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Research Article

DEMONSTRATION OF PRECLINICAL ANTIDERMATOPHYTIC ACTIVITY BY SEED KERNELS OF *PONGAMIA PINNATA* (LINN.)

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ABSTRACT

The aim of the present study was to evaluate the *in vitro* pharmaceutical efficacy of different extracts and partially purified phytochemical substances of *Pongamia pinnata* (Linn.) for their antifungal properties. The seeds of *P. pinnata* (Linn.) were processed by standard procedures so as to obtain aqueous and ethanolic extracts. These were further independently subjected to seitz filtration, lyophilization and column chromatography to prepare appropriate working whole and constituent extracts. These extracts were tested by CLSI prescribed microdilution method (M38-P) for their antidermatophytic activity against the clinical isolates and standard strains of *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. As a control study standard antifungal agents were also tested by similar procedure. The aqueous seed extract exhibited complete inhibitory activity against both clinical and standard strains at concentrations ≥12.5µg/ml except for *T. mentagrophytes* (≥25µg/ml). While the constituent chemical compound 'C' inhibited all the organisms at concentrations ≥12.5µg/ml, the compound 'D' could inhibit only *T. mentagrophytes*. The drug Fluconazole exhibited total antidermatophytic activity with profound action on *M. gypseum* (≥6.25µg/ml). Overall, the study portrayed the efficacious antidermatophytic property of seitz filtered-lyophilized aqueous extract of seed kernel of *Pongamia pinnata* (Linn.) and advocates the potentiality of the plant as a source of alternative medicine.

Keywords: *Pongamia pinnata*, Antifungal, Dermatophytes, Seitz filtered-lyophilized extract.

1. INTRODUCTION

The 'mycoses' – infections caused by fungi are categorized into four major groups viz., superficial, subcutaneous, systemic and opportunistic. The dermatophytic fungi cause superficial mycoses, which affect keratinized tissues (hair, nails & skin) leading to dermatophytosis, which is more common in immunocompromised patients[1-3]. The dermatophytic fungi are ubiquitous in nature and possess extraordinary characteristics in surviving extreme conditions. The dermatophytes are implicated with frequent infections in tropical countries known for hot and humid climatic conditions. The morbidity caused by these agents is viewed as a public health problem in most of the countries. The socioeconomic conditions such as overcrowding, malnutrition, poor sanitation and improper practice of personal hygiene greatly influence the occurrence of this pathogen[4-7].

Pongamia pinnata (Linn.) is a moderate sized, semi-evergreen tree of the family Papilionaceae and popularly called as 'Karanj.' The plant is rich in flavonoids compounds and reported to exhibit antimicrobial properties. The powdered seed is valued as febrifuge and used for controlling bronchitis and whooping cough. A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for healing ulcers in gonorrhea and scrofulous enlargement[8]. The seed extract has been demonstrated to possess potential antiviral properties against Herpes Simplex Viruses (HSV) *in vitro*[9].

In the Ayurvedic literature of India, different parts of this plant have been recommended for use in treating various ailments and have been traditionally employed in preparing medicines for bronchitis, whooping cough, rheumatic joints and diabetes. Pharmacognostic characterizations had been established with respect to organoleptic, macroscopic, microscopic, fluorescence analysis and physicochemical parameters of the stem bark of *P. pinnata* (Linn.)[10]. Different phytochemical componds including those of β-sitosterol, β-sitosteryl acetate, β-sitosteryl galactoside, stigmasterol, stigmasteryl galactoside and sucrose had been extracted from the seed of *P. pinnata* (Linn.)[11]. The seeds have been used for treating various inflammatory and infectious diseases such as Leucoderma, Lumbago, Muscular and Auricular Rheumatism and Lepropsy[12]. The leaves had been reported to confer various pharmacological properties such as digestive, laxative, antibacterial, anticandidal (seed kernel)**,** antiviral and antigiardial activities[13-14].

As this is the modern era of post antibiotic discovery and renewed interest in traditional medicine, there is an immense need to explore naturally occurring products to combat emerging and re-emerging infectious diseases. In order to achieve such a goal follow of standard and universally acceptable scientific evaluation procedures are mandatory. The Clinical Laboratory Standards Institute (CLSI) protocols are considered vital in evaluating the efficacy of antimicrobial agents[15]. Thus the present study has been conducted with the aim of extracting and isolating potential components of the Indian Medicinal Plant, *Pongamia pinnata* (Linn.) so as to investigate its antifungal activity against commonly occurring dermatophytic agents.

2. MATERIAL AND METHODS

2.1. Preparation of seed kernel extracts of P. pinnata (Linn.)

2.1.1. Preparation of Seitz filtered and lyophilized whole extracts

Healthy dried seeds of *P. pinnata* (Linn.) were collected from the Guindy campus of University of Madras, Chennai, India. The seeds were washed twice with double distilled water and then surface sterilized with 70% ethanol. It was ground into a paste, which was mixed in appropriate diluents or solvent so as to obtain desirable viscosity i.e., aqueous and ethanol extracts were prepared by mixing the paste in water (1:5) or ethanol (EtoH) (70%) respectively[16]. The extract paste thus obtained was then placed on sterile gauze cloth and the liquid portion of it was collected by gentle hand pressure. This extract was further centrifuged at 3000rpm for 10 min. The supernatant was collected and filtered through sterile seitz filter (0.2μm pore size) (Biomed Technologies, India) and the filtrate was collected in a sterile conical flask by negative pressure. The pH of the extract filtrate was measured as 6.2 (aqueous) and 6.9 (ethanolic) using pH meter (Hanna Instruments, India). The sterility of this extract was tested by placing a drop of it on sterile Nutrient agar and Sabouraud's Dextrose Agar **(**SDA) plates (HiMedia Laboratories, India). The sterile seitz filtered extract was then transferred to lyophilization flask and kept for freezing at -80^{o}C in deep freezer (Blue star, India). The frozen sample was then loaded onto the lyophilizer (Patel Scientific Instrument, India) to remove the water content at -55^{o}C for 24 h. At the end of freeze-drying the powdered extract was transferred to sterile vials and stored at -20^{o} C until further use**.**

2.1.2. Partial purification of Phytoconstituents of the seed kernel of P. pinnata (Linn.)[17-18]

For the purpose of extraction of oil from *P. pinnata* (Linn.) the seed kernels were shade dried and ground. This powder (1 kg) was treated with petroleum ether (3 liters) at 85^{o}C for 8 h. The residue (considered to possess Pongam oil) was separated from the aqueous portion by filtration. The Pongam oil (260 ml) was mixed with methanol (150 ml) and water (150 ml) and left at room temperature for 2 h to achieve the separation of methanol layer (150 ml) and aqueous layer (410 ml). The aqueous layer was dissolved in petroleum ether with subsequent addition of acetic acid (180 ml) and water (180 ml). From this, aqueous acetic acid layer and petroleum ether layer (230 ml) were separated. The petroleum ether layer was separated and packed in Column Chromatography. The purification of the petroleum ether layer (50 ml) yielded four

fractions presumed possess potential phytochemical compounds and were named as A, B, C and D.

2.1.3. Characterisation of Phytoconstituent fractions (C & D)

The physico-chemical characterization of setiz filtered and lyophilized whole extract of the seed kernel such as physical nature and their solubility were performed using standard procedures[19].

2.2. Test organisms and antifungal drugs for antidermatophytic assay

2.2.1. Test organisms

The study included three clinical isolates of dermatophytes namely, *Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum*. In order to conduct a control study in parallel, standard strains such as *M. gypseum* (ATCC 24102), *T. mentagrophytes* (ATCC 9533) and *T. rubrum* (ATCC 28188) (HiMEDIA Laboratories, India) were maintained as control organisms. These cultures were subjected to standard testing procedures to confirm their identity[20].

2.2.2. Standard antifungal drugs

Commercially available standard drugs namely Fluconazole and Itraconazole (HiMedia Laboratories, India) were employed in the study for comparative analysis of antidermatophytic activities. The fresh working solution of each drug was prepared by dissolving it in 100% dimethyl sulfoxide (DMSO) as per standard CLSI guidelines.

2.3. Testing of seed kernel extracts for antidermatophytic property

2.3.1. Serial two-fold dilution of drugs

Serial two-fold dilutions were carried out according to CLSI guidelines (M38-P). Series of eight test tubes were taken and the first tube was filled with 2ml of sterile RPMI-1640 medium (ATCC, cat. No. 30-2001) and the others were filled with 1ml of medium. Fixed quantity (200µg) of whole lyophilized extract was dissolved in the first tube and mixed well. From the first tube 1ml was transferred to the 2nd tube and then it was serially diluted through the 8th tube following aseptic procedure. The resultant dilutions of the extract so obtained per ml were 25µg, 12.5µg, 6.25µg, 3.125µg, 1.56µg, 0.78µg, 0.39µg and 0.165µg. Similarly, the standard antifungal agents and partially purified chemical components (C and D) were weighed (100µg), dissolved in dimethyl sulfoxide (HiMedia Laboratories, India) and serially diluted. The resultant dilutions of these extracts per ml were 12.5µg, 6.25µg, 3.125µg, 1.56µg, 0.78µg, 0.39µg, 0.165µg and 0.0975µg respectively.

2.3.2. Preparation of inoculum of test organis

Inoculum was prepared by aseptically suspending the fungal colonies in 5ml of sterile physiological saline and adjusting its transmittance equal to that of 0.5 McFarland standard at 530nm (1-5x10^6 CFU/ ml). It was further diluted (1:50) using RPMI-1640 medium and vortexed for 15 min. The resultant suspension was transferred aseptically into sterile tubes and kept undisturbed to allow the separation of homogenous conidial suspension as upper layer. The density of spore was adjusted spectrophotometrically at 530nm equivalent to that of 0.5 Mc Farland standard so as to achieve 0.5-5.0x10^6 of spores/ml. This spore suspension was finally diluted 1:50 using RPMI-1640 medium and quantified by plating on SDA with chloramphenicol and observed for the growth after sufficient incubation.

2.2.5. In vitro Antifungal Assay

The *in vitro* antidermatophytic assay was performed by broth microdilution technique as per CLSI (M38-P) guidelines[15]. Three controls were maintained in microtitre well plate viz., control 1 (positive control - dry free RPMI-1640 medium (200µl) with 100µl of fungal inoculums), control 2 (Blank - which consisted of dry free medium (200µl) without fungal inoculums) and control 3 (inoculums mixture - 200µl of medium with 100µl of DMSO and 100µl of fungal inoculums). Concurrently equal number of wells each filled with RPMI-1640 medium was maintained. Fixed quantities (100 µl) of prepared serial two-fold diluted drugs (standard / test) were added to the respective wells. Subsequently 100 µl of the standard fungal inoculum was dispensed into all the wells except blank. The microtitre plates were incubated at 24°C for dermatophytes and examined after 21 - 26, 46 - 50 and 70 -74 h of incubation. The minimum fungicidal concentration (MFC) was determined by examining the inhibition of growth with the aid of a reading mirror[21].

2.2.6. Determination of MFC

The minimum fungicidal concentration (MFC) was determined by Sub-culture Recovery technique, where in 20µl of suspension from the dilution well of microtitre plate was taken at the end of incubation and lawn cultured on SDA plate. The plates were incubated at 24°C

for 72 h and examined for presence or absence of fungal growth[22].

3. RESULTS

3.1. Characterization of whole extracts of seed kernel

The results of physico-chemical characterization of aqueous and ethanol extracts of both Setiz filtered and lyophilized whole extract of the seed kernel is presented in table 1. Both the extracts exhibited similar characteristics excepting their pH. The Phytochemical constituents of the extracts were insoluble in the sterile distilled water, partially miscible in 50% ethanol and completely in soluble in 100% DMSO (Table 1).

3.2. Antifungal activity of standard drugs

The standard antifungal drugs varied in exhibiting their activities on the test organisms. Itraconazole, the commonly prescribed drug for dermatophytes did not show any inhibitory effect on both standard and clinical isolates at lower concentrations. At higher concentration (12.5μg/ml) it inhibited the organisms excepting *M. gypseum*. The other agent, Fluconazole, promising to be efficacious for emerging resistant strains, interestingly was found to be effective against *M. gypseum* even at lower concentration. But it could inhibit all the dermatophytes at the concentration of ≥12.5μg/ml (Table 2).

3.3. Antifungal activity of seed kernel extracts of P. pinnata (Linn.)

The seitz filtered-lyophilized ethanolic extract of seed kernel when screened for antidermatophytic activity did not show any inhibitory activity. Hence it was excluded from further studies. Promisingly the aqueous seed extract exhibited complete inhibitory activity against both clinical and standard strains at the concentrations 12.5μg/ml (except *M. gypseum*) and 25μg/ml (against all tested) (Table 3).

The column chromatography of the aqueous extract yielded four fractions at different retention times namely, A to D. Among the partially purified phytochemical compounds, the 'A' and 'B', presumed to contain crude substances, were retained for future studies and not processed in the current study. The compound 'C' was observed to be potential in causing complete inhibition of all the three dermatophytes (Standard and Clinical strains) at a concentration of ≥12.5μg/ml. The compound 'D' exhibited comparatively least inhibitory effect and shown to be effective only against *T. rubrum* at concentration ≥12.5μg/ml (Table 4).

4. DISCUSSION

The dermatophytic fungal pathogens are considered to be most important infectious agents in the study of mycoses of human origin. These pathogens are endowed with an array of virulent characteristics such as *Arthroderma benhamiae, Trichophyton verrucosum* which enable them to be successful agents capable of invading susceptible individuals[23]. In recent years there have been more reports on the prevalence of dermatophytic mycoses among immunocompromised individuals[24], patients with malignancies[25] and people under therapy with immunosuppressive drugs[26]. As these organisms are increasingly gaining resistance to commonly used antifungal agents[27], there occurs a desperate need for finding an alternative and natural drug in controlling them.

The Indian Medicinal Plant, *Pongamia pinnata* (Linn,) has been a most sought after source of various drugs since ancient times owing to its nature of exhibiting extraordinary pharmacological properties[28]. This study has made a maiden attempt in exploring the antidermatophytic property of this plant by testing the seitz filtered-lyophilized extract and partially purified phytochemical of seed kernel. The preclinical screenings of natural products for antidermatophytic activity constitute a vital step in pharmaceutical evaluation their properties.

In this study, we followed the standard protocol (M38-P) prescribed by CLSI in order to determine the MIC values of whole and partially purified seed kernel extracts of *P. pinnata* (Linn.) and compare them with that of commonly used commercial antifungal agents. The *in vitro* antifungal assay by broth microdilution method (M38-P), although most commonly followed in research studies across the world[29-30], the literature indicate only scanty of work carried out in India employing this method[31]. Thus the present study could be considered as the first of its kind in terms of testing seitz filtered-lyophilized whole seed extracts of *P. pinnata* (Linn,) for evaluating the antidermatophytic activity employing broth microdilution method.

The antifungal drug Fluconazole exhibited that relatively higher MFC value (6.25μg/ml) in respect of inhibiting *M. gypseum*. The present study demonstrated its antidermatophytic potency at concentrations (MFC) of ≥12.5μg/ml against all the test organisms (both clinical and standard strains) (table 2).

Many research works had been carried out to portray the *in vitro* susceptibilities of dermatophytes to variety of naturally occurring compounds[32-33]. Marine sponges have been reported to possess certain unique natural bio-components. The marine sponge *Sigmadocia carnosa* had been shown to be efficacious against *Trichophyton mentagrophytes, Trichophyton rubrum, Epidermophyton floccosum* and *Microsporum gypseum* with MIC values of 125, 250, 250 and 250 µg/mL respectively when evaluated by disc diffusion method[34].

The *in vitro* antifungal activity of different type of extracts viz., chloroform, methanol and water of *P. pinnata* (Linn.) had been studied on five strains of dermatophytes namely, *Trichophyton rubrum, T. mentagrophytes, T. tonsurans, Microsporum gypseum* and *M. fulvum*. The MIC values of these extracts as determined by broth microdilution method were in the range from 1.25 to 10 mg/mL with the least value was recorded in water extract[35]. Interestingly, the present study recorded better antidermatophytic activity with aqueous whole extract (table 3) than ethanol extract. This could be due to the seitz filtration and lyophilization of extract causing augmentation of expression of antimicrobial compounds. The seitz filtered-lyophilized aqueous seed extract tested in the present study, although spared *T. mentagrophytes* at lower concentration (12.5µg/ml) caused complete inhibition of all the dermatophytes tested at concentration ≥25µg/ml which was further confirmed by subculture recovery techniques. Thus these results advocate the necessity to further explore the drug potential of whole aqueous extract of seed kernel of *P. pinnata* (Linn.).

The results of the present study in demonstrating the antidermatophytic activities of partially purified phytochemical compounds of seed kernel of *P. pinnata* (Linn.) gain significance as the compound 'C' seemed to be toxic for all the test organisms a concentration of ≥12.5µg/ml (table 4).

According to the available literatures, till date there are no studies to demonstrate the antifungal properties of seitz filtered-lyophilized extracts of seed kernel of *P. pinnata* (Linn.) against dermatophytes. However, present research group had reported the anticandidal potential of seitz filtered- lyophilized extracts of this plant[13]. Hence the present study could be viewed as an earnest pioneering attempt to portray the antidermatophytic potential of seitz filtered-lyophilized seed extracts of this plant and comparing them with that of partially purified phytochemical compounds.

Although the field of medicine concerned with infectious diseases is impacted by emerging and re-emerging pathogenic microbes, there has been always a search for finding an efficacious drug to successfully manage the issues afflicting the health of human. Owing to the appreciable fungicidal properties the seed kernel of *P. pinnata* (Linn.) possesses, its therapeutic use could be explored by further *in vitro* and *in vivo* studies. This may help the authorities for approval of drug sourced from this plant and implementing regulation for pharmaceutical production of the same as a savior of human ailing with dermatophytic mycoses.

Conflict of interest statement

We declare that we have no conflict of interest.

Table 1: Characteristics of seed kernel extracts of P*. pinnata* (Linn.)

Extract tested	Characteristics Examined	Type of extract	
		Aqueous extract	Ethanol extract
Seitz Filtered Extract	Part used	Dried seed	Dried seed
	Taste	bitter	bitter
	Colour	Pale yellow	Pale yellow
	Transparency	Transparent	Transparent
	Dilution	1:5	1:5
	Viscosity	Non-viscous	Non-viscous
	P^H	6.2	6.9
Lyophilized Extract	Colour	Dusty yellow	Dusty yellow
	Appearance	Flat crystals	Flat crystals
	Physical nature	sticky	sticky

Table 2: Antifungal Activity of the standard drugs

S. No.	Organisms	Concentration (µg/ml) and action on the test and standard organisms			
		Fluconazole		Itraconazole	
		12.5	6.25	12.5	6.25
1.	*Microsporum gypseum* ATCC 24102	+	+	_	_
2.	*Microsporum gypseum*	+	+	_	_
3.	*T. mentagrophytes* ATCC 9533	+		+	
4.	*T. mentagrophytes*	+	_	+	_
5.	*T. rubrum* ATCC 28188.	+	_	+	_
6.	*T. rubrum*	+	_	+	_

(+) Inhibition; (-) No inhibition

Table 3: Antifungal activity of the seitz filtered-lyophilized whole aqueous seed extract of *P. pinnata* (Linn.)

S. No.	Organisms	Concentration (µg/ml) and action on the test and standard organisms							
		25	12.5	6.25	3.125	1.56	0.78	0.39	0.195
1.	*Microsporum gypseum* ATCC 24102	+	+	_	_	_	_	_	_
2.	*Microsporum gypseum* (clinical isolate)	+	+	_	_	_	_	_	_
3.	*T. mentagrophytes* ATCC 9533	+	_	_	_	_	_	_	_
4.	*T. mentagrophytes*(clinical isolate)	+	_	_	_	_	_	_	_
5.	*T. rubrum* ATCC 28188.	+	+	_	_	_	_	_	_
6.	*T. rubrum* (clinical isolate)	+	+	_	_	_	_	_	_

(+) Inhibition; (-) No inhibition

Table 4: Antifungal Activity of the partially purified phytochemicals 'C' & 'D'

S. No.	Organisms	Chemical compound tested	Concentration (µg/ml) and action on the test and standard organisms							
			12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097
1.	*Microsporum gypseum* (clinical / standard)	C	+	_	_	_	_	_	_	_
		D	-	_	_	_	_	_	_	_
2.	*Trichophyton mentagrophytes* (clinical / standard)	C	+	_	_	_	_	_	_	_
		D	-	_	_	_	_	_	_	_
3.	*T. rubrum* (clinical / standard)	C	+	_	_	_	_	_	_	_
		D	+	_	_	_	_	_	_	_

(+) Inhibition; (-) No inhibition

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