ORIGINAL RESEARCH PAPER | Biological Science | Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Evaluation of Piper Betle L. (Local Cultivar Karpoora Variety) Leaf Extracts for Antifungal Activity Against Selected Fungal Pathogens

KEYWORDS

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ABSTRACT *The antifungal potential of the Piper betle extracts were investigated in the present study. Different extracts of P. betle leaves viz., Methanol, Ethanol, Acetone, Chloroform and Water were used against six human pathogenic fungi. Among the extracts tested, methanol extracts prepared from Karpoori variety of Piper betle collected from Tamil Nadu exerted good antifungal activity against test pathogens. The yeast pathogens viz., Candida albicans, Cryptococcus neoformans, and Histoplasma capsulatum were exhibited greater susceptibility towards methanol extract of betel leaves. The results clearly indicated that there was a marked difference among the same variety of P. betle cultivated under two different regions in their antimicrobial nature.*

Introduction

Fungi are ubiquitous in nature. Of the thousands of fungal species, a few could cause disease in humans. Fungal infections may be categorized as mild (minor skin infections) to life- threatening (Systemic) diseases. Some species are opportunistic pathogens, they normally present in the body without causing any infection, when the person become immunodeficient (or) immunocompromized, the existing fungi can cause opportunistic infection. The fungal infections are more common among the persons taking immunosuppressive therapies, use of indwelling intravenous devices *etc.*

In recent years, there has been an increasing search for new antifungal agents due to the emergence of resistance against already available antifungal agents. Since many of the available antifungal drugs have undesirable side effects or are very toxic (amphotericin- B), produce recurrence, show drug- drug interactions (azoles) or lead to the development of resistance (fluconazole, 5- flucytosine), some shows ineffectiveness (White *et al.*, 1998; Muschietti *et al.*, 2005) and have become therefore less successful in therapeutic strategies. Therefore it is necessary to search for more effective and less toxic novel antifungal agents that would overcome these disadvantages.

Medicinal plants have been a source of wide variety of novel compounds they can be used extentively as crude material or as pure compound for treating various diseases (Arif *et al.*, 2011). It is estimated that about 25% of modern medicines are directly derived from higher plants. It is interesting to note that about 60% of the antitumor and antimicrobial medicines currently available on the market are derived from natural products, mainly from higher plants (Kirtikar and Basu.1999).

The plant *P.betle* has many traditional medicinal uses and their antiseptic property has been known since from 600B.C (Sudewo, 2011).The *P. betel* (Tamil: Vetrilai) is a perennial dioecious glabrous climbing vine belonging to the family Piperaceae. Leaves are simple, alternate, and yellowish to bright green in colour (Sharma *et al.*, 2006; Jayaweera, 1982).The plant grows well in warm, humid climates (Nanmayakkara *et al.*, 2014). Eventhough numerous research has been done on various medicinal uses of *P. betle* leaves, the comparative study between leaf varieties grow under different climate is limited. Therefore, objective of the present study was to compare antifungal properties of betel leaf Karpoora variety collected from Tamil Nadu and Kerala state against selected fungal pathogens.

Materials and methods

Collection of plants

The *P. betle* L. (Local cultivar Karpoora) variety leaves cultivated round Dindigul (Dt), Tamil Nadu and Palakkad (Dt), Kerala were selected for the present study. Healthy and well grown young greenish leaves were collected directly from the cultivated fields in sterile polythene bags and transported in to the laboratory. The leaves were washed alternatively with tap water and distillted water, then surface sterilization was done with 10% concentrated sodium hypochloride solution to prevent the growth of microbes, rinsed again with sterile distilled water and shade dried at room temperature.

Authentication of plant materials

The *Piper betle* leaves collected from Dindigul area authenticated by Dr.R. Ramasubbu, Assistant Professor, Department of Biology, The Gandhigram Rural Institute- Deemed University, Dindigul, Tamil Nadu. The betel leaves collected from Palakkad were authenticated by Dr. V. Suresh, Assistant Professor, Department of Botany, Government Victoria College, Palakkad, Kerala. The voucher specimen of the leaf varieties were deposited at the herbarium of the above said colleges for further reference.

Preparation of plant extracts

Dried leaves were homogenized into a fine powder by using mixer – grinder. Five different solvents *viz.*, Methanol, Ethanol, Acetone, Chloroform and Water were used as solvents to extract bioactive compounds from the sample. About 50 gram of powdered samples were loaded in Soxhlet apparatus with 250ml of respective solvent separately and extracted for about 72hours. The extraction was continued until the extractive become colorless. Finally, all the successive extracts were evaporated in rotary vacuum evaporator at 40°C. The crude extract thus obtained were transferred into glass vials and stored at 4°C until it is required.

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Test organisms used
The fungal Pathogens used in the present study were procured from Institute of Microbial Technology (IMTech), Chandigarh. The lyophilized cultures were revived by inoculating the strains in to Sabourad's Dextrose Broth. The stock culture was maintained on Sabourad's Dextrose Agar slants and stored at 4°C in a refrigerator.

Preparation of Inoculum
The yeast and mold culture were grown on PDA plates at 28°C for 48 hours(for yeast) and 7 days (for fungi). Spore suspensions were prepared in sterile distilled water containing 0.5% Tween 20 and the densities adjusted with a spectrophotometer at a wavelength of 530nm to yield an inoculums of $1.0x10^6$CFU/ml (Prabhu *et al.*, 1995).

Antifungal assay
For the assay, the fungal broth culture was macerated and homogenized under sterile condition. About 0.1ml of fungal culture with known spore count was uniformly seeded with sterile cotton swab on SDA plates. The extract loaded whatman paper discs were placed on the fungal seeded plates with sterile forceps under aseptic conditions. The plates were incubated at 28***± 2°C*** for 72 hours (parekh and Chenda, 2007). The standard antifungal antibiotic Amphotericin-B (10µg/disc) was also placed to compare the results. After incubation, the zone of inhibition was measured in mm unit.

Minimum Inhibitory Concentration (MIC)
For the determination of MIC two fold serial dilution method was followed with Muller Hinton Broth (Ericsson and Sherriel, 1971). The plant extracts were diluted to the concentration ranging from 3.9 to1000µg/ml. The MH broth tube inoculated with respective culture was served as a positive control, whereas uninoculated broth served as negative control. The tubes were incubated at 28°C for 72 hours and the results were recorded. The MIC was the lowest concentration of the extract that did not permit any visible growth on the inoculated tubes.

Minimum Fungicidal Concentration (MFC)
The MFC of the extracts was determined by plating a loopful of culture onto SDA plates and incubated under aseptic condition for 72 hours at 28°C. The lowest concentration of the extract that showed no visible growth on solid media was recorded as the MFC.

Results and Discussion
As like antibacterial property of betel leaves, it is equally important to explore the antifungal property resides in the betel leaves. Their antifungal potency has not been adequately studied by the researchers. Hence, in the present study antifungal properties of the different extracts obtained from two varieties of *P. betle* leaves were studied against some human pathogenic fungi using disc diffusion method. As stated by Kishore *et al*. (2001) paper disc diffusion method gives qualitative information on the efficiency of test compound and it can be used routinely to demonstrate antifungal activity of extracts.

Ali *et al*. (2010) have analyzed *in vitro* antifungal activity of hydroxychavicol obtained from the chloroform extraction of the aqueous leaf extract of *P. betle* L. (Piperaceae). Of 124 strains of fungi tested, dermatophytes were found to be most susceptible species to hydroxychavicol. MIC values ranged between 15.62 to 500 µg/ml for yeast, 125 to 500 µg/ml for *Aspergillus* species and 7.81 to 62.5 µg/ml for dermatophytes whereas the MFCs were found to be similar or two fold greater than the MIC. Evans *et al*. (1984), demonstrated antifungal activity and isolated hydroxychavicol, chavicol, chavibetol and chavicol acetate from chloroform extracts of *P. betle*.

In the present study, the methanolic extracts prepared from Local cultivar P. betle (Karpoore variety) collected from Tamil Nadu exerted good antifungal activity. The yeast strains such as C. albicans and Cryptococcus neoformans were highly susceptible and showed 15.8±0.3mm zone of inhibition against methanolic extracts of P. betle collected from Dindigul, Tamil Nadu. This was followed by Histoplasma capsulatum (14.9±0.5mm), C. glabarata (13.9±0.3mm), A. flavus (12.3±0.5mm) and A. niger (10.8±0.3) at 300 µg/ml concentration of methanolic extracts. The MIC values ranged between 62.5 to 125µg/ml, where as the MFCs were two fold greater than the MICs (Table-1). The P. betle (local cultivar Karpoore variety)from Palakad of Kerala, showed lesser inhibitory activity when compared with Tamil Nadu variety against selected fungal pathogens (Table-2).

About 12 species of local Malaysian plants were screened for antifungal activity against 5 strains of medically important fungi by disc diffusion method. Among the plants tested, *Piper betle* extracts showed better antifungal property against 4 to 5 strains of fungus. Maximum inhibitory property was noted against *Trichophyton rubrum*, least activity was noted against *C. albicans* (9mm) compared to other fungi (Nazmul *et al.*, 2011). On the contrary, *C. albicans* was the most susceptible pathogen against betel leaf extracts used in the present study.

Trakranrungsie *et al.*, (2006), formulated 10% *P. betle* cream and subjected to physical and microbial limit tests, and evaluated for its effect against zoonotic dermatophytes *in vitro*, *P.betle* cream exhibited greater inhibitory effect towards the tested dermatophytes *viz.*, *Microsporum canis, M. gypseum* and *Trichophyton mentagrophyte*.

Kawsud *et al*. (2014) evaluated certain herbs for their anticandidal and antibiofilm activity by determining MIC and MFC by microdilution method. Puspitasari and Apriasari (2012) compared antifungal test against *C. albicans* which was given 35% extract of *P. betle* Linn. Leaf and 0.2% chlorhexidine. The extract of 35% *P. betle* leaf had antifungal activity against *C. albicans* due to phenol content but the activity was lesser than 0.2% chlorhexidine (positive control).

The antibacterial activity of *P. betle* Karpoori variety leaves from Tamil Nadu and Kerala was reported in our previous studies (Aruljothi *et al.*, 2016). The results of the present study also showed a significant remark in the antifungal activities of the same variety of betel leaves grown under different locations. Our results are in accordance with the findings of Ramalakshmi *et al.*, (2002). According to them, the constituent in the betel oil may vary qualitatively and quantitatively based on different factors like plant variety, soil, climate and the agronomic practices followed to raise the crop *etc*. Most of the studies revealed that plant the grows under stress condition may found to possess effective phytochemicals both qualitatively and quantitatively. This has been confirmed in our study, the plant leaves collected from Tamil Nadu recorded good antimicrobial activity than those collected from Kerala. Further study in this regard might be useful to isolate potent antifungal compound from betel leaves to develop drug against fungal pathogens.

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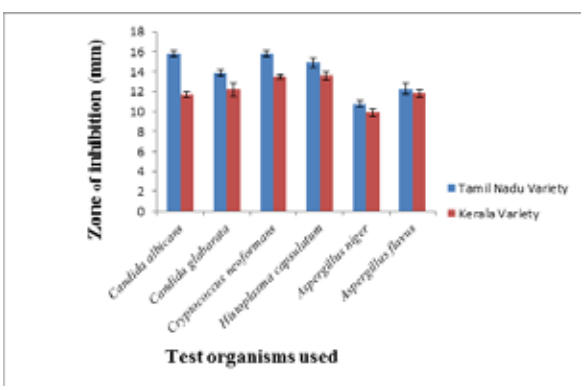
Table:1. Antifungal activity of Karpoora variety of Piper betle leaves collected from Tamil Nadu

S.No	Fungal pathogens used	Zone of inhibition(mm) Concentrations used (µg/ml)			MIC (µg/ml)	MFC (µg/ml)	StandardAmphotericine -B (5 µg/disc)
		100	200	300			
1.	Candida albicans	9.5 ±0.4	11.9 ±0.4	15.8 ±0.3	62.5	125	24.5 ±0.4
2.	Candida glabarata	9.3 ±0.5	12.2 ±0.4	13.9 ±0.3	125	250	27.6 ±0.5
3.	Cryptococcus neoformans	9.6 ±0.4	11.9 ±0.5	15.8 ±0.3	125	250	20.7 ±0.3
4.	Histoplasma capsulatum	10.9 ±0.4	12.8 ±0.3	14.9 ±0.5	62.5	125	26.7 ±0.5
5.	Aspergillus niger	8.5 ±0.5	9.7 ±0.5	10.8 ±0.3	62.5	125	26.7 ±0.6
6.	Aspergillus flavus	10.4 ±0.4	10.8 ±0.3	12.3 ±0.5	62.5	125	23.5 ±0.4

Table:2. Antifungal activity of Karpoora variety of Piper betle leaves collected from Kerala.

S.No	Fungal pathogens used	Zone of inhibition(mm) Concentrations used (µg/ml)			MIC (µg/ml)	MFC (µg/ml)	Standard Amphotericine -B µg/mL (5 µg/disc)
		100µg/ml	200µg/ml	300µg/ml			
1.	Candida albicans	8.9±0.5	9.6±0.4	11.7±0.3	125	250	24.5 ±0.4
2.	Candida glabarata	9.9±0.4	11.8±0.5	12.2±0.6	125	250	27.6 ±0.5
3.	Cryptococcus neoformans	9.9±0.3	12.8±0.5	13.5±0.2	125	250	20.7 ±0.3
4.	Histoplasma capsulatum	10.7±0.4	12.8±0.5	13.6±0.4	125	250	26.7 ±0.5
5.	Aspergillus niger	8.5±0.4	9.3±0.3	9.9±0.4	125	250	26.7 ±0.6
6.	Aspergillus flavus	8.5±0.5	10.9±0.3	11.8±0.4	125	250	23.5 ±0.4

Fig: 1. Antifungal activity of methanolic extracts of Karpoora variety Piper betle leaf extracts against selected pathogens



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