Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(8):136-141

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

In vitro antifungal activities of *Croton sparsiflorus*

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ABSTRACT

Plants are the basis of many of the modern pharmaceuticals used today for our various ailments. The aim of the present study is to evaluate the in-vitro antifungal activities of Croton Sparsiflorus plant extract in different organic solvents like hexane, chloroform, ethyl acetate and methanol. The antifungal activities of the extract were studied against the three fungal organisms like Aspergillus niger, Candida albicans and Candida tropicalis by agar well cut diffusion method. The results were compared with Ketoconazole which was used as a standard. All of the extracts exhibited varied antifungal activities at different concentration and the inhibition efficiency varied from solvent to solvent depending on the type of the species. The antifungal activities increased as the concentration of the extract increased. At the maximum concentration of 10 µl, the methanol extract of the root and stem showed the highest inhibition activity around 30 mm against Candida albicans and Candida tropicalis.

Keywords: Sparsiflorus, Antifungal, Microdilution, Ketoconazole, Diffusion.

INTRODUCTION

Plants are used medicinally in different countries and are a source of many powerful drugs [1]. The use of medicinal plants as a source of relief from illness can be traced back to the written documents of the early civilization in India. For centuries, plants have been used by indigenous people to produce medicines that were used to treat different kinds of ailments [2]. According to WHO, upto 80% of populations rely only on medicinal plants [3, 4]. Traditional doctors and elders use several plants to treat different types of infections including those caused by fungal organisms. The preferred parts of specific medicinal plants are collected each at its favorable harvest season, dried by directly exposing them to sunlight, pounded using pestle and mortar and then stored in containers in powder form for future usage [5]. The parts of the plant used vary among species and traditional healers and also depends on the nature and state of the disease [6]. Many potent drugs including anti-malarial, anti-bacterial and anti-diabetic compounds have been purified from medicinal plants in the past [7]. Biologically active compounds present in plants have always been of great interest. In recent years, interest to evaluate plants possessing antibacterial activity for various microbes is growing [8,9]. The traditional methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries and moreover the use of herbal remedies has increased in the developed countries in the last decades. In this connection, plants continue to be a rich source of therapeutic agents. The remarkable contribution of plants to the drug industry was possible because of the large number of phytochemical and biological studies all over the world [10]. Acquired Immune Deficiency Syndrome (AIDS) caused by the Human Immunodeficiency Virus (HIV) is a devastating epidemic disease in many countries. It has been reported that HIV/AIDS is the fast growing disease in the world [11]. Up to 90% of all HIV patients contract fungal infections during the course of the disease, of which 10 - 20% dies as a direct consequence of fungal infections [12]. *Candida albicans* is the most common opportunistic infections in HIV/AIDS patients and management of such infection is faced with difficulties. Therefore, there is a need to search for alternative control methods for treating HIV/AIDS patients. The utilization of medicinal plants by the local populations constitutes an

important source of new active and renewable antifungal drugs [13]. Commercially available antifungal drugs are expensive and some fungal species are developing resistance and therefore screening for more medicinal plants with antifungal activities can help in the identification and development of more active and affordable drugs [14]. *Aspergillus niger* is a saprophytic and filamentous fungus found in soil, forage, organic debris and food product, causing black mould of onion, Shallot and boll rot of Cotton; spoilage of cashew kernels, dates, figs, vanilla pods and dried prune[15,16]. Previous studies on medicinal plants have concentrated on the antibacterial activity and very few studies have targeted the antifungal activities of medicinal plants [17,18]. *Croton sparsiflorus* belonging to family Euphorbiaceae is found to spread over the waste lands of Southern India. Although many research works were carried out on other medicinal plants, the thorough literature survey shows that there is no literature available on the species *Croton sparsiflorus.* Hence the present study is aimed to investigate the antifungal activities of *Croton sparciflorus* against *Aspergillus niger, Candida albicans and Candida tropicalis.*

EXPERIMENTAL SECTION

Collection of plant and extraction

Croton Sparsiflorus Morong (Syn. *C. Bonplandium*), is an herb that grows throughout India as a wild weed in fields and forest. The plant was collected in the month of August from Auxilium College campus, Vellore, Tamil Nadu, India and was taxonomically identified by the Department of Botany, Auxilium College, Vellore, Tamil Nadu, India and a voucher specimen is retained as an herbarium in the department for future reference.

The freshly cut plants were sorted out as root, stem, leaf, inflorescences and shade dried in the room with active ventilation at ambient temperature and pulverized to a coarse powder using mechanical grinder and sieved with the help of 40 mesh size. The different parts of the plant were separately percolated using different solvents like hexane, chloroform, ethyl acetate and methanol as solvent. The miscella was then concentrated using a vacuum rotary evaporator under reduced pressure. The dark brownish semisolid extract was preserved in tightly closed container and used for the analysis.

Fungal strains

The clinical fungal test organisms used for study are *Candida albicans(*ATCC10231), *Aspergillus niger (*ATCC16404) and *Candida tropicalis* (ATCC10435) were procured from National Chemical Laboratory (NCL), Pune, India. The standard strains of the microbes used are incubated at 25ºC for 24 - 48 h. Each of the pure cultures was suspended in a Roux bottle containing 5 ml of physiological saline. Each suspension of microorganisms was standardized to 25% transmittance at 560 nm using an ultraviolet (UV) - visible spectrophotometer.

Agar well cut diffusion method

The fungal isolate was suspended in Brain Heart Infusion (BHI) broth and diluted to approximately 10^5 colony forming unit (CFU) per ml. They were flood-inoculated onto the surface of BHI agar and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer and the sample of 500 mg was dissolved in 1ml DMSO. The different volumes of the extract like 2.5µl (12.5mg), 5µl (25 mg) and 10 µl (50 mg) were transferred into the wells. The plates were incubated for 18 h at room temperature. Antifungal activity was evaluated by measuring the diameter of the zone of inhibition in millimeter against the tested microorganisms. Ketoconazole was used as a positive control and DMSO was used as a negative control. The tests were carried out in triplicates and the values are expressed as mean inhibition zone (mm) ± S.D [19].

Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was determined by micro dilution method by using 96 well micro titration plates. Briefly, 185µl of the broth was added into each well in the first row of micro titration plate and 100 µl to the rest of the wells from the second row downwards. 15µl of the plant extracts was then added into each well on the first row (row A), starting with the positive control (Nystatin, Roche), followed by the negative control (20% DMSO used to dissolve the plant extracts) and the plant extracts in the rest of the wells on that row. A twofold serial dilution was done by mixing the contents in each well of the first row and transferring 100µl to the second well of the same column and the same was done up to the last well of the same column and the last 100 µl from the last well was discarded. Then 100 µl of yeast suspensions was added. The results were observed after 24 h incubation at 37°C followed by the addition of 40 µl of a 0.2% Iodo Nitro Tetrazolium (INT) solution after a further incubation of 4 h at 37°C. The wells that did not show any colour change after INT was added indicating the concentration of the plant extract that was able to inhibit fungal growth whereas the pink colour change indicated fungal growth.[20]

Minimum Fungicidal Concentration

The Minimum Fungicidal Concentration (MFC) was determined by inoculating the contents from the MIC plates onto SDA plates and the results were observed after 24 h incubation at 37°C. The presence of the fungal colonies on

agar plates was an indication that the plant extract inhibited the growth of the fungi without killing them and the absence indicated that the plant extract was able to kill the fungal organisms. The lowest concentration of the plant extract that was able to kill the microorganisms was considered as the minimum fungicidal concentration [21].

Data analysis

The tests were carried out in triplicates and the results are expressed as mean inhibition zone (mm) ± S.D. Regression analysis was followed to check the linearity for the mean absorbance for all concentration. ANOVA was used to study the significance of the concentrations.

RESULTS AND DISCUSSION

Over the last two decades, fungal infections have become important public health concerns [22]. The fungal infections are the most common opportunistic infections in the immuno-compromised individuals causing various illnesses [23]. The antifungal drugs used to cure infections make the organisms to become resistant towards it [24]. Because of this reason new drugs are to be discovered to control the disease causing pathogens. In this regard higher plants play an important role by providing antibiotic compounds and they are rich in active principles, which are used as therapeutic drugs [25] In India, variety of medicinal plants are used to control a number of diseases in folk medicine. But only few of them were studied for their antimicrobial activities [26]. The Antifungal activities of the different part of the plant in Hexane extract were studied and the results are presented in table 1. From the result it is evident that the root, stem, leaf and inflorescences showed a maximum inhibition zone with the values of 21±0.22, 12±0.77, 20±0.33 and 08±0.44 mm against *Aspergillus Niger*. The *Candida albicans* showed the activities of 28±0.89, 19±0.33, 21±0.22 and 14±0.66 mm and *Candida tropicalis* exhibited 22±0.67, 14±0.56, 11±0.12 and 12±0.29 mm inhibitory effect at the concentration of 10µl which is comparable with the standard Ketoconazole. Among the three fungal organisms, maximum growth suppression is observed in *Candida albicans* than that in both *Aspergillus niger and Candida tropicalis*.

The results of the antifungal activities of the different parts of the plant in Chloroform extract are shown in table 2 and the results show that the root, stem, leaf and inflorescences exhibited a maximum inhibition zone of 21±0.56, 18±0.54, 08±0.77 and 07±0.88 mm against *Aspergillus Niger*. The fungi *Candida albicans* was inhibited by the chloroform plant extract whose zone of inhibition values are 26±0.25, 14±0.78, 21±0.34 and 08±0.21 mm and *Candida tropicalis* was inhibited with zone of inhibition 18±0.46, 11±0.36, 12±0.36 and 10±0.27 mm at a concentration of 10µl which are comparable with the standard Ketoconazole. Among the three fungal organisms, maximum growth suppression is observed in Candida albicans than that in both *Aspergillus niger and Candida tropicalis*.

The Antifungal activities of the different parts of the plant in ethyl acetate were studied and results are presented in table 3. The root of ethyl acetate extract is highly effective against *Aspergillus Niger* and *Candida albicans* with a zone of inhibition of 21±0.26mm and 26±0.57 respectively. The stem showed the inhibition efficiency of 27±0.38 mm at a concentration of 10µl. The antifungal activities of the other parts of the plant varied depending on the concentration and fungal organisms.

The Antifungal activities of the different parts of the plant in Methanol extract was shown in table 4. From the result it is evident that the zone of inhibition against all the tested fungi increases with increase in concentration. The inflorescences showed the least inhibition activity of 07± 0.44mm at 2.5µl and found to increase 21±0.67 mm at 10µl which is closer to standard Ketoconazole whose zone of inhibition is 22±0.45. The root, stem and leaf showed a maximum inhibition zones of 24±0.44, 24±0.18, 25±0.67 mm against *Aspergillus Niger*, 29±0.25, 23±0.47, 28±0.17 mm against *Candida albicans* and 31±0.19, 29±0.27, 29±0.13 mm against *Candida tropicalis* at a concentration of 10µl whose values are comparable with the standard Ketoconazole. Among the three fungal organisms, maximum growth suppression is observed in Candida tropicalis than that in both *Aspergillus niger and* Candida albicans.

From the above results it is evident that the root showed the highest zone of inhibition against all the fungi in different organic solvents. When compared with the other extracts the methanolic extracts showed exceptionally prominent activity and the activity of the extracts decreased in the order of: Ethyl acetate> Chloroform> Hexane based on the polarity of the solvents. The exemplary activity of the methanolic extract was due to the phenolic and flavonoid content in the extract and it can be suggested that methanolic extracts of screened plants would be helpful in treating fungal diseases. [27]

The MICs of the extracts against the three fungal organisms were calculated and the results are given in fig 1. The lowest MICs are obtained with all the extracts of inflorencence with the value of 0.46 mg/ml against *Aspergillus niger*. The methanolic extract of Leaf against *Candida albicans* and *Candida tropicalis* is found to be 1.00 mg/ml and 2.00 mg/ml. The hexane extract of stem, inflorescence and root are not as active as the other extracts against *Candida tropicalis* (MIC 7.0 mg/ml) but more active against other species like *Aspergillus niger* and *Candida albicans.*

The capacity of the plant extracts to kill the fungal organisms instead of inhibiting their growth is measured and reported in Fig 2. The MFC value of the plant extract in different organic solvents varied from 2.00 mg/ml to 9.00 mg/ml.

Table: 1 Antifungal activities of the different part of the plant in Hexane extract

Microorganisms	Concentration (µl)	Zone of inhibition (mm)				
		Root	Stem	Leaf	Inflorescence	Ketoconazole
Aspergillus niger	2.5	07±0.45	-	-	-	22±0.45
	5.0	15±0.32	-	12±0.24	-	22±0.45
	10	21±0.22	12±0.77	20±0.33	08±0.44	22±0.45
Candida albicans	2.5	09±0.13	-	-	-	30±0.36
	5.0	16±0.23	11±0.45	14±0.14	09±0.23	30±0.36
	10	28±0.89	19±0.33	21±0.22	14±0.66	30±0.36
Candida tropicalis	2.5	07±0.56	-	-	-	27±0.78
	5	14±0.12	-	-	7±0.44	27±0.78
	10	22±0.67	14±0.56	11±0.12	12±0.29	27±0.78

Values are mean inhibition zone (mm) ±S.D of tripl icates.

Table: 2 Antifungal activities of the different part of the plant in Chloroform extract

Microorganisms	Concentration (µl)	Zone of inhibition (mm)				
		Root	Stem	Leaf	Inflorescence	Ketoconazole
Aspergillus niger	2.5	07±0.12	07±0.33	-	-	22±0.45
	5	16±0.33	11±0.22	-	-	22±0.45
	10	21±0.56	18±0.54	08±0.77	07±0.88	22±0.45
Candida albicans	2.5	11±0.67	-	09±0.66	-	30±0.36
	5	20±0.46	-	14±0.39	-	30±0.36
	10	26±0.25	14±0.78	21±0.34	08±0.21	30±0.36
Candida tropicalis	2.5	09±0.11	-	-	-	27±0.78
	5	12±0.89	-	-	-	27±0.78
	10	18±0.46	11±0.36	12±0.36	10±0.27	27±0.78

Values are mean inhibition zone (mm) ±S.D of tripl icates.

Table: 3 Antifungal activities of the different part of the plant in Ethyl Acetate extract

Microorganisms	Concentration (µl)	Zone of inhibition(mm)				
		Root	Stem	Leaf	Inflorescence	Ketoconazole
Aspergillus niger	2.5	07±0.34	-	-	-	-
	5	16±0.57	-	19±0.48	05±0.67	22±0.45
	10	21±0.26	13±0.28	26±0.37	09±0.22	22±0.45
Candida albicans	2.5	11±0.25	-	07±0.55	-	-
	5	20±0.33	11±0.45	13±0.36	07±0.33	30±0.36
	10	26±0.57	25±0.32	21±0.17	15±0.55	30±0.36
Candida tropicalis	2.5	09±0.79	07±0.33	09±0.25	07±0.78	27±0.78
	5	12±0.44	15±0.47	11±0.12	13±0.45	27±0.78
	10	18±0.36	27±0.38	20±0.11	20±0.17	27±0.78

Values are mean inhibition zone (mm) ±S.D of tripl icates.

Table: 4 Antifungal activities of the different part of the plant in Methanol extract

Microorganisms	Concentration (µl)	Zone of inhibition (mm)				
		Root	Stem	Leaf	Inflorescence	Ketoconazole
Aspergillus niger	2.5	08±0.33	-	07±0.22	07±0.44	22±0.45
	5	17±0.35	11±0.66	13±0.17	13±0.77	22±0.45
	10	24±0.44	24±0.18	25±0.67	21±0.67	22±0.45
Candida albicans	2.5	07±0.11	07±0.26	09±0.25	-	30±0.36
	5	16±0.23	12±0.44	15±0.28	11±0.49	30±0.36
	10	29±0.25	23±0.47	28±0.17	23±0.56	30±0.36
Candida tropicalis	2.5	11±0.28	11±0.44	11±0.15	9±0.19	27±0.78
	5	23±0.28	19±0.35	16±0.15	15±0.29	27±0.78
	10	31±0.19	29±0.27	29±0.13	26±0.17	27±0.78

Values are mean inhibition zone (mm) ±S.D of tripl icates.

Fig 1 MIC of the different parts of the plant in different organic solvents

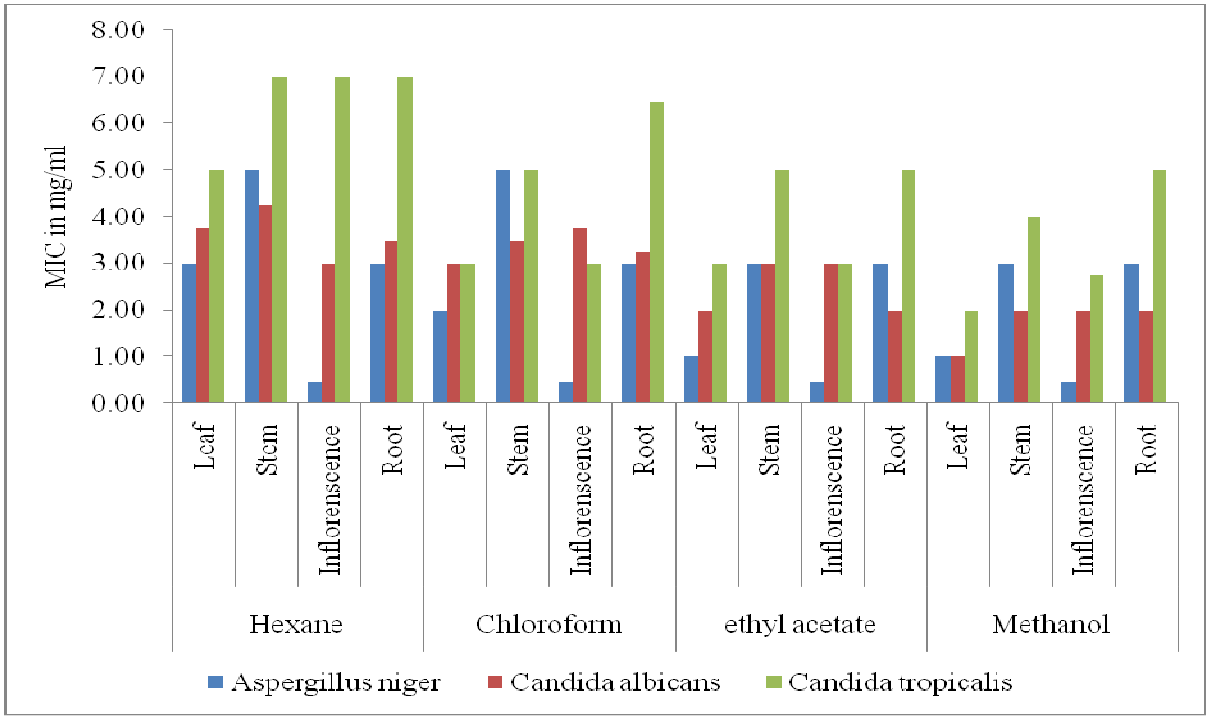
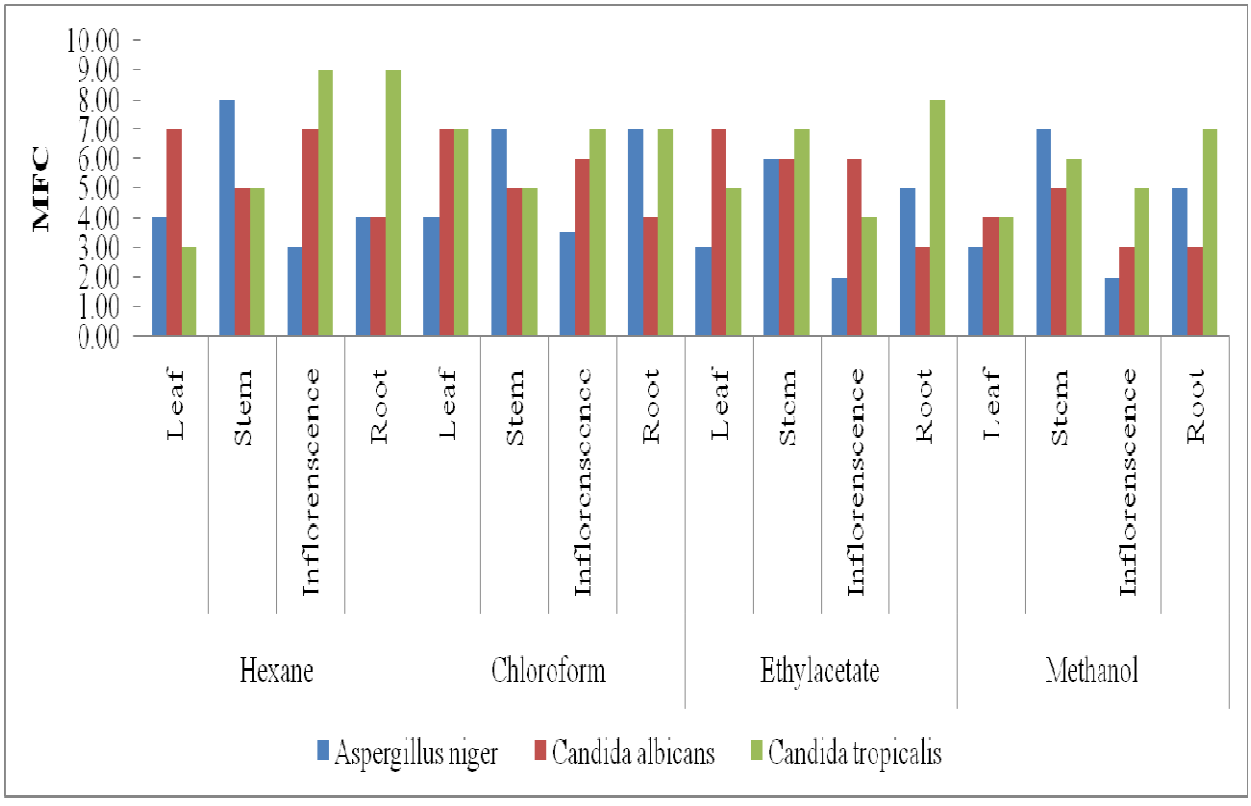


Figure 2 MFC of the different parts of the plant in different organic solvents



CONCLUSION

This study provides new scientific information on antifungal activity of Croton Sparsiflorus plant against Aspergillus niger, Candida albicans and Candida tropicalis. The results confirm that the antifungal activity increases with the concentration of the different organic extract. At the highest concentration of 10µl the zone of inhibition is found to be closer to the standard Ketoconazole. The inhibition activity varied depending on the organic solvents

and on the type of the species studied. However, further work may be extended to identify the active ingredients present in these extracts, which will be useful for laboratory synthesis and production of natural fungicides.

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