been recognized as an important factor in plant disease resistance (Mahadevan, 1982). Such compounds, being biodegradable and selective in their toxicity, are considered valuable for controlling plant diseases (Singh and Dwivedi, 1987).

Materials and Methods

Survey and collection of plant samples

Healthy plant samples such as fresh leaves of *Azadirachta indica*, *Lantana camara* and fresh leaves as well as rhizome of *Curcuma longa* were collected from four different locations viz. R.S. Pura, Miran Sahib, Chatha, Chakrohi of Jammu Division (Appendix-I). The collected plant parts were brought to the laboratory in sterile zip lock polythene bags for further studies.

Processing of plant samples

The collected samples (leaves and rhizome) were washed thoroughly 2-3 times with running water and once with sterile distilled water to remove the adhering soil and other impurities. The cleaned plant part material were air dried on sterile blotter under shade and blended to form a fine powder and stored in airtight containers for further analysis (Salvamohan *et al.*, 2012).

Preparation of plant extract

Preparation of leaf extract

Methanolic and aqueous extracts of all the three medicinal plants were prepared by dissolving 10gm of fine powder of each medicinal plant separately in 50ml of methanol and water respectively. Extracts were kept in orbital shaker for 48 hours. Then the extracts were filtered and dried in hot air oven at 40°C. The extract then stored under refrigeration at 4°C for further studies (Salvamohan *et al.*, 2012).

Preparation of Rhizome extract

Fresh *Curcuma longa* rhizome washed with distilled water to remove soil and other impurities, cut into small pieces of ¼ inches size and air dried for 2 days. The dried sample was again dried in hot air oven at 50°C for 24 h, then grounded into powder and pass through sieve with nominal mesh size of 2mm diameter (Harit *et al.*, 2013). Methanolic and aqueous extract of *Curcuma longa* (rhizome) was prepared similarly as discussed earlier in 3.3.1.

Growth and maintenance of test microorganisms for antifungal studies

Five fungal pathogens, *Curvularia lunata*, *Bipolaris specifera*, *Rhizoctonia solani*,

Macrophomina phaseolina and *Alternaria alternata* were obtained from the Division of Plant Pathology, SKUAST-J, Chatha. These fungal cultures were purified on potato dextrose agar medium by subculturing and were stored in the refrigerator as slants at 10°C for the assay and further use (Jeyasakthy *et al.*, 2013)

Preparation of standard fungal suspensions

The fungal cultures (*Curvularia lunata*, *Bipolaris specifera*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Alternaria alternata*) were maintained on potato dextrose agar, incubated at 25±2°C for four days.

The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100ml) of sterile normal saline and the suspension was maintained for further use (Grover *et al.*, 2011).

Screening of plant extracts against fungal pathogens

Antifungal activity of plant extracts against fungal pathogen was done by agar well diffusion method (Perez *et al.*, 1990). The PDA medium were poured into the sterile Petriplates and allowed to solidify. The fungal suspensions were evenly spread over the media by sterile cotton swabs. Wells (6mm) were made in the medium using sterile cork borer and various concentrations (50,100,150,200µl/ml) of each plant extracts were added to the wells. The plates were incubated at 25±2°C for 48-72 hrs. After incubation the plates were observed for formation of clear incubation zones around the well indicated the presence of antifungal activity. The zones of inhibition were calculated with the help of scale. Each experiment was repeated thrice and the mean value was taken (Prince and Prabakaran, 2011).

Preliminary phytochemical test

The aqueous and methanol extracts of leaves of *Azadirachta indica*, *Lantana camara* and leaves as well as rhizome of *Curcuma longa* were screened to determine the presence of the following chemical constituents (viz. Alkaloid, Flavonoid, Tannin, Saponin, Steroid, Carbohydrate, anthocyanin etc.) through preliminary Phytochemical test by their colour reaction (Sawant and Godghate, 2013; Singh and Chauhan, 2014).

Test for Alkaloid

For detection of alkaloid, to 1ml of the filtrate, add 2ml of Dragendorff's reagent, it showed turbid orange colour which confirmed the presence of alkaloid.

Test for tannin: Ferric chloride test

To 2ml of the filtrate, 1-2 drops of 5% ferric chloride solution was added.

A dark green colour indicated the presence of tannin.

Test for saponin: Foam test

For detection of saponin:

5ml of extract was mixed with 20ml of distilled water

Then agitated in graduated cylinder for 15 mins.

A thin layer of foam measuring 1cm was formed which indicated the presence of saponin.

Test for steroid: Salkowski test

For detection of Steroid:

1ml of extract was dissolved in 10ml of chloroform

Equal volume of concentrated H_2SO_4 (Sulphuric acid) was added from the side of test-tube.

The upper layer turned red and H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroid.

Test for anthocyanin

For detection of anthocyanin:

To 1ml of filtrate add 5ml of dilute HCL

It showed the presence of pale pink colour which confirmed the presence of anthocyanin.

Test for carbohydrates: Benedict's test

Equal volume of reagent was added to equal volume of filtrate

Heat it to boil in a water bath for 3-5 mins

Occurrence of green colour showed the presence of reducing sugar

Test for phenolic flavonoid: Lead acetate test

To 1ml of filtrate, add 2ml of 10% lead acetate

Brown precipitate formed which confirmed the presence of phenolic flavonoid

Test for flavonoid: Alkaline reagent test

To 1ml of the filtrate, add 2ml dilute NaOH

Golden yellow colour formed showed the presence of flavonoid

Statistical analysis

Experimental data obtained from the investigation was analysed by using Factorial CRD (Completely Randomized Design) (Sheoran *et al.*, 1998).

Results and Discussion

Screening of plant extracts against plant pathogens

The result presented in Table 1 and 2 showed that Extracts of *Curcuma longa* rhizome showed antifungal activity against all the plant pathogens. The highest inhibition was recorded in *Rhizoctonia solani* (11.00mm) followed by *Bipolaris specifera* (8.00mm) and the lowest activity was recorded in *Macrophomina phaseolina* (7.00mm) whereas extract of *Curcuma longa* leaves showed antifungal activity against four pathogens. The highest activity was recorded in *Curvularia lunata* (10.00mm) followed by *Bipolaris specifera* (7.00mm) Our result are combats with the results of (Mahalingam *et al.*, 2011; Saxena and Sahu, 2012; Rathaur *et al.*, 2012), they observed the presence of various acids (O-coumaric acid, protocatechuic acid, synergic acid and vanillic acid) and bioactive compounds in *Curcuma longa* leaves and rhizome that possess antibacterial, antifungal, anti-carcinogenic, anti-diabetic, anti-viral activity. Leaf extract of *Azadirachta indica* significantly inhibited the mycelia growth of *Alternaria alternata* (10.66mm), *Rhizoctonia solani* (7.33mm), *Curvularia lunata* (3.13mm) and *Macrophomina phaseolina* (2.83mm).

The result of our studies are in confirmatory with Aslam *et al.*, (2010) who observed that *Azadirachta indica* leaf extract inhibited the fungal growth because of the presence of certain secondary metabolites (quercetin and β -sitosterol) in the leaves that possess antimicrobial properties (Subapriya and

Nagini., 2005; Mahmoud *et al.*, 2011) whereas the leaf extract of *Lantana camara* showed antifungal activity against *Curvularia lunata* (5.63mm) only (Plate 1&2). Our results combats with (Saraf *et al.*, 2011) they reported that *Lantana camara* showed antifungal activity against various fungal pathogens including *C. lunata* because of its broad antimicrobial spectrum, (ferulic acid, anisic acid, ambrosin etc.).

The Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate (Ncube *et al.*, 2008). In present study, fresh and healthy plant samples of *Azadirachta indica* leaves, *Lantana camara* leaves, *Curcuma longa* leaves as well as rhizome were taken and the extracts were prepared by using two different solvents viz., methanol and aqueous and the result revealed that methanolic extracts showed the best results over aqueous extracts.

Using organic solvent showed more consistent antimicrobial activity as compared to water (Parekh *et al.*, 2005) as the aqueous extracts showed the least activity against plant pathogens because when plant materials are grounded in water, some phenolases and hydrolases are released and could have modulating effects on the activity of the compounds in the extracts and it could also be due to incomplete extraction of the active principles (Mahmood *et al.*, 2008).

Phytochemical tests

The methanolic and aqueous extracts of the plants revealed the presence or absence of certain phytochemicals (viz. Tannin, saponin, steroid, phenolic flavonoid, flavonoid, carbohydrate, anthocyanin, alkaloid etc.) in the selected botanicals (Table 3).

Table.1 Growth inhibition of *Curvularia lunata*, *Bipolaris specifera* and *Rhizoctonia solani* by the methanolic and aqueous extract of different plants

Plant Part	Pathogens																							
	<i>Curvularia lunata</i>								<i>Bipolaris specifera</i>								<i>Rhizoctonia solani</i>							
	Mean Inhibition Zone(mm)								Mean Inhibition Zone(mm)								Mean Inhibition Zone(mm)							
	Methanol (µl/ml)				Aqueous (µl/ml)				Methanol (µl/ml)				Aqueous (µl/ml)				Methanol (µl/ml)				Aqueous (µl/ml)			
	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200
<i>Azadirachta indica</i> (leaves)	0.00	0.00	1.8	3.13	0.00	0.00	0.00	3.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.46	3.93	5.4	7.33	0.00	0.00	0.00	0.00
<i>Lantana camara</i> (leaves)	0.00	0.00	0.00	5.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Curcuma longa</i> (leaves)	0.00	3.83	9.00	10.00	0.00	0.00	0.00	0.00	0.00	4.83	5.46	7.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Curcuma longa</i> (rhizome)	0.00	2.73	4.46	7.66	0.00	0.00	0.00	0.00	1.96	5.7	7.00	8.00	0.00	0.00	0.00	0.00	3.33	6.5	7.66	11.00	0.00	0.00	0.00	0.00
C.D. (p=0.05) Plant extract	0.40				N.S				0.17				N.S				0.41				N.S			
C.D.(p=0.05) Concen.	0.40				N.S				0.17				N.S				0.41				N.S			
C.D. (p=0.05) Interaction	0.80				N.S				0.155				N.S				0.82				N.S			

Table.2 Growth inhibition of *Macrophomina Phaseolina* and *Alternaria alternata* by the methanolic and aqueous extract of different plants

Plant Part	Pathogens															
	<i>Macrophomina Phaseolina</i>								<i>Alternaria alternata</i>							
	Mean Inhibition Zone(mm)								Mean Inhibition Zone(mm)							
	Methanol(µl/ml)				Aqueous(µl/ml)				Methanol(µl/ml)				Aqueous(µl/ml)			
	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200
<i>Azadirachta indica</i> (leaves)	0.00	0.00	0.00	2.83	0.00	0.00	0.00	0.00	0.00	2.93	7.66	10.66	0.00	0.00	0.00	0.00
<i>Lantana camara</i> (leaves)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Curcuma longa</i> (leaves)	0.00	0.00	3.83	5.5	0.00	0.00	0.00	0.00	0.00	0.00	5.33	6.66	0.00	0.00	0.00	0.00
<i>Curcuma longa</i> (rhizome)	0.00	1.7	6.00	7.00	0.00	0.00	0.00	0.00	0.00	0.00	3.66	8.33	0.00	0.00	0.00	0.00
C.D. (p= 0.05) Plant extract	0.40				N.S				0.42				N.S			
C.D.(p=0.05) Concentration	0.40				N.S				0.42				N.S			
C.D. (p= 0.05) Interaction	0.80				N.S				0.83				N.S			

Table.3 Phytochemical analysis of botanicals

Phytochemical	Test	Methanol				Aqueous			
		<i>Azadirachta indica</i>	<i>Lantana camara</i>	<i>Curcuma longa</i> (Leaves)	<i>Curcuma longa</i> (Rhizome)	<i>Azadirachta indica</i>	<i>Lantana camara</i>	<i>Curcuma longa</i> (Leaves)	<i>Curcuma longa</i> (Rhizome)
Alkaloid	Dragendroff's reagent test	+	-	-	+	-	-	-	-
Tannin	Ferric chloride test	+	+	+	-	-	-	-	-
Saponin	Foam test	+	-	-	+	+	-	-	-
Steroid	Salkowski test	-	-	-	+	-	-	-	-
Anthocyanin	Hydrochloric-acid test	-	-	+	-	-	-	-	-
Carbohydrate	Benedict's Reagent test	-	+	-	-	-	+	-	-
Phenolic Flavonoid	Lead acetate test	-	-	-	-	-	-	-	-
Flavonoid	Alkaline Reagent test	-	-	+	-	-	-	-	-

+ ~ present, - ~ Absent

Plate.1

Efficacy of methanolic plant extracts against *Curvularia lunata*



Efficacy of methanolic plant extracts against *Macrophomina phaseolina*



Efficacy of methanolic plant extracts against *Alternaria alternata*

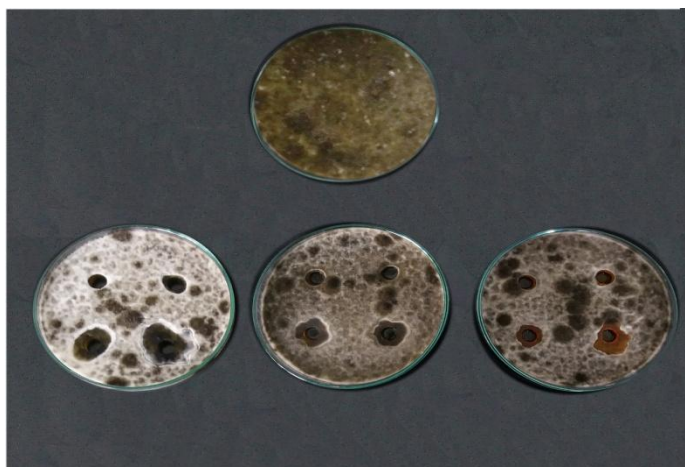
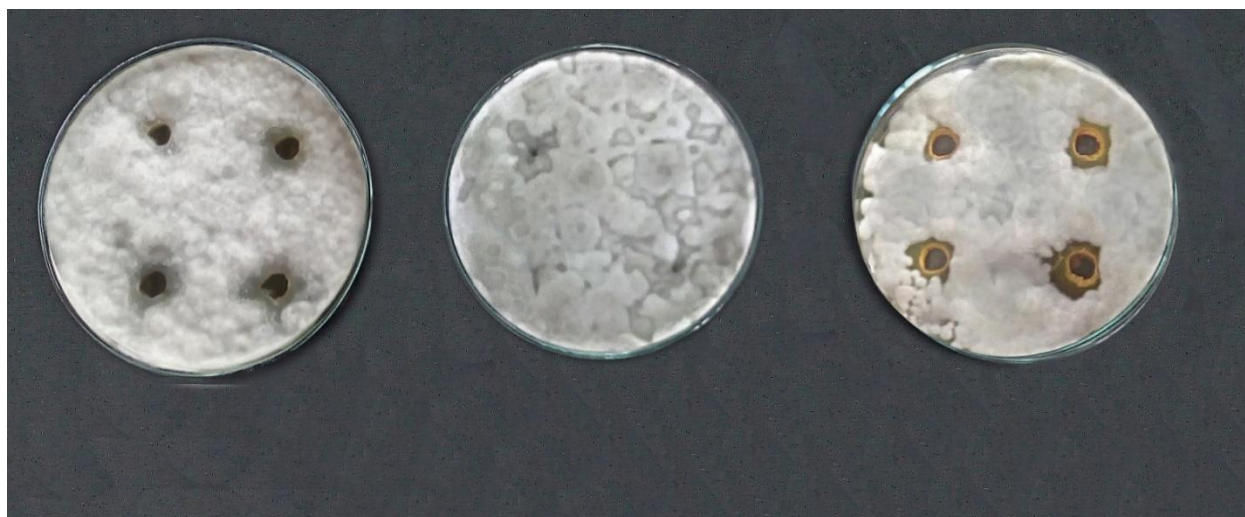


Plate.2

Efficacy of methanolic plant extracts against *Bipolaris spicifera*



Efficacy of methanolic plant extracts against *Rhizoctonia solani*

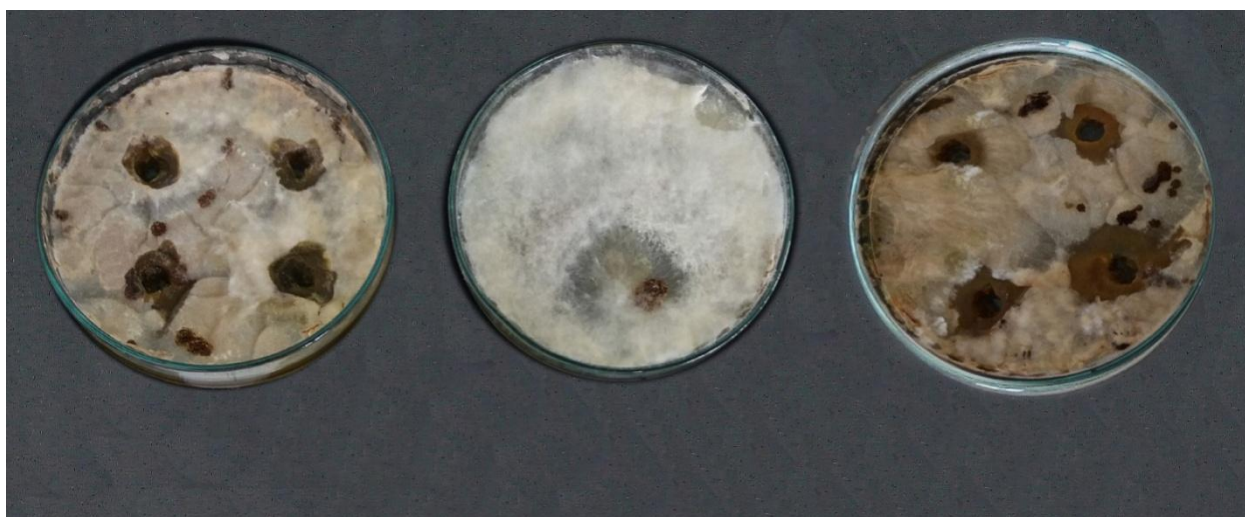
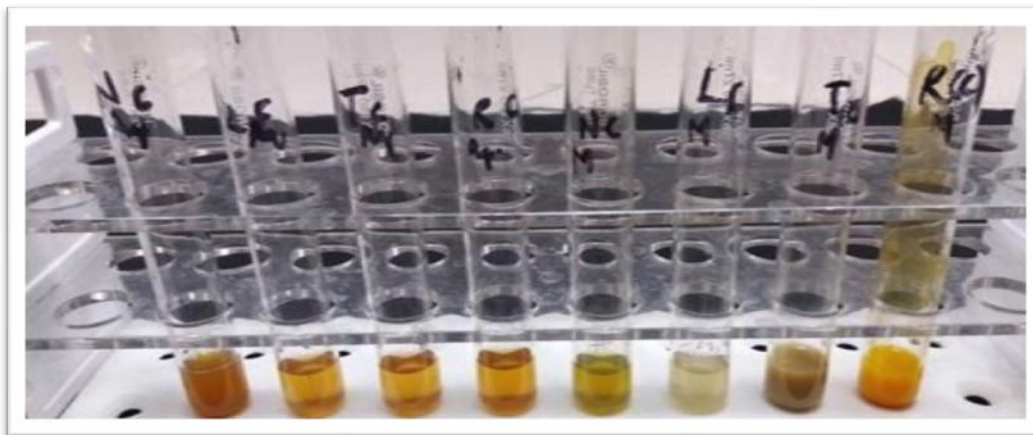
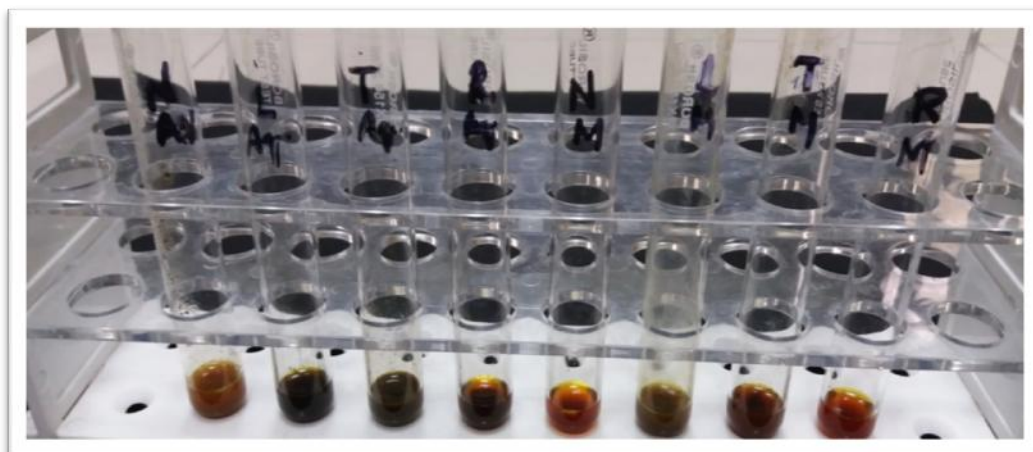


Plate.3 Phytochemical analysis test

Control



Dragendroff's reagent test



Ferric chloride test

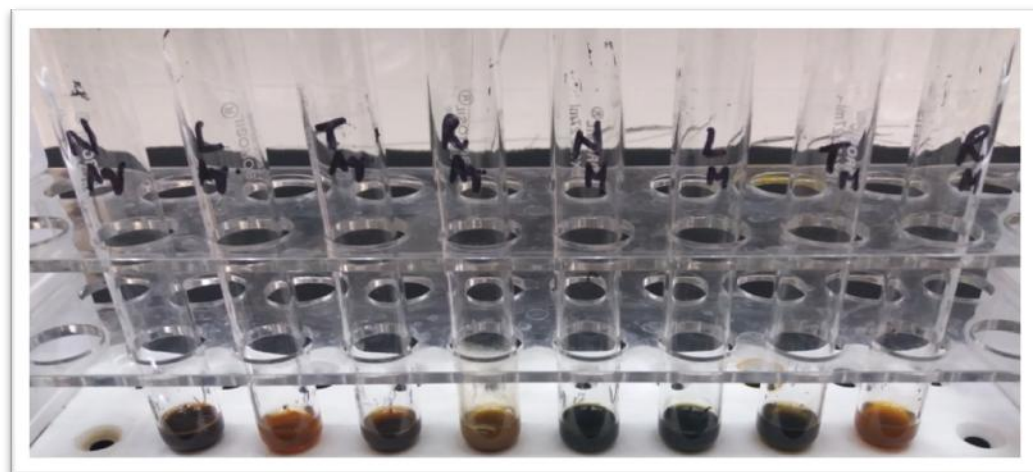
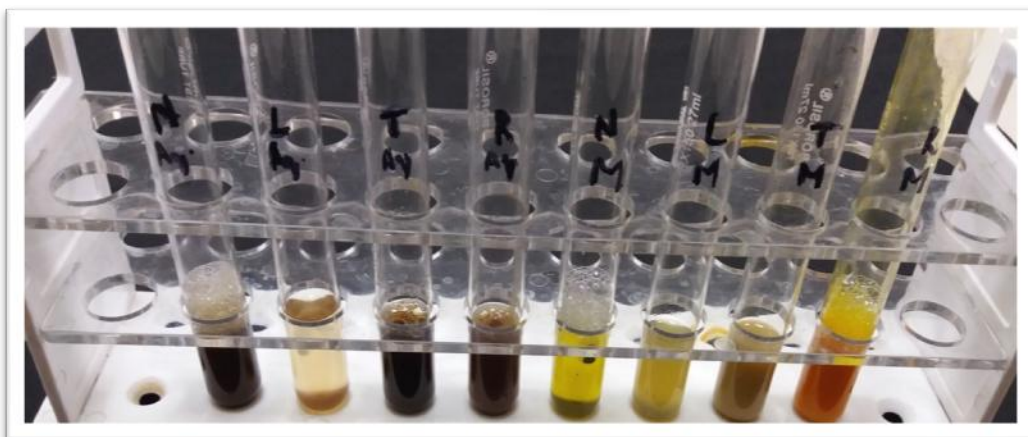
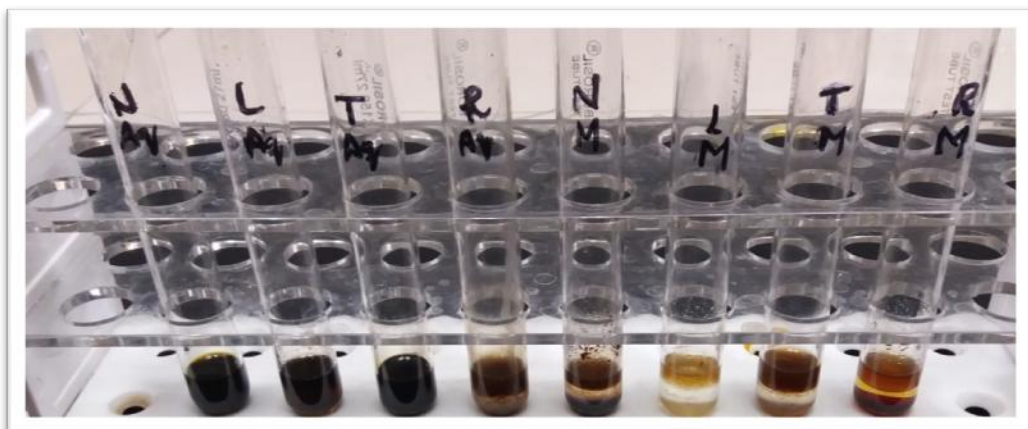


Plate.3 Phytochemical analysis test

Foam test



Salkowski test



Hydrochloric- acid test

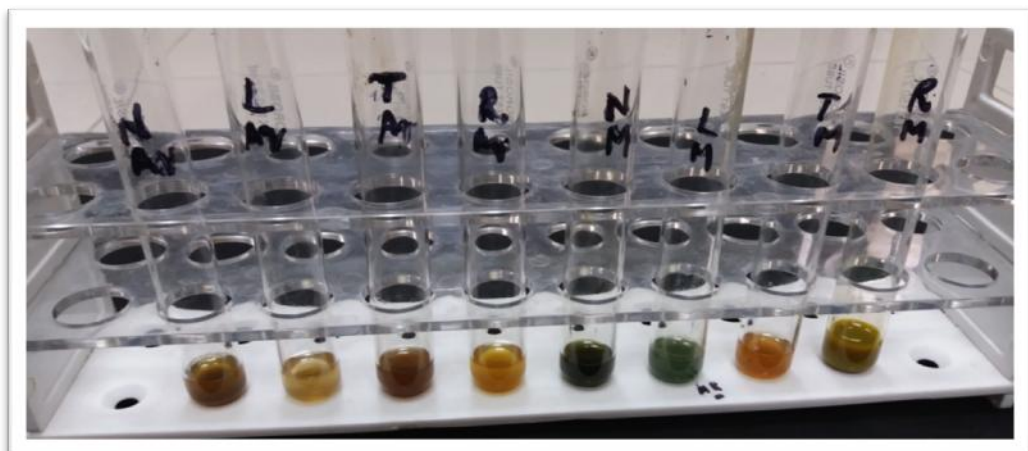
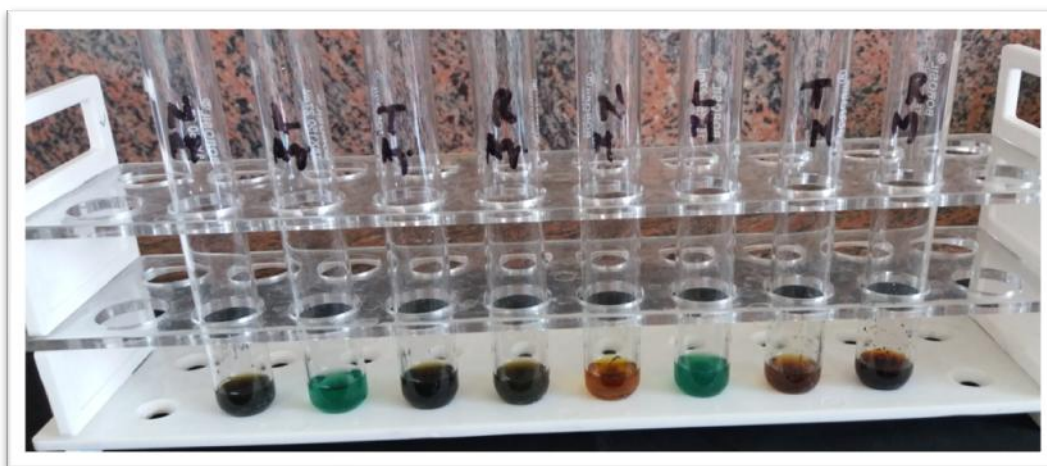
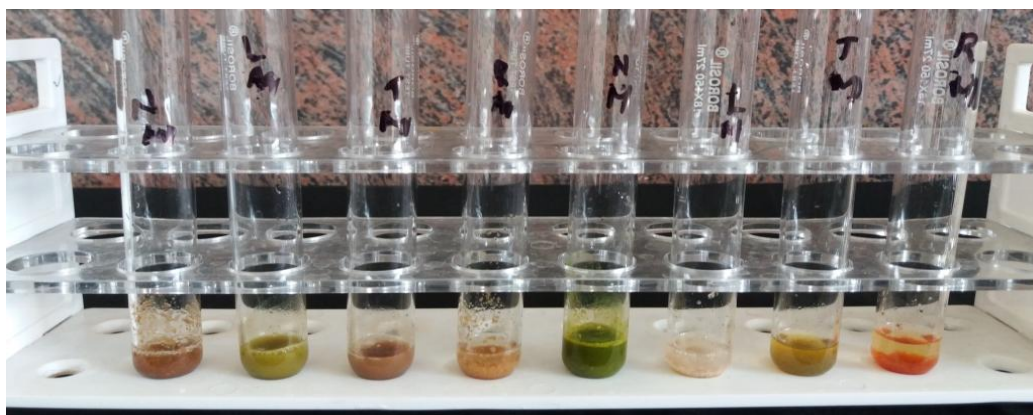


Plate.3 Phytochemical analysis test

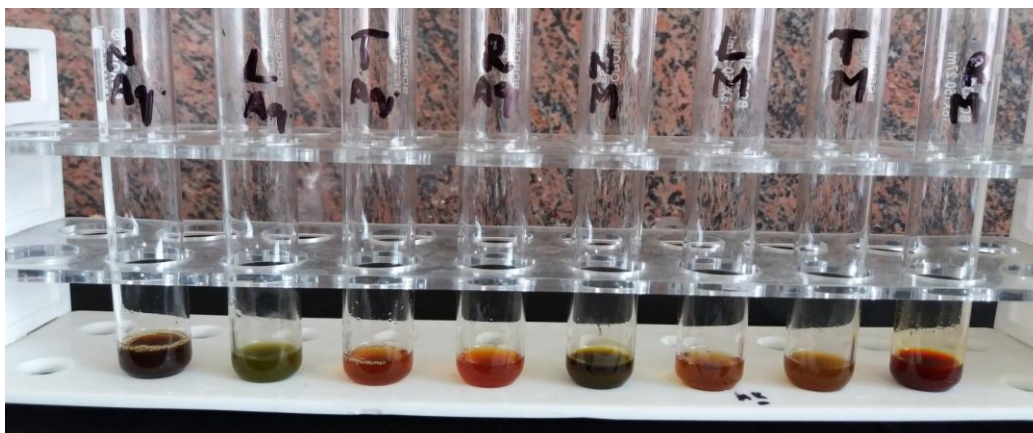
Benedict's reagent test



Lead acetate test



Alkaline reagent test



The results from the phytochemical screening of the studied medicinal plants extract have shown that alkaloid were present in two of the four plants, *Azadirachta indica* and rhizome extract of *curcuma longa*. Tannin was found in leaf extract of *Azadirachta indica*, *Lantana camara* and *C. longa* whereas saponin was present in rhizome of *Curcuma longa* and methanolic and aqueous leaf extract of *Azadirachta indica*. Steroid is present in rhizome of *Curcuma longa* whereas anthocyanin and flavonoid was found in *Curcuma longa* leaf extract.

Carbohydrate was found in aqueous and methanolic leaf extract of *Lantana camara*, whereas phenolic flavonoid was found to be absent in all the extracts (Plate 3). Our studies are in confirmatory with the studies of (Usman *et al.*, 2005; Seema and Parminderjit, 2016; Saxena and Sahu, 2012; Shah *et al.*, 2011) they reported various secondary metabolites present in *Azadirachta indica*, *Curcuma longa* leaves as well as rhizome and *Lantana camara* leaves. The antimicrobial activity possessed by flavonoids, alkaloids, saponins, tannins etc. was reported by (Newman *et al.*, 2000; Omulokoli *et al.*, 1997).

These phytoconstituents strongly inhibited the spore germination and mycelial growth of the fungi (Singh *et al.*, 2008; William, 2008), or to inhibit the growth and development of microbes by protein precipitation or by restricting the availability of nutritional proteins to microbes (Sodipo *et al.*, 1991).

So, the present study revealed that antifungal activity vary with the species of the plants and plant material used Plants contain potential antifungal components that would lead to the establishment of some valuable compounds that has to be used to formulate new, different and more potent antifungal drugs of natural origin. Since, Further studies are needed to determine the chemical identity of the

bioactive compounds responsible for the observed antifungal activity.

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How to cite this article:

Sakshi Bhagat, Upma Dutta and Tanika Mahajan. 2019. Antifungal Activity of Important Botanicals against Plant Pathogens. *Int.J.Curr.Microbiol.App.Sci*. 8(10): 531-545.
doi: <https://doi.org/10.20546/ijcmas.2019.810.058>