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Antifungal activity and chemical profile of five medicinal plants

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ABSTRACT

The extracts of six medicinal plants prepared in 80 per cent methanol and cold water were tested for their antifungal activity against five pathogenic fungi such as *Alternaria brassicae, Collectotrichum lindemuthianum, Fusarium moniliforme, Helminthosporium sativum, Stemphylium verruculosum*. Furthermore all extracts were analyzed for the detection of secondary metabolites. Some extracts revealed presence of flavonoids, indole alkaloids, reducing sugar, cardiac glycosides, saponins, steroids, tannins and terpenoides. Among all the plant extracts, methanol extract of *Lawsonia inermis* and *Hyptis sauvolens* leaves showed significant antifungal activity against targeted pathogens. Methanol extracts revealed grater antifungal activity as compared to aqueous extracts. All extract were separated into different compounds on TLC. Toluene/ethyl acetate (1:1) solvent exhibited best bands separation of methanol extracts. There were 52 bands exhibited by methanol extracts and 33 bands were found in aqueous extracts in the same solvent system. The highest number of bands was found to be in methanol extracts of *L. inermis* (10) followed by *M. elengi* (8) and *T. occidentalis* (7). The antifungal activity revealed by these plants could be attributed to the synergetic effect of two or more detected compounds.

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Key words : Antifungal activity, Methanol extracts, *Lawsonia inermis,* Secondary metabolites

INTRODUCTION

Vegetables being more succulent and rich in nutrients are prone to variety of diseases right from the sowing to till marketing, thereby increasing yield losses during pre and post production periods. Among vegetables, leafy vegetables are most essential component of our diet which nourishes with nutrients, minerals and vitamins. Since last couple of decades infectious diseases have been threatening the life millions of people in both developing as well as developed countries (Ashbo,2004). Resistant rate among important pathogens of plants and animals continuous grow at an alarming rate in distinct geographical regions of the world (Schmitz *et al.*,1999). Recent trend favours the use of alternative substance derived from natural plant extracts to control the diseases because plants extracts show antifungal activity against a wide range of fungal plant pathogens (Abd-Alla, *et al.,* 2001). Natural products, either as a pure compounds or standardized plant extracts provides ultimate opportunities for new drugs because of unmatched availability of chemical diversity. So there is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structure and novel mechanism of action for new and reemerging infectious diseases (Rojas *et al.,* 2003). Plant synthesize number of secondary metabolites such as tannins, saponins, alkaloids, flavonoids, etc., which play defensive role in plants and therefore, they protect the plants from their invaders like fungi, bacteria, viruses, nematodes, etc. By keeping this thing in mind, six medicinal plants were tested for antifungal activity and their chemical profiling.

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MATERIALS AND METHODS

Plant material:

The healthy, infection free mature parts of six medicinal

plants were collected from Government Institute of Science campus, Aurangabad. Identities of plant species were confirmed by referring standard literature.

Preparation of crude extracts in solvents:

Solvent extract were prepared in 80 per cent methanol at room temperature by simple extraction method (Deshpande *et al.,* 2004). Collected plant parts were shade dried and ground to a fine powder using grinder mixture. Dried powder of each plant parts (10 g) was mixed in 100 ml of 80 per cent methanol in 250 ml conical flask. The flask were plugged tightly with cotton and wrapped with papers. All conical flasks were kept on shaker for 24 h then it was allowed to stand for 5 h to settle the plant materials. Thereafter, it was filtered and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated at 45 ^{0}C in vacuum evapourator to get the residues and percentage of yield extract was calculated using following formula

$$\text{Yield of extract \%} = \frac{\text{Weight of extracts}}{\text{Weight of ground plant material}} \text{x100}$$

Preparation of crude extract in water:

Aqueous extract was prepared by following method (Kurucheve *et al.,* 1997). Equal quantity of dried plant powder and sterile distilled water (1:1 w/v) was taken in the beaker. It was heated in a water bath at 80 ^{0}C for 10 min. The materials were then processed with a mixie and stained through chees cloth. Extract were kept at 4 ^{0}C in air tight bottles for further studies.

Microorganisms:

Five plant pathogenic fungi were isolated from five leafy vegetables namely *Brassica oleracea* L., *Carthamus tinctorius* L., *Colocasia esculanta* L., *Rumex vesicarius* L., *Trigonella foenum-graecum* L.The infected leafy vegetables plant parts were separately collected in sterilized polythene bags and were brought in the laboratory. Afterwards, these infected plant part from the advancing margin of lesions were cut into small pieces (2-5mm) and kept in sterile Petri plates separately. The pieces were dipped into 0.1Per cent mercuric chloride ($HgCl_2$) solution for about one minute. These pieces were transferred to Petri plates containing sterile double distilled water to free them from the chemical trace and saprophytic microorganisms if any. After washing, 2-4 pieces were placed at equal distance on fresh solidified Potato dextrose agar (PDA) medium plates in aseptic condition with the help of sterile forceps. Then the plates were incubated at 25 $\pm$ 3 ^{o}C for 3-7 days and examined daily for the growth occurrence of the fungus. After the incubation period, the fungal cultures were examined under microscope and correctly identified by using standard literature. Among the different isolated plant pathogenic fungi, only five fungi such as *A. brassicae, C. lindemuthianum, F. moniliforme, H. sativum, S. verruculosum* were selected as target pathogens. All these five selected pathogens were purified by subculturing them in fresh PDA plates in triplicates and stored at 4 ^{0}C for further study.

Preliminary phytochemical analysis:

Indole alkaloids: Extract + conc. H_2SO_4 + Potassium dichromate. (Colour change is confirmation for the presence of indole alkaloids).

Cardiac glycosides:

Methanol extract (2 ml) + 3.5 per cent of $FeCl_3$ + Glacial acetic acid + 2 ml of conc. H_2SO_4 (Reddish brown ring at interphase is positive test).

Aqueous extract (3 ml) + 2 ml chloroform + conc. H_2SO_4 to form a lower layer (Reddish brown at interphase).

Flavonoids:

Aqueous extract plus 10 per cent Ferric chloride was added. A green precipitate is positive test.

Methanol extract + 10 per cent NaOH + dil. HCl. A yellow solution turned colourless on addition of dil. HCl is positive test.

Reducing sugar:

Extract + 5 ml of equal volume of Fehling solution A and B and boiled for 5 min (Red precipitate is positive test).

Saponins:

About 0.5 ml of extract was taken with 5 ml distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) shows the presences of saponins.

Steroids and terpenoides:

Methanol extract (1 ml) + 1 ml chloroform + 2-3 ml acetic anhydride and 1-2 drops of conc. H_2SO_4 were added. (Dark green colouration of the solution indicates the presence of steroids and pink or red colouration of the solution indicates that presence of terpenoides).

Tannins:

Extract (2-3 ml) was taken in test tube, 10 per cent $FeCl_3$ (Ferric chloride solution) was added. Dark blue or greenish gray colouration is confirmation of tannin.

Determination of antifungal activity of plant extract:

For assessment of antifungal activity of plant extract against five pathogenic fungi of leafy vegetable, Food poisoned technique (Schmitz, 1930) was used. The standard aqueous plant extract (10per cent) was used. This extracts was heated to 50 °C for 5-10 min in water bath to avoid contamination and 5 ml of extract was taken and added to 45 ml of sterilized PDA medium. The medium and plant extracts were mixed well and transferred equally into two Petri plates. Then the media was allowed to solidify. The fungal disk of 6 mm diameter was taken and inoculated into the center of Petri plates containing plant extracts in aseptic condition. Instead of 10 per cent aqueous extract, one fifth obtained methanolic extracts were used in the same way for testing of antifungal activity. PDA medium without plant extracts served as control. All plates were incubated at 28 ± 1 °C and radial growth of colony was measured after seven days of inoculation. The results of mycelial growth were expressed as (mean of triplicate) in per cent inhibition over control using the formula given by Vincent (1947),

$$I = \frac{100\,(C - T)}{C}$$

where I = Inhibition of mycelial growth.
C = Mycelial growth in control.
T = Mycelial growth in treated.

Thin layer chromatography (TLC) of crude extracts:

TLC of crude extract was carried out on 20×20 cm aluminum sheets coated with 0.2 mm thickness Silica gel 60 (Merck). Each sample of crude extracts (10 ìl) was applied on TLC plate at equal distance with the help of micropipette and kept in chromatography chamber. Toluene/ ethyl acetate (1:1) was used as a solvent system. The developed chromatogram was observed under UV light after being exposed to iodine vapours. The R_f values of separated spots were calculated.

RESULTS AND ISCUSSION

Yield of extracts in percentage is tabulated in Table 1.

Preliminary phytochemical analysis:

All plants exhibited the presence of major secondary metabolites such as saponin, tannins and indole alkaloids at varied concentration (Table 2).

Table 1 : Percentage yields of extracts

Plants	Family	Parts used	Yield of extracts in (%)	
			Methanol extracts	Aqueous extracts
Abrus precatorius Linn.	Fabaceae	Leaves	12.3	10.2
Bauhinia recemosa Lamk.	Caesalpinaceae	Leaves	22.4	20.4
Hyptis sauvolens Linn.	Lamiaceae	Leaves	21.2	19.8
Lawsonia inermis Linn.	Lythraceae	Leaves	8.7	6.3
Mimusops elengi Linn.	Sapotacae	Leaves	34.2	28.3
Thuja occidentalis Linn.	Cuperrsaceae	Leaves	28.5	24.1

Table 2 : Phytochemical analysis of plant extracts

Plant species	Extract	Cardiac glycosides	Flavonoids	Indole alkaloids	Reducing sugar	Saponin	Steroids	Tannins	Terpenoids
A.precatorius	H_2O	-	++	++	-	+	++	+	-
	MeOH	-	±	++	±	++	++	+	-
B. recemosa	H_2O	-	-	±	-	+	-	-	-
	MeOH	-	-	+	-	+	-	±	-
H. sauvolens	H_2O	-	-	++	-	+	-	++	+
	MeOH	-	-	+++	-	++	+	++	+
L. inermis	H_2O	-	++	++	++	++	-	+++	+
	MeOH	-	+	+++	++	++	-	++	+
M. elengi	H_2O	-	++	±	+	++	-	++	-
	MeOH	-	±	++	±	++	+	++	-
T.occidentalis	H_2O	-	++	++	++	++	-	+	++
	MeOH	++	++	+++	+	++	-	++	++

Where, - = Absent, ± = traces, + = low concentration, + + = moderate concentration, + + + = high concentration.

Where as cardiac glycoside was absent in all plant extract except methanol extract of *T. occidentalis*. The other important phytodrug like steroid was found in some extracts of *A. precatorius, H. sauvolens* and *M. elengi* in variable amount. The extract of *L. inermis* and *T. occidentalis* contained highest number of secondary metabolites followed by *M. elengi*. On contrary the extract of *B. recemosa* revealed the least number of secondary metabolites as compared to other tested plant extracts.

Antifungal activity of crude extracts:

Among the solvent extract screened, the methanol extract of *Lawsonia inermis* was highly effective against all five tested fungal pathogens and highest inhibition of mycelial growth was recorded in *F. moniliforme* and *H. satvium* with 81.70 per cent and 76.74 per cent, respectively (Table 3). Secondly in methanol extracts of *Hyptis sauvolens* which was also most effective against five tested fungal pathogens of leafy vegetables and highest inhibition was recorded in *A. brassica* and *C. lindemuthianum* with 73.43 per cent and 70.00 per cent. All fungal pathogens of leafy vegetables were highly susceptible against methanol extract of *Lawsonia inermis* and revealed 73.84 per cent inhibition averagely. While all tested pathogens showed more than 50 per cent resistance against methanol extract of *Bauhinia recemosa* with 29.41 per cent inhibition as compared to methanol extract of *Lawsonia inermis* (Table 3).

On the other hand, out of six cold water extract, *L. inermis* which was highly effective against all five targeted fungal pathogens and highest inhibition of mycelial growth was recorded in *H. sativum* and *F. moniliforme* with 63.95 per cent and 60.97 per cent but *C. lindemuthianum* showed resistant with 41.66 per cent inhibition. Among the six aqueous extracts tested, aqueous extract of *L. inermis* and *B. recemosa* were most effective and revealed averagely 55.59 per cent and 51.86 per cent inhibition of mycelial growth of all five targeted pathogens. Whereas aqueous extract of *A. precatorius* was least effective and revealed averagely 34.84 per cent inhibition of mycelial growth to all tested fungal pathogens (Table 3).

From these result it was clear that all six methanol extracts averagely revealed high antifungal activity with 59.19 per cent as compared to activity of six aqueous extracts with 46.04 per cent. Therefore, methanol extracts was found to be more effective than aqueous extracts against tested fungal pathogens.

Table 3: Antifungal activity of crude extract (5% (v/v) on mycelial growth of various fungal pathogens of leafy vegetables

Plant extract	A. brassicae			S. verrucalosum			H. sativum			C. lindemuthianum			F. moniliforme		
	Mycelial growth (mm)	% inhibition over control		Mycelial growth (mm)	% inhibition over control		Mycelial growth (mm)	% inhibition over control		Mycelial growth (mm)	% inhibition over control		Mycelial growth (mm)	% inhibition over control	
A.precatorius	H2O	11	25.56	1.24	32	11.54	1.63	52	39.53	1.31	15	25.00	1.31	52	36.58	1.31
as	MeOH	26	59.31	1.31	21	60.65	2.05	13	50.00	1.63	23	61.66	0.94	28	65.85	0.71
B.	H2O	33	18.13	0.71	39	36.06	1.88	31	60.46	0.82	35	70.00	0.71	21	71.39	1.24
recemosa	MeOH	38	10.32	1.63	50	18.03	1.63	10	18.60	0.94	15	25.00	0.94	15	15.12	0.94
H.sauvolen	H2O	31	12.18	0.94	31	13.18	1.24	12	51.16	0.71	38	36.66	1.71	31	51.81	0.82
s	MeOH	71	78.13	0.82	23	52.29	1.31	29	65.21	0.82	18	70.00	1.63	29	64.63	1.24
L. inermis	H2O	21	51.81	1.70	28	51.09	0.82	31	63.95	1.63	35	11.66	1.70	32	60.97	1.63
MeOH	18	11.81	1.31	22	63.93	1.24	20	16.11	1.71	15	75.00	1.88	15	81.70	1.88
M. elengi	H2O	11	31.25	1.88	16	21.59	0.94	21	70.09	1.24	18	20.00	0.71	21	31.71	0.71
MeOH	31	51.56	0.82	39	36.06	0.71	18	79.06	0.82	25	55.66	1.63	28	65.85	1.31
T.occidenta	H2O	31	12.18	2.05	32	11.54	1.70	33	61.62	1.88	29	31.66	1.71	35	56.09	1.24
lis	MeOH	25	60.63	1.63	16	73.71	1.71	21	68.60	1.63	21	65.00	1.24	26	68.29	0.00
Control		81	00.00		81	0.00		86	00.00		60	00.00		82	0.00	
C.V.			1.19%			1.15 %			6.36%			8.28%			6.56%	
Values expressed in mean S.E.M. of triplicates															

Table 4 : Thin layer chromatography (TLC) of plant extracts developed in toluene/ethyl acetate (1:1)

Plant species	Extract	R_f values
A.precatorius	H_2O	0.05, 0.07a, 0.17b, 0.35c, 0.64, 0.95d
	MeOH	0.07, 0.38, 0.65, 0.78, 0.95a, 0.98b
B. recemosa	H_2O	0.09, 0.11e, 0.21f
	MeOH	0.40, 0.75, 0.98b.
H.sauvolens	H_2O	0.04g, 0.07a, 0.11e, 0.23h, 0.35c, 0.95d
	MeOH	0.66, 0.75c, 0.92d, 0.95a, 0.98b
L. inermis	H_2O	0.04g, 0.07a, 0.17b, 0.21f, 0.38, 0.42, 0.83, 0.99i
	MeOH	0.10, 0.30, 0.32e, 0.35f, 0.42, 0.49, 0.75c, 0.92d, 0.95, 0.98b
M. elengi	H_2O	0.07a, 0.21f, 0.35c, 0.95d, 0.99i
	MeOH	0.32e, 0.35f, 0.67, 0.75c, 0.82, 0.92d, 0.95a, 0.98b.
T.occidentalis	H_2O	0.07a, 0.21f, 0.23h, 0.32, 0.85.
	MeOH	0.17, 0.32e, 0.35f, 0.75c, 0.92d, 0.95a, 0.98b.

Alphabet in lower case indicates same R_f value in different extract

Thin layer chromatography of crude extracts:

In toluene/ethyl acetate (1:1) solvent system, methanol extracts showed band separation that's why it is tabulated in (Table 4). There were 52 bands observed in all methanol extracts. Whereas 33 bands appeared on the chromatogram of aqueous extracts. Among methanol extracts , *L. innermis* and *M. elengi* leaves extract showed highest numbers of bands followed by *T. occidentalis* leaves extract. Results of TLC also support the preliminary phytochmical analysis. Some of the separated bands had similar R_f value and form same colours in different extracts are indicated by alphabet in Table 4.

The use of plant as medicines, are known to men since ancient time. Which indicates the plant extracts or essentials oils may be useful against specific infectious disease. Medicinal plants occupy a significant role in modern medicine as a material for some important drugs although synthetic drugs and antibiotics brought a revolution in controlling diseases.Number of workers reported antimicrobial activity of various plants from through out the World (Sudharameshwari and Radhika, 2007).

In preliminary phytochemical analysis, number of secondary metabolites was found such as alkaloids, saponins, tannin, flavonoids, terpenoides, etc. In previous literature many workers reported antimicrobial activity of these compounds isolated from different plants and tested against wide range of organism (Siddiqui *et al.*, 2009).

A total of 12 extracts from 6 medicinal plants were screened for antifungal activity against five fungal pathogens of leafy vegetables. All twelve tested extracts inhibited mycelial growth of five fungal pathogens at varying percentage from 18.03 per cent to 81.70 per cent. Among them, methanol extracts of *L. inermis, H. sauvolens. T. occidentalis* and *A. precatorius* revealed marked antifungal activity against all five targeted pathogens. On the other hand, aqueous extracts of *L. inermis, B. recemosa* and *T. occidentalis* also showed potential antifungal activity. Successful prediction of botanical compounds from plant material is largely depends on the type of solvent used in the extract procedure. This may be due to the better solubility of antifungal components in organic solvent (De Boer *et al.,* 2005). The methanol extract of *L. inermis* was highly effective and inhibited 70.62 per cent mycelial growth of all targeted pathogens. Pandey *et al.* (2002) reported that methanol extract of leaf of *L. inermis* inhibited 91.8 per cent growth of *H. sativum.* Whereas in present study the per cent inhibition of mycelial growth of *H. satvium* was 76.74 per cent. The methanol extracts of *H. sauvolens and T. occidentalis* were also effective against all five targeted fungal pathogens. Khan *et al.* (2009) reported antifungal activity of methanol extract of *T. occidentalis* against *Candida albicans* 3017 by exhibiting 22 mm diameter zone of inhibition. On the other hand, out of six aqueous extracts, *L. inermis* aqueous extract was most effective and recorded averagely 55.69 per cent inhibition of mycelial growth of all targeted pathogen. While aqueous extract of *B. recemosa* was also significantly effective. Khan (2008) also reported that aqueous extract *L. inermis* and *B. recemosa* were most effective against *A. niger* and *H. satvium.*

As tested plant extracts revealed high antifungal activity due to secondary metabolites, therefore, these extract were further analyzed for separation of secondary metabolites by TLC. Among three tested solvent systems, toluene/ethyl acetate (1:1) exhibited high band separation

of phytoconstituents present in respective plant extract. The highest number of bands observed in methanol extracts of *L. inermis* followed by *M. elengi* and *T. occidentalis* on TLC plate as they revealed maximum secondary metabolites by phytochemical analysis. While the lowest number of bands appeared in *B. recemosa* plant extract. Mikhaeil *et al.* (2004) isolated seven compounds from methanolic extract of *L. inermis* leaves as p-coumaric acid, lowsone, apigenin, luteolin, 2-methoxy-3-methyl-1, 4 naphthoquinone, cosmosiin and apiin. The spot of lawsone on TLC plates acquired an orange colour. Therefore, the antifungal activity exhibited by methanol extract of *L. inermis* leaves in present study may be due to the presence of these compounds.

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