Investigation of Antifungal Properties of *Lantana camara* Stem Bark Extract and its Bioassay Guided Fractionation

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ABSTRACT

Medicinal plants represent a rich source of anti-microbial agents.In this study *L.camara* was selected due to lack of information in our region about the antifungal activity of its stem bark and the selected fungi were plant pathogens. Objective of the study was to carry out fractionation of crude extract and to investigate their antifungal activity. The air dried stem bark (100g) of *L.camara* was powdered and extracted with ethyl acetate (2× 200mL) at room temperature and the resulting extract was concentrated under reduced pressure to give 4.2g of crude extract. By usingVacuumLiquid Chromatography (VLC), the ethyl acetate crude was divided into two fractions (A and B) by the Thin Layer Chromatography (TLC)analysis. The antifungal bioassay was done to the above two fractions against *Aspergillus* sp.,*Alternaria* sp.,*Fusarium* sp.,*Trichoderma* sp. and *Penicillium* sp. The diameter of the inhibition zone was measured after 24, 48, 72 and 96 hour incubation periods. Zone of inhibition of extracts were compared with synthetic antifungal agent Mancozeb(positive control) and the solvent ethyl acetate (negativecontrol).Fraction B showed higher antifungal activity than fraction A. Therefore fraction B was subjected to further chromatographic fractionation. By using column chromatography, the fraction B was divided into two fractions X and Y and gave single spot based on TLC analysis. The antifungal bioassay was also done to fractions X and Y against same fungi. Both fractions X and Y showed highest inhibition on *Fusarium* sp. were 25mm and 32mm and on *Penicillium* sp. were 26mm and 34mm respectively after 48 hours of incubation. Further studies should be carried out to find out pure antifungal compounds,since this work contributed as a primary platform.

Key words: Antifungal compounds, Vacuum Liquid Chromatography, Column Chromatography

1. INTRODUCTION

Medicinal plants were used as excellent antimicrobial agents because it possess a variety of chemical constituents. In many parts of the world, medicinal plants are used for their antibacterial, antifungal and antiviral activities. The uses of plant derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance[1]. Pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including after harvesting. Generally, phytopathogenic fungi are controlled by synthetic fungicides. However, the use of these is restricted due to the harmful effects on human health and the environment.

Fusarium sp. is a filamentous fungi widely distributed in soil and in association with plants. Some species produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain. The fungal pathogen *Fusarium oxysporum* affects a wide variety of hosts of any stage. Tomato,tobacco, legumes, cucurbits and banana are a few of the most susceptible plants. *Fusarium oxysporum* generally produces symptoms, wilting, chlorosis, necrosis,prematureleafdrop, browning of the vascular system, stunting and damping-off.

Alternaria sp. is a genus of ascomycete fungi. It generally attacks the aerial parts of host. Important host plants include variety of crops such as potato, tomato, brinjal,apple, carrot and many ornamental plants[2].

Aspergillus is a member of thedeuteromycetesfungi. Aflatoxins are highly toxic and carcinogenic metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus*[3].

The Verbenaceae family plants are well known for their uses in the traditional medicinal systems of various

countries since ancient time. *Lantana camara* becomes under the family Verbenaceae. *Lantana* is a flowering plant native to tropical regions of the America and Africa [4]. Tamil name of this plant is Unnichedi and commonnames are Coronitas, *Lantana* weed, Wild sage, and Shrub Verbena.

This plant grows up to 1 to 3 meters and it can spread to 2.5 meter in width. The leaves are 2-10cm long with toothed edge and green in colour. Leaves and stem are covered with rough hairs. They grow opposite one another along the stems. Small flowers are held in clusters that are typically 2.5-5.1cm across. The different coloured flowers are presenton the same cluster. Fruit is fleshy, greenish- blue to black and berry–like with each containing one seed.

In this study *L. camara* was selected due to lack of information in our region about the antifungal activity of its stem bark and the selected fungi were plant pathogens. Objective of the study was to carry out fractionation of crude extract and to investigate their antifungal activity.

2. MATERIALS AND METHODS

Collection of plant material and extraction

Stembarkof *Lantanacamara* was collected from Kanderodai in Jaffna Peninsula. The plant material was washed and air dried. Then ground into fine powder.100g of powdersample was taken into a 500ml stoppered bottle. 200ml of ethyl acetate was added and soaked for 24 hourswith occasional shaking and filtered it. This procedure was repeated and the filtrates were combined. Then the solventwas evaporated under reduced pressure by using rotatory evaporator. The remaining samples were opened to air dry. The sample of ethyl acetate crude was weighed [3].

Preparation of slurry for VLC analysis

3.0 g of the stem bark of the ethyl acetate crude was dissolved in minimum amount of ethyl acetate and 6.0g of silica gel 60 A particle size 70 – 200 micron was added to that and mixed thoroughly. Then the solvent was evaporated under reduced pressure by using the rotator evaporator and the slurry was obtained. Initially the Buchner funnel was packed with filter paper and then a layer of cotton wool was packed above it. Silica gel 60A was packedto about 1cm thick. The slurry was then packed above that Silica gel and covered with a layer of cotton wool. Then the selected solvent systems were passed through the funnel by using micro pipette and the eluent was collected in the Buchner flask. After each elution the flask was disconnected and each fractions were collected separately[5].

Collection of fungal cultures

Tested fungi, *Aspergillus* sp., *Alternaria* sp., *Fusarium* sp., *Trichoderma* sp., *Penicillium* sp.were obtained from the culture collection of the Department of Botany, University ofJaffna. These fungal cultures were sub cultured on potato dextrose agar medium. Culture plates were incubated at roomtemperature for 2-3 days.

Preparation of fungal spore suspension

0.85% of NaCl solution was prepared. Then 9.0 ml of the saline water was transferred into medium size McCartney bottles and those bottles were sterilized by an autoclave. A loopful of each spore was taken by a sterile loop and suspended into a sterile saline water under aseptic condition. SeparatelySpore concentration was determined by the haemocytometer. Then the suspension was stirred well and serially diluted to 10^7 number of spores/ml.

Determination of antifungal activity

FractionsA&B were obtained via VLC analysis respectively. The synthetic antifungal agent Mancozeb (Dithane M-45) wasused as standard. The solvent ethylacetate was used to dissolve the crude was used as control.

0.1 ml of each fungal suspension was spread on the entire surface of PDA plate using a sterile spreader. 9 mm diameter wells were made using by a sterile cork borer. 150 microlitre of test solutions, standard (Mancozeb) and control (ethyl acetate) were added into well separately by using sterile micro titer pipettes. Plates were incubated at room temperature for 4-5 days and the zone of inhibition around the well was measured at various time of incubation such as 24 hours, 48 hours, 72 hours and 96 hours. Each experiment was repeated thrice and the meanvalue was taken. The above procedure was repeated to each fungus [6].

Column preparation

Silica gel 60A particle size 70-200 micron was packed in the column. The crude sample obtained, after concentrated using rotatory evaporator, was dissolved in minimum amount of suitable solvent. Then it was carefully appliedto the top of the column. Then the column was eluted with different solvent system [3].

3.RESULTS AND DISCUSSION

Table 1: Antifungal activity of fraction A on tested fungi

Fungus	Mean diameter of inhibition zone(mm)			
	24 hrs	48 hrs	72 hrs	96 hrs
*Aspergillus*sp.				
50 ppm	-	13	12	10
25 ppm	-	12	11	-
12.5 pm	-	10	10	-
Ethyl acetate	-	-	-	-
Mancozeb	-	14	14	10
*Penicillium*sp.				
50 ppm	-	17	16	16
25 ppm	-	11	10	10
12.5 pm	-	10	10	
Ethyl acetate	-	14	12	14
Mancozeb	-	11	10	10
*Trichoderma*sp.				
50 ppm	-	15	14	12
25 ppm	-	12	11	11
12.5 pm	-	10	10	-
Ethyl acetate	-	10	10	-
Mancozeb	-	17	15	13
*Fusarium*sp				
50 ppm	-	16	14	12
25 ppm	-	13	11	11
12.5 pm	-	-	-	-
Ethyl acetate	-	-	-	-
Mancozeb	-	11	11	10
*Alternaria*sp.				
50 ppm	-	19	15	12
25 ppm	-	16	14	11
12.5 pm	-	14	12	10
Ethyl acetate	-	11	10	-
Mancozeb		12	10	-

The negative sign indicates therewas no clear zone.

Many fractions were obtained through VLC analysis by using different polarity of ethyl acetate/hexane solvent system. These fractions were analyzed through the Thin Layer Chromatography (TLC),then similar Rf value fractions were mixed and labeled as Fraction A and Fraction B. 50ppm, 25ppm, 12.5ppm concentration of test solutions were prepared for fraction A and fraction B respectively for the antifungal assay.

Table 2: Antifungal activity of fraction B on tested fungi

Fungus	Mean diameter of inhibition zone(mm)			
	24 hrs	48 hrs	72 hrs	96 hrs
*Aspergillus*sp.				
50 ppm	-	16	14	11
25 ppm	-	14	12	11
12.5 pm	-	12	11	10
Ethyl acetate	-	11	10	-
Mancozeb	-	16	14	12
*Penicillium*sp				
50 ppm	-	24	22	13
25 ppm	-	20	20	11
12.5 pm	-	19	17	10
Ethyl acetate	-	10	-	-
Mancozeb	-	11	11	10
*Trichoderma*sp.				
50 ppm	-	16	14	13
25 ppm	-	14	11	10
12.5 pm	-	12	10	10
Ethyl acetate	-	10	10	10
Mancozeb	-	14	14	13
*Fusarium*sp.				
50 ppm	-	21	19	18
25 ppm	-	18	15	14
12.5 pm	-	13	11	11
Ethyl acetate	-	-	-	-
Mancozeb	-	10	10	-
*Alternaria*sp.				
50 ppm	-	18	18	17
25 ppm	-	17	17	17
12.5 pm	-	13	13	13
Ethyl acetate	-	-	-	-
Mancozeb		12	10	10

The negative sign indicates therewas no clear zone.

Fraction A, test solution of 50 ppm showed high antifungal activity than 25 ppm and 12.5 ppm test solutions. This indicates that the increased concentration have higheramount of antifungal compounds. Effect of ethyl acetate (control) and Mancozeb(standard) was almost same against thegrowth of *Fusarium sp*. and *Alternaria* sp. But, the effect ofstandard was higher than thecontrol against fungi *Aspergillus*sp.and *Trichoderma* sp. The effect of control was higher than the standard, against fungi *Penicillum* sp.Antifungal activity of the 50 ppm test solution was high on almost all tested fungi except *Trichoderma* sp.and*A spergillus* sp. compared with control (Table 1).

Fraction B, higher concentration of test solution exhibited high antifungal activity rather than low concentration against fungi tested. Effectof ethyl acetate (control) and Mancozeb (standard) was almost had same effect against the growth of *Penicillium* sp. and *Trichoderma* sp. But, the effect of standard was higher

when compared with fungi *Aspergillus* sp., *Fusarium* sp.and *Alternaria* sp.

Table 3: Antifungal activities for fraction X on tested fungi

Fungus	Mean diameter of inhibition zone(mm)			
	24 hrs	48 hrs	72 hrs	96 hrs
*Aspergillus*sp.				
50 ppm	-	18	16	13
25 ppm	-	16	14	12
12.5 pm	-	16	13	10
Ethyl acetate	-	11	10	-
Mancozeb	-	20	18	18
*Penicillium*sp.				
50 ppm	-	26	24	22
25 ppm	-	24	20	20
12.5 pm	-	21	18	17
Ethyl acetate	-	18	17	15
Mancozeb	-	32	31	30
*Trichoderma*sp.				
50 ppm	-	16	14	10
25 ppm	-	14	13	12
12.5 pm	-	11	10	10
Ethyl acetate	-	13	12	10
Mancozeb	-	11	11	10
*Fusarium*sp.				
50 ppm	-	25	22	20
25 ppm	-	20	15	14
12.5 pm	-	19	16	15
Ethyl acetate	-	11	10	10
Mancozeb	-	26	26	24
*Alternaria*sp.				
50 ppm	-	-	-	-
25 ppm	-	-	-	-
12.5 pm	-	-	-	-
Ethyl acetate	-	-	-	-
Mancozeb		-	-	-

The negative sign indicates therewas no clear zone.

Over all,fraction B showed significantly higher antifungal activity than fraction A. Hence,fraction B was run through the column. 52 fractions were obtained through column chromatography analysis by using 5,10,15,20,25, 30, 35, 40,50 and 100% of ethyl acetate/ hexane solvent system. These fractions were analyzed in TLC, then similar Rf value fractions were mixed according to the TLC analysis further fraction was done and labeled as fraction X and fraction Y. The above crude fractions X and Y may contain pure compounds. In future, these compounds haveto be analyzed whether they are pure or not through NMR studies and mass spectrometry for the determination of the structure. From the NMR studies, further antifungal analysis can be carried out to the above crudefractions X and Y and from that it can be reconfirmed that these compounds have certain antifungal potential and labeled as fraction X and fraction Y.

Table 4: Antifungal activities for fraction Y on tested fungi

Fungus	Mean diameter of inhibition zone(mm)			
	24 hrs	48 hrs	72 hrs	96 hrs
*Aspergillus*sp.				
50 ppm	-	16	15	14
25 ppm	-	15	13	12
12.5 pm	-	12	11	10
Ethyl acetate	-	17	15	14
Mancozeb	-	22	20	18
*Penicillium*sp.				
50 ppm	-	34	32	32
25 ppm	-	32	30	28
12.5 pm	-	28	28	26
Ethyl acetate	-	17	14	14
Mancozeb	-	32	28	28
*Trichoderma*sp.				
50 ppm	-	15	14	13
25 ppm	-	13	13	12
12.5 pm	-	12	11	10
Ethyl acetate	-	10	10	-
Mancozeb	-	14	12	11
*Fusarium*sp.				
50 ppm	-	32	31	30
25 ppm	-	26	25	24
12.5 pm	-	25	23	20
Ethyl acetate	-	17	15	14
Mancozeb	-	28	25	23
*Alternaria*sp.				
50 ppm	-	11	11	10
25 ppm	-	11	10	-
12.5 pm	-	10	-	-
Ethyl acetate	-	12	11	-
Mancozeb		15	14	12

The negative sign indicates therewas no clear zone.

The 50 ppm concentrated test solution of fraction X exhibited more antifungal activity on *Aspergillus* sp. rather than fraction Y. However, standard Mancozeb showed higher antifungal activity compared with fraction Y & ethyl acetate. But conversely, in case of *Penicillum* sp. 50ppm concentrated fraction Y highly inhibited the growth of the fungi than fraction X. In addition to that, it showed higher antifungal activity compared with standard. On the other hand, at 50ppm concentration, both fractions X&Y revealed more inhibitory effect against *Fusarium*sp. rather than Mancozeb X& control, whereas fraction X showed better activity among all tested solutions. Hence, the growth of *Trichoderma* sp. highly reduced by fraction Y than fraction X and the standard Mancozeb.There was no antifungal activity on *Alternaria* sp. by fraction X at all tested concentrations. But fraction Y exhibited antifungal activity in all concentrations.

4. CONCLUSION

We were able to successfully isolate the components present in the stem bark of *Lantana camara*. Then we did the bioassay of the different concentration of fractions against the fungi *Aspergillus* sp*., Alternaria* sp*., Trichoderma* sp*., Penicillium* sp.and *Fusarium* sp.We were happy to note that the crude of the stem bark of *Lantana camara* showed inhibitory effect against all the fungi under investigation.

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