Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Archives of Applied Science Research, 2013, 5 (6):214-221
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-508X
CODEN (USA) AASRC9

Antifungal activity and qualitative phytochemical analysis of extracts obtained by sequential extraction of some medicinal plants in Jaffna peninsula

Jeyasakthy S.[1], Jeyadevan J. P.[1], Thavaranjit A. C.[2], Manoranjan T.[1], Srikaran R.[1] and Krishnapillai N.[2]

[1]Department of Chemistry, University of Jaffna, Sri Lanka
[2]Department of Botany, University of Jaffna, Sri Lanka

ABSTRACT

Antifungal activity of sequentially extracted different cold organic solvents extracts of some common medicinal plants found in Jaffna peninsula, such as Cassia alata, Azadirachta indica, Lantana camara and Mangifera indica against four phytopathogenic fungi, Fusarium sp, Alternaria sp, Aspergillus sp and Pythium sp by agar well diffusion method were studied .. Synthetic fungicide Dithane M-45 (mancozeb) and ethyl acetate were used as standard and control repectively. Overall, A.indica showed higher degree of antifungal activity compared to other tested plants. Hexane extracts of M.indica and L.camara did not exhibit activity at all. However hexane extracts of C.alata and A.indica only revealed the antifungal activity even though which had lower activity than the other tested extracts. In which the inhibitory effect of ethylacetate of stem bark of C.alata on Pythium sp was higher (32 mm) than the other extracts after 48 hours. Both ethyl extracts of root bark of M.indica and stem bark of L.camara revealed significantly highest inhibition on Alternaria spp were 29 mm and 25 mm respectively after 48 hours incubation. Besides this, approximately similar inhibition zone (29 mm) was produced by ethylacetate extract of root bark of A.indica and leaf of A.indica against Pythium sp at same concentration. Moreover, this study revealed that the leaf extracts produced prominent inhibitory effect against fungi. Phytochemical analysis revealed the presence of various bioactive compounds such as alkaloids, cardiac glycosides, flavonoids, phlobatannins, saponins, tannins, terpenoids and steroids in tested extracts which were differ quantitatively. However, further studies should be performed for the isolation and characterization of the active compounds.

Key words: Medicinal plants, sequential extraction, antifungal activity, well diffusion method.

INTRODUCTION

Phytopathogenic fungi causes severe losses in plants and crop production, significant reduction in seed germination, seedling emergence and tuber yield. It is therefore necessary to reach for control measures that are cheap, ecologically found and environmentally safe to eliminate or reduce the incidence of these economic important pathogens. A series of molecules with antifungal activity have been found in plants, which are of great importance to human and plants. Plants are rich source of bioactive secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, reported to have *invitro* antifungal properties. [1]

Among the reports on natural products, two fungicidal compounds have been isolated from the uncrushed green leaves of *Azadirachta indica*. The effect of neem leaf extracts on *Aspergillus parasiticus* growth and aflatoxin biosynthesis was investigated [2]. Several species of *Aspergillus* cause decay or deterioration in grains and legumes after harvest, during storage or transit. *Aspergillus flavus* often infects corn kernels and ground nuts in the field. Aflatoxins are produced by several species of *Aspergillus* [3].

Alternaria species affect the leaves, stem, flowers and fruits of primarily annual plants. *Alternaria* diseases appear usually as leaves spot and blights. But they may also caused damping off of seedlings, stem rots, tuber and fruit rots. Several species of *Fusarium* cause vascular wilts, rotting of seeds and seedlings, rotting of roots, stems and crowns, rots of corns, bulbs and tubes. They affect different kinds of vegetables, flower and field crops [3. The efficacy of leaf extracts of neem against two tomato pathogenic fungi *Alternaria solani* and *Fusarium oxysporum* were carried out the casual agents of early blight and wilt diseases of tomato plants respectively. Evaluation of the activity of the cold expeller neem oil and the fractions derived through solvent partitioning, against *Drechsleri aryzae*, *Fusarium oxysporum* and *Alternaria tenius* showed that the active antifungal fraction is a mixture of tetranortriterpenoids.

The sap or the extract of the plant *Cassia alata* has been reported to possess some medicinal value, for example, the leaves have been reported to have a laxative effect and are also used against ringworm, scabies, ulcers and other skin diseases [6].

Aqueous extracts of *Lantana camara* had antifungal activity and the alcoholic one was shown to possess insecticidal properties [4]. Various *Pythium* species cause seed rot, seedling, damping-off on tomato, mustard, tobacco, ginger, papaya and wheat and root rot of all types of plants. Also it causes soft rot of fleshy fruits in contact with the soil [3].

The present study was carried out to evaluate the *in vitro* antifungal activity of sequentially extracted different cold organic solvent extracts of stem bark, root bark and leaves of *Cassia alata*, *Azadirachta indica*, *Mangifera indica* and *Lantana camara* against plant pathogenic fungi such as *Aspergillus* spp, *Alternaria* spp, *Fusarium* spp and *Pythium* spp and to elucidate the available phytochemicals in the test extracts

MATERIALS AND METHODS

Collection of plant materials

Plant materials of *Cassia alata (*leaf , stem bark), *Azadirachta indica*(leaf, stem bark, root bark), *Mangifera indica(*leaf, stem bark, root bark) and *Lantana camara* (leaf, stem bark) were collected from botanical garden of the Department of Botany, University of Jaffna, Sri Lanka and identified by taxonomist based on herbarium records in the Department.

Preparation of plant extract

The plant material was air dried and ground into fine power using an electric blender. 100 g of powder was soaked in 200 ml of hexane with intermittent shaking for 3 days. The supernatant was filtered in sterile condition. This procedure was repeated twice to ensure the complete separation of all constituents. The solvent was evaporated under reduced pressure by using rotatory evaporator. The remaining samples were allowed to air dry. Finally the sample of hexane crude was weighed. The above procedure was repeated to the each dried plant material by using ethylacetate and methanol solvent for sequential extraction method respectively.

Test Fungi

The fungal pathogens, *Aspergillus* spp, *Alternaria* spp, *Pythium* spp and *Fusarium* spp were isolated from diseased plant materials and identified based on their morphological, reproductive features and pathogenicity under laboratory conditions. These fungal cultures were purified on potato dextrose agar medium by subculturing technique and were stored in the refrigerator as slants at 10 ^{o}C for the assay and future use.

Antifungal assay

Preparation of fungal spore suspension

0.85 g NaCl was weighed and it was dissolved in 100 ml of distilled water in a volumetric flask. Then 9.0 ml of the saline water was transferred into Mac Cartney bottles and those bottles were sterilized by an autoclave. A loopful of spores was taken by a sterile loop and suspended into sterile saline water under aseptic condition. Spore concentration was determined by the Haemocytometer. Then the suspension was stirred well and serially diluted to 10^5 number of spores/ml.

Preparation of Standard and control solutions

The synthetic antifungal agent Mancozeb (Dithane M-45) was prepared in 0.3 mg/150 µl concentration as standard. The solvent used to prepare the crude was used as control.

Agar well diffusion method

0.1ml of each fungal spore suspension was spread uniformly on the surface of PDA plate by using a sterile glass spreader. 8mm diameter wells were made by using a sterile cork borer. 100 µl of each test extracts were

administered into each well separately. Mancozeb and ethyacetate were also used as standard and control respectively. Plates were incubated at room temperature for 3-5 days and the zone of inhibition around the well was measured at various time intervals (24, 48, 72 and 96 hours). Each experiment was repeated thrice and the mean value was taken [6].

Phytochemical analysis of plant extracts
The phytochemical analysis of crude extracts were carried out to determine the presence of the following biomolecules using standard procedures [7, 8, 9, 10].

Test for tannins
About 0.01g of the crude extract was boiled in 20 ml of water in a boiling tube. Few drops of 0.1 % of $FeCl_3$ were added. Formation of brownish green or a blue black colouration indicated the presence of tannins.

Test for saponins
About 0.01 g of the crude extract was boiled in 20 ml of distilled water in a water bath. Then it was mixed with 5 ml of distilled water and it was shaken well. Stable persistent froth indicated the presence of saponins.

Test for phlobatanins
About 0.01 g of the crude extract was boiled with 1 % aqueous hydrochloric acid. A deposition of a red precipitate indicated the presence of phlobatanins.

Test for flavanoids
About 0.01 g of the crude extract was dissolved in 2 ml of ethanol solvent. Con. HCl and Mg turnings were added. Formation of yellow colour indicated the presence of flavanoids.

Test for steroids
About 0.01 g of the crude extract was dissolved in 2 ml of ethanol solvent. 2 ml of aceticanhydride and 2 ml of con H_2SO_4 were added. A colour change from violet to blue or green indicated the presence of steroid

Test for cardiac glycosides
0.01g of the crude extract was dissolved in 2 ml of ethanol and then 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 ml of con. H_2SO_4. Appearance of brown ring indicated the presence of the cardiac glycosides.

Test for alkaloids
About 0.01 g of crude extract was dissolved in ethanol and it was divided into two parts.

Few drops of Mayer's reagent were added to one part. A creamy white precipitate indicated the presence of alkaloids. Few drops of Wagner's reagent were added to other part. A red-brown colour precipitate indicated the presence of alkaloids.

RESULTS AND DISCUSSION

3.1 Extraction of plant materials
Powdered plant materials were sequentially extracted with cold hexane, ethylacetate and methanol. The sequential extraction method ensured the extraction of active compounds from plant material according to their polarity and also reduced the antagonistic effect of compounds in the extract.

3.2 Antifungal activity of plant extracts
The results indicated that all tested plants, *Cassia alata, Azadirachta indica, Mangifera indica and Lantana camara* showed antifungal activity against all tested fungi at least in one solvent.

Interestingly, all solvent extracts of *C. alata* exhibited antifungal activity against *Pythium* spp and *Fusarium* spp from 48 hrs to 96 hrs of incubation, while there was no inhibition on *Alternaria* spp at any incubation period. Unfortunately, hexane, ethylacetate and methanol extracts of leaf and stem bark of *C. alata* did not show any inhibitory effect against all fungi after 24 hrs of incubation.

Crude methanol extracts from leaves of *Cassia alata*, *Cassia fistula* and *Cassia tora* were investigated for their antifungal activities on *Microsporum gypseum*, *Tricophyton rubrum* and *Penicillium marneffei*. Among three species, *Cassia alata* was the most effective leaf extract against *T.rubrum* and *M.gypseum* [5].

In our study, among the leaf extracts, methanol extract revealed significantly higher inhibitory effect (30 mm) against *Pythium sp* after 48 hrs which was followed by ethylacetate extract of *C.alata* leaf (19 mm) at the same incubation period. Moreover, ethylacetate extracts showed the equal antifungal activity (18 mm) against *Aspergillus* spp and *Alternaria* spp after 48 hrs. Whereas *Pythium* spp and *Alternaria* spp had no significant different between their activity after 72 hrs. On the other hand, methanol extracts revealed the same activity on *Alternaria sp* and *Fusarium sp*.

Previous study also proved that the methanol, ethanol and petroleum ether extracts of leaves of *C.alata* exhibited antifungal activity against *Aspergillus* spp at 70 mg/ml concentration which were 4 mm, 3mm and 3.2 mm respectively [12]. But in our case *Aspergillus sp* revealed higher activity (9 mm) rather than previous one. This difference may be due to different concentration and mode of sequential extraction, since some antifungal compounds may partially dissolve in intermediate solvent, which was used for methanol.

Table 3.1: Antifungal activity of *Cassia alata*

Parts of Plant	Solvents	Time Hours	Aspergillus sp	Pythium sp	Alternaria sp	Fusarium sp
Leaf	Hexane	24	-	-	-	-
		48	14	11	-	10
		72	10	9	-	10
		96	-	9	-	9
	Ethylacetate	24	-	-	-	-
		48	18	19	18	13
		72	14	11	11	9
		96	-	10	-	9
	Methanol	24	-	-	-	-
		48	9	30	16	13
		72	-	13	12	12
		96	-	9	9	9
Stem bark	Hexane	24	-	-	-	-
		48	10	12	16	22
		72	9	9	12	20
		96	8	9	9	16
	Ethylacetate	24	-	-	-	-
		48	19	32	30	10
		72	17	27	25	9
		96	10	20	20	9
	Methanol	24	-	-	-	-
		48	-	24	25	18
		72	-	18	19	15
		96	-	15	17	10

In the case of stem bark, mostly ethylacetate exhibited most significant antifungal effect on all tested fungi except *Fusarium* spp (10 mm) after 48 hrs of incubation. Fortunately both leaf and stem ethylacetate extracts showed prominent activity on *Pythium* spp after 48 hrs of incubation

In all cases, the antifungal activity exhibited by all extracts decreased with increasing incubation period. Moreover, there was significant differences among the activity of each extracts may be due to the different extraction using different solvents. (Table 3.1)

The result indicated that all solvent extracts of *A.indica* showed antifungal activity against all tested fungi from 48 to 96 hrs of incubation, while there was no inhibition zone observed against any of the tested fungi till 24 hrs.

Ethylacetate extract of leaf and root bark revealed significantly higher inhibitory (29 mm) against *Pythium* spp after 48 hrs. But, ethylacetate extract of stem bark of *A.indica* showed higher inhibition zone (26 mm) against *Alternaria* spp than other extracts of plant parts. Whereas the highest inhibition zone (28 mm) against *Fusarium* spp was observed in root bark of ethylacetate extract at 48 hrs.

In addition to that, methanol extract of leaf and stem bark of *A.indica* exhibited equal antifungal activity (9 mm) against *Aspergillus spp* and *Alternaria* spp and *Aspergillus* spp and *Pythium* spp respectively after 96 hrs. Likewise hexane extract of root bark revealed the same activity (19 mm) against *Alternaria* spp and *Fusarium* spp.

Nevertheless, there was no activity exhibited by hexane extract of stem bark and root bark on *Aspergillus* spp. Hence, the same extract of root bark did not show any activity on *Alternaria* spp. And also methanol extract of root bark had no activity on *Pythium spp* as well. (Table 3.2)

The previous study (Usha et al,2009) indicated that neem showed antifungal activity against *Fusarium* spp [13]. From the investigation of Sunita Bansod and Mahendra Rai (2008), *A.indica* inhibited *Aspergillus niger* at 0.5 % (v/v) using agar well diffusion method. Inhibition zone for antifungal activity of *A.indica* was 16 mm at 100 µg concentration of neem oil [14].

Table 3.2: Antifungal activity of *Azadiracta indica*

Parts of Plant	Solvents	Time Hours	Aspergillus sp	Pythium sp	Alternaria sp	Fusarium sp
Leaf	Hexane	24	-	-	-	-
		48	10	14	18	13
		72	9	13	14	10
		96	8	10	11	9
	Ethylacetate	24	-	-	-	-
		48	11	29	20	14
		72	10	19	14	12
		96	9	15	10	11
	Methanol	24	-	-	-	-
		48	12	25	11	16
		72	11	18	10	14
		96	9	15	9	10
Stem bark	Hexane	24	-	-	-	-
		48	9	20	25	13
		72	9	16	18	11
		96	-	10	12	9
	Ethylacetate	24	-	-	-	-
		48	13	17	26	17
		72	11	12	21	16
		96	9	10	17	14
	Methanol	24	-	-	-	-
		48	12	18	16	22
		72	11	13	12	20
		96	9	9	10	17
Root bark	Hexane	24	-	-	-	-
		48	10	14	19	19
		72	-	12	10	15
		96	-	9	-	11
	Ethylacetate	24	-	-	-	-
		48	12	29	17	28
		72	10	19	14	23
		96	9	9	12	10
	Methanol	24	-	-	-	-
		48	11	14	18	14
		72	10	11	14	12
		96	9	-	11	9

Another study was carried out by Singh et al(1993) on aqueous leaves extracts of fifteen locally available plants *in vitro* against *Alternaria lini*, which caused leaf and bud blight in linseed, Maximum inhibition (67.7 %) was recorded with *Azadirachta indica* [4].

Root bark and leaf extracts of the *M.indica* exhibited significant antifungal activity on most of thetested fungi rather than the stem bark extract. Ethylacetate and methanol extracts showed higher antifungal activity (29 mm) on *Alternaria* spp after 48 hrs. As well as the highest inhibition zones were observed after 48 hrs incubation for ethylacetate leaf extract, methanol root bark extract and ethylacetate root bark extract of *M.indica* were 22 mm, 17 mm and 12 mm respectively. Hexane extracts had no effect on the growth of *Aspergillus* spp, *Pythium* spp and *Alternaria* spp.(Table 3.3)

Ethylacetate extract of leaf of *L.camara* had significant effect on the growth of all fungi. Ethylacetate extract of stem bark of *L.camara* exhibited highest inhibition zone (25 mm) after 48 hrs on *Alternaria* spp. Conversely hexane extracts of all plant parts had no inhibitory effect on all fungi tested. Methanol extracts of stem bark and leaf of *L.camara* only inhibited the growth of *Alternaria* spp and *Pythium* spp respectively.(Table 3.4)

3.3 Results of phytochemical analysis

In this study, the qualitative test for the presence of phytochemicals revealed the presence of various types of phytochemicals in sequentially extracted hexane, ethylacetate and methanol extracts.

Extracts of *C.alata* had saponins, tannins, flavonoids, terpenoids and alkaloids in above sequential extracts. Only flavonoids were present in the stem bark hexane extract of *C.alata*. Ethylacetate leaf extract of *C.alata* revealed the presence of both flavonoids and alkaloids. Ethylacetate stem bark extract of *C.alata* had flavonoids only. On the other hand both tannins and flavonoids were found in the methanol extract of *C.alata* stem bark. In addition to the above phytochemicals methanol extract of leaf of *C.alata* exhibited the presence of saponins and terpenoids.

The extracts of *A.indica* showed positive results to saponins, flavonoids, terpenoids, cardiac glycosides and alkaloids in above sequential extracts.. Hexane extract of *A. indica* root bark had both cardiac glycosides and alkaloids. Both alkaloids and flavonoids were in the ethyl acetate leaf extract of *A. indica*. But ethyl acetate extract of stem bark and root bark had flavonoids only. Tannins, saponins, flavonoids and terpenoids were present in the methanol extracts of leaf of *C. alata* and *A. indica* Tannins, saponins and terpenoids were in the extracts of both stem bark and root bark of *A. indica*.

Tannins, saponins, phlobatannins, steroids, flavonoids, terpenoids and alkaloids were present in the different solvent extracts of *M.indica.* Even though, the chemical constituents of both stem bark and root bark of methanol extract were similar in *M. indica* ,Cardiac glycosides were not present in any extracts . Flavonoids were in the hexane leaf and root bark extracts of *M. indica* which was similar to the stem bark hexane extract of *C. alata*.

Hexane leaf extract of *L. camara* had both terpenoids and cardiac glycosides. But hexane stem bark extract of *L. camara* had cardiac glycosides and flavonoids.

Plants *C .alata*, *M. indica*, *A. indica* and *L. camara* had different profile in chemical constituents. Even in a particular plant, different plant parts had different compounds and for a particular plant part of different plants had different compounds as well. The variation in the result of these compounds was also determined by the mode of solvent extraction.

Table 3.3: Antifungal activity of *Mangifera indica*

Parts of Plant	Solvents	Time Hours	Aspergillus sp	Pythium sp	Alternaria sp	Fusarium sp
Leaf	Hexane	24	-	-	-	-
		48	-	-	-	9
		72	-	-	-	-
		96	-	-	-	-
	Ethylacetate	24	-	-	-	-
		48	9	22	25	9
		72	-	20	21	-
		96	-	11	11	-
	Methanol	24	-	-	-	-
		48	-	18	18	12
		72	-	16	13	11
		96	-	-	-	-
Stem bark	Hexane	24	-	-	-	-
		48	-	-	-	-
		72	-	-	-	-
		96	-	-	-	-
	Ethylacetate	24	-	-	-	-
		48	-	11	-	-
		72	-	-	-	-
		96	-	-	-	-
	Methanol	24	-	-	-	-
		48	-	11	-	-
		72	-	-	-	-
		96	-	-	-	-
Root bark	Hexane	24	-	-	-	-
		48	-	-	-	-
		72	-	-	-	12
		96	-	-	-	-
	Ethylacetate	24	-	-	-	-
		48	12	14	29	14
		72	10	11	26	13
		96	-	-	17	-
	Methanol	24	-	-	-	-
		48	-	11	29	17
		72	-	-	24	-
		96	-	-	15	-

Table 3.4: Antifungal activity of *Lantana camara*

Parts of Plant	Solvents	Time Hours	Aspergillus sp	Pythium sp	Alternaria sp	Fusarium sp
Leaf	Hexane	24	-	-	-	-
		48	-	-	-	-
		72	-	-	-	-
		96	-	-	-	-
	Ethylacetate	24	-	-	-	-
		48	10	22	18	12
		72	-	18	16	9
		96	-	9	10	-
	Methanol	24	-	-	-	-
		48	-	12	-	-
		72	-	10	-	-
		96	-	-	-	-
Stem bark	Hexane	24	-	-	-	-
		48	-	-	-	-
		72	-	-	-	-
		96	-	-	-	-
	Ethylacetate	24	-	-	-	-
		48	11	-	25	-
		72	9	-	22	-
		96	-	-	13	-
	Methanol	24	-	-	-	-
		48	-	-	16	11
		72	-	-	12	-
		96	-	-	-	-

The mean diameter of inhibition zone values were measured in mm, the negative sign indicate there was no clear zone was observed.

CONCLUSION

Different plants exhibited different degree of antifungal activity among tested fungi. Hence, different plant parts extracts of a particular plant had different antifungal effects. *A.indica* showed higher degree of antifungal activity than the other tested plants. Hexane plants part extracts of *M.indica* and *L.camara* did not exhibit activity at all. However hexane extracts of *C.alata* and *A.indica* only revealed antifungal activity even though which had lower activity than the other tested extracts. Leaf extracts produced prominent inhibitory effect against tested fungi compared to other parts of tested medicinal plants. Therefore, these medicinal plants could be used for further identification of bio active ingredients which are responsible for the antifungal activity.

Acknowledgement
The authors wish to thank the National Research council, Sri Lanka for providing financial assistance (Grant No 08 / 29) for this work.

REFERENCES

[01] Nwachukwu, E.O; Umchuruba, C.I. *Journal of Applied Science and Environmental Management*. **2001**, Vol.5 (1).:29-32.
[02] Deepar, B;Susan,P.mcCormick. *Journal of the American oil Chemists' society*. **1987**, Vol.65 (7):;1166-1168.
[03] George, N.Agrios. Plant pathology. Fifth Edition, Elsevier Academic Press Publications. **2005**.
[04] Singh, H.N.P; Prasad, M.M; Surha, K.K. *Letters in applied Microbiology* **1993**, 17, 269- 271.
[05] Phongpaichit, S. Pujenjob, N. Rukachaisirikul, V. Ongsakul, M. *J. Sci. Technol*. **2005**, 27(2);; 517-523.
[06] Clark, AM; EI-Feraly, FS; LiWS. *Journal of Pharmaceutical Sciences*, **1981**, vol.70:951-952.
[07] Matos, FJA;. Introducaoa fitoquimica experimental. Imprensa Universitaria, Universidade Federaldo Ceara, **1988**, Folrtaleza.pp 128.
[08] Sofowara AE, Medicinal plants and traditional medicine in Africa, part II, **1982**, Pitman Press Ltd, London pp 128-146.
[09] Treas, GE and Evans, WC. A text book of pharmacognosy, 12th Edition, **1989**, Bailliere Tinall Ltd, London pp 333-337.
[10] Harbone JB, Phytochemical methods: A guide to Modern Techniques of plant analysis, **1973**, Chapman and Hall, London pp 199-204.
[11] Makinde AA; Igoli JO; Ama LTA; Shaibu SJ and Garba A. *African Journal of Biotechnology*. **2007**, Vol.6 (13): 1509-1510.

[12] Owoyale JA; Olatunji GA and Oguntoye SO . *Journal of Applied Sciences and Environmental Management.* **2005**, Vol.9 (4): 105-107.
[13] Usha K; Singh B; Praseetha P; Deepa N; Agarwal DK; Agarwal R and Nagaraja A *European Journal of Plant Pathology*. **2009**, 124 (4): 637-657.
[14] Sunita B; Mahendra R *World Journal of Medical Sciences*. **2008**, 3(2): 81-88.
[15] Govindachari TR; Suresh G; Geetha G; balaganeshan B and Masilamani S. *Phytoparasitica*. **1998**, 26(2): 109-116.