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IN VITRO SUSCEPTIBILITY CLINICAL ISOLATES AND STRANDED STRAIN TO LYOPHILIZED HERBAL EXTRACTS (AQUEOUS AND ETHANOLIC) OF *PONGAMIA PINNATA, TERMINALIA CHEBULA* AND *AZARDIRACHTA INDICA* IN COMPARISON WITH FLUCONAZOLE AND ITRACONAZOLE.

C. Natarajan , N. Arunagirinathan and S. Rajarajan

Abstract

Herbs are considered as nature's gift to mankind. All major civilization of the old world has given as rich reference of herbs and other forms of naturally occurring substance and "anti-infectious" regardless of the agents associated with it. And the process of searching "new drugs" is still on progress. WHO (1973) has emphasized the need to scientifically evaluate the therapeutic value of medicinal plants. The practice of Ayurvedha, Siddha, Unani and Homoeopathy dated back since ancient times which utilize a large number of plants for the treatment of human diseases. The present study was conducted to evaluate the In Vitro Antifungal Susceptibility Testing Against Seitz Filtered Lyophilized Herbal Extracts (Pongamia Pinnata, Terminalia Chebula And Azardirachta indica) In Comparison With Two Standard Antifungal Agents Namely Fluconazole And Itraconazole. The cytotoxic free concentration of plant extract showed minimal inhibitory concentration.

Keywords :*Clinical Isolates And Stranded Strain , Pongamia Pinnata, Terminalia Chebula And Azardirachta indica.*

Short Profile

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INTRODUCTION:

Like Bacteria, Fungi are also responsible for a variety of infection in human beings. They are a group of Eukaryotic and Heterotrophic organism. Fungal infections of the skin are also known as 'mycoses'. They are common and generally mild. However, in very sick or other wise immune suppressed people, fungi can sometimes cause serious disease. These can further be classified into four groups viz. superficial, subcutaneous, and systemic and opportunistic. There is a need of herbal plant extract for certain fungai due to resistance. The efficacy of Lyophlized Aqueous & Ethanolic extracts was tested by 96 well micro titre plate Pongamia pinnata L. is one among the medicinal herbs used world wide. The plant is a medium seized evergreen or briefly deciduous, glabrous shrub or tree 15-25 m high, with straight or crooked trunk 50-80 cm or more in diameter. Leaves alternate, imparipinnate with long slender leafstalk, hairless, pinkish-red when young, glossy dark green above and dull green with prominent veins beneath when mature. Leaflets 5-9, paired at end, short-stalked, ovate elliptical or oblong, 5-25 x 2.5-1.5cm. Seed compressed ovoid or elliptical, bean-like, 1.5-2.5 x 1.2-2 x 0.8 cm, with a brittle coat long, flattened, dark brown, oily. This species has been placed alone in its genus Pongamia, derived from the Malabar local name (pongam).

Azadirachta indica, popularly called as "Neem", is a large, evergreen tree, with long, speeding branches forming a broad pinnate

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leaves and is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka. Its fruits and seeds are the source of neem oil.

Terminalia Chebula L., It is a deciduous tree growing to 30 metre (98 ft) tall,with a trunk up to 1 meter (3 ft 3 in) in diameter. The leaves are alternate to subopposite in arrangement,oval in shape. The fruit is drupe like, 2-4.5 centimeter, blackish, with five longitudinal ridges. Terminalia ,native to southern Asia from India and Nepal east to southwestern China.

MATERIALS AND METHODS

Plants collection

The plant parts are collected in and around Chennai and specimens were identified.

Preparation of plant extracts

The healthy parts were surface sterilized with 70% ethanol. The parts were ground into powder. The fine powder was collected by sieving. 20 gms of the powder was soaked as follows

Ethanolic (95%) Extract

20 gms of the specified powdered plant part was soaked with 100 ml Ethanol and stored overnight at 4º C. Filtered and centrifuged to get clarified extract. This method repeated 3 times more, all extracts pooled together and concentrated, Seitz Filtered and lyophilized.

Aqueous Extract

20 gms of the specified powdered plant part was soaked with 100 ml water and stored overnight in 4º C. Filtered and centrifuged to get clarified extract. This method repeated 3 times more, all extracts pooled together, concentrated, Seitz Filtered and lyophilized.

Lyophilisation:

The sterile extract was transferred to lyophilisation flask & freezed at -80^{0}C in deep freezer. The frozened extract was loaded to Lyophilizer. The lyophilised extract was stored in – 20^0 C till bioevaluation.

DEMONSTRATION OF FUNGI FROM CLINICAL ISOLATES

LACTOPHENOL COTTON BLUE STAINING

Lacto phenol Cotton Blue Stain is formulated with lacto phenol, which serves as a mounting fluid, and cotton blue. Organisms suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lysed. Cotton blue is an acid dye that stains the chitin present in the cell walls of fungi

A drop of Lacto phenol Cotton Blue Stain was placed in the center of a clean glass slide.

⇩

A fragment of the fungus colony was removed 2-3mm from the colony edge using an inoculating or teasing needle.

⇩

The fragment was placed in the drop of stain and teased gently.

⇩

Cover slip was placed over this.

⇩

.Examine the preparation under low and high magnification for the presence of characteristic mycelia and reproductive structures.

PREPARATION OF WORKING MEDIA FOR ANTIFUNGAL ACTIVITY:

Preparation of RPMI1640 for antifungal activity

RPMI -1640 (supplemented with phenol red as an indicator) with Glutamine without sodium bicarbonate and with Ph indicator MOPS buffered with0.615mol/liter and is

stirred till dissolution and in the mean time the pH was adjusted to 7.0 at 25°C using 1mol/liter Sodium Hydroxide. Filtered and sterilized through a 0.22µm Seitz filter pad in Seitz filter apparatus and dispensed aseptically in air tightened bottles and stored at 4°c until use.

MC FARLAND STANDARD PREPARATION:

Solution A was prepared by dissolving 1.175g of Barium Chloride in 100ml sterile double distilled water.

Solution B was prepared by adding 1 ml of 0.3 N Sulphuric acid to 100ml of sterile distilled water.

0.5ml of solution A added to 99.5ml of solution B and mixed well .It was then transferred to test tube of same dimension as that of the tube containing the inoculums.

The standard was stored at ambient temperature in the dark with the test tube plugged tightly to avoid evaporation and was agitated vigorously in a vortex before use.

INOCULUM PREPARATION (NCCLS METHOD M 27A)

Inoculum suspension of *Candida albicans* was prepared by picking five colonies of 1mm diameter from fresh subcultures on Sabourard's dextrose agar plates and it is dissolved in 5 ml of sterile 0.85%Nacl. The transmittance was adjusted to that of 0.5 MC FARLAND standards at 530nm. This suspension contained 1-5×10^6 Cfu/ml.

INOCULUM PREPARATION FOR FILAMENTOUS FUNGI:

Conidial / Spore suspension of each of the filamentous fungus was obtained by the addition of 2 ml of saline into seven days old culture tube. The 2ml of saline with conidial / spore was transferred into test tube and the content was allowed to settle for few minutes and then upper homogenous suspension containing conidia was mixed for 15 seconds by a vortex.

This transmittance was adjusted to that of 0.5 MC FARLAND standards at 530nm. Inoculums suspension of the yeast &the filamentous fungi were individually diluted with RPMI 1640along with MOPS in the ratio of 1:2000. As result the yeast suspension contained about 0.5-2.5×10^3Cfu/ml and conidial suspension approximately 0.5-5×10^4Cfu/ml.

SERIAL TWO FOLD DILUTION OF DRUGS (STANDARD/TEST):

PREPARATION OF STOCK SOLUTIONS

One ml of sterile RPMI-1640 medium was taken. To this 100 µg of standard and isolated compounds were added in individual tubes.

One ml of sterile RPMI-1640 medium was taken. To this 200 µg of lyophilized extracts were added in individual tubes.

Dilution

Amphotericin B Itraconazole, fluconazole & chemical compounds were serially diluted from the stock solution in 7 sterile test tubes at 2 fold dilution with a concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78µg/ml

Test Pongamia pinnata Lyophilized extracts were serially diluted from the stock solution in 7 sterile test tubes at 2 fold dilution with a concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56µg/ml.

BROTH MICRODILUTION METHOD (FROM M38-P DOCUMENT) FOR ANTIFUNGAL ACTIVITY:

The growth control well contained 200 ?l of sterile drug free medium (RPMI-1640) and were inoculated with 100µl of the corresponding diluted inoculum suspension. Sterility control was performed by including 100µl of uninoculated, drug free medium.

In addition 200µl of sterile drug free medium was added in a separate well 100µl of DMSO was added to check the efficacy of DMSO and 100 µl of the corresponding diluted

inoculum suspension was added.

Each micro titre well containing 200µl of sterile RPMI-1640 medium and 100µl of the diluted(two fold) standard drug and isolated compound at a concentration from 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78µg/ml and lyophilized extracts at a concentration from 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56µg/ml respectively.

100µl of the standardized (M 38- P) Fungal inoculum suspension was inoculated.

All micro dilution trays were incubated at 24°C and examined after 21 to 26, 46 to 50 and 70 to 74 hrs of incubation.MIC determination requires the visual examination of growth inhibition. The growth in each MIC well was compared with that of the growth control with the aid of a reading mirror.

SUB CULTURE RECOVERY TECHNIQUE

Subculture recovery technique was performed to confirm the complete inhibition in The visible clear dilutions by lawn subculture on Sabourard's Dextrose Agar plate. The plates were incubated at 37°C For 48 hrs for *Candida albicans* and at 24°C for 72 hrs for Dermatophytes. The plates were observed after the specific period of incubation. Absence of colony formation indicates the fungicidal activity of the drug.

RESULTS

INVITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AMPHOTERCIN B

TABLE-1

In vitro antifungal susceptibilities results for standard ATCC and clinical strains of Candida albicans

S.No.	Organisms	Inhibitory Effects of Amphotercin B (µg/ml)							
		100	50	25	12.5	6.25	3.125	1.56	0.78
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Candida albicans clinical strain	+	-	-	-	-	-	-	-

Standard drug 'Amphotercin B' showed inhibitory activities at a concentration of 100 µg/ml for the both strains of Candida albicans.

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR ITRACONAZOLE

TABLE-2

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of Itraconazole (µg/ml)							
		100	50	25	12.5	6.25	3.125	1.56	0.78
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	+	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	+	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR ITRACONAZOL

TABLE-3
In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of Itraconazol (µg/ml)							
		100	50	25	12.5	6.25	3.125	1.56	0.78
1.	Candida albicans	+	-	-	-	-	-	-	-
2.	Microsporum gypseum	+	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	+	-	-	-	-	-	-	-
4.	T. rubrum	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR FLUCONAZOLE

TABLE-4
In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of Fluconazole (µg/ml)							
		100	50	25	12.5	6.25	3.125	1.56	0.78
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	+	+	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	+	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR FLUCONAZOLE

TABLE-5

In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of fluconazole (µg/ml)							
		100	50	25	12.5	6.25	3.125	1.56	0.78
1.	Candida albicans	+	-	-	-	-	-	-	-
2.	Microsporum gypseum	+	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	+	-	-	-	-	-	-	-
4.	T. rubrum	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED SEED EXTRACT OF PONGAMIA PINNATA (LINN.,)

TABLE-6

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of Pongamia pinnata (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	+	+	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	+	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED SEEDS EXTRACTS FROM PONGAMIA PINNATA (LINN.,)

TABLE-7

In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of Amphotercin B (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans	+	-	-	-	-	-	-	-
2.	Microsporum gypseum	+	+	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	+	-	-	-	-	-	-	-
4.	T. rubrum	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR 70% ETHANOLIC SEITZ FILTERED LYOPHILIZED SEEDS EXTRACTS FROM PONGAMIA PINNATA(LINN.,)

TABLE-8

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of Pongamia pinnata (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	-	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR 70% ETHANOLIC SEITZ FILTERED LYOPHILIZED SEEDS EXTRACTS FROM PONGAMIA PINNATA (LINN.,)

TABLE-9

In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of Pongamia pinnata (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans	±	-	-	-	-	-	-	-
2.	Microsporum gypseum	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	-	-	-	-	-	-	-	-
4.	T. rubrum	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED LEAVES EXTRACT IN VITRO OF *AZARDIRACHTA INDICA*

TABLE 10

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of Azardirachta indica (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans ATCC 90029	+	+	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	+	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED SEED EXTRACT OF *AZARDIRACHTA INDICA*

TABLE-11

In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of *Azardirachta indica* (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans	-	-	-	-	-	-	-	-
2.	Microsporum gypseum	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	+	-	-	-	-	-	-	-
4.	T. rubrum	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

MICRODILUTION METHOD FOR 70% ETHANOLIC SEITZ FILTERED LYOPHILIZED SEEDS EXTRACTS FROM *AZARDIRACHTA INDICA*

IN VITRO ANTIFUNGAL ASSAY BY BROTH

TABLE-12

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of *Azardirachta indica* (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans ATCC 90029	+	-	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	-	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR 70% ETHANOLIC SEITZ FILTERED LYOPHILIZED SEEDS EXTRACTS FROM *AZARDIRACHTA INDICA*

TABLE-13

In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of *Azardirachta indica* (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans	+	-	-	-	-	-	-	-
2.	Microsporum gypseum	-	-	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	-	-	-	-	-	-	-	-
4.	T. rubrum	-	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED SEED EXTRACT OF *TERMINALIA CHEBULA*

TABLE-14

In vitro antifungal susceptibilities results for standard ATCC strains

S.No.	Organisms	Inhibitory Effects of *Terminalia Chebula* (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans ATCC 90029	+	+	-	-	-	-	-	-
2.	Microsporum gypseum ATCC 24104	+	+	-	-	-	-	-	-
3.	Trichophyton mentragrophytes ATCC 9533	+	-	-	-	-	-	-	-
4.	T. rubrum ATCC 28188	+	-	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

IN VITRO ANTIFUNGAL ASSAY BY BROTH MICRODILUTION METHOD FOR AQUEOUS SEITZ FILTERED LYOPHILIZED SEED EXTRACT OF *TERMINALIA CHEBULA*

TABLE-15
In vitro antifungal susceptibilities results for clinical isolates

S.No.	Organisms	Inhibitory Effects of *Terminalia Chebula* (µg/ml)							
		200	100	50	25	12.5	6.25	3.125	1.56
1.	Candida albicans	+	+	-	-	-	-	-	-
2.	Microsporum gypseum	+	+	-	-	-	-	-	-
3.	Trichophyton mentragrophytes	+	-	-	-	-	-	-	-
4.	T. rubrum	+	+	-	-	-	-	-	-

+ symbol indicates inhibitory effects, - symbol indicates absence of inhibitory effects

DISCUSSION

Amphotricin B was found to posses complete Inhibitory activity at 100µg/ml. The stranded & clinical strain on Candida. However, this could be administered only intraveneously. Itraconazole which is often preventable by dermatologist was found to effect at 100µg concentrate on ¾ of organism. Yet Tricophyton rubrum which is not common dermatological agent is as not susceptible to Itraconazole. Fluconazole which has been reported to loosing grade due to reports of resistant strains to Fluconazole was uniformly effective. Agents all the form of fungi and MIC value have been estimated. Interesting and encouraging feautures of the results was the uniform inhibition activity of Lyophilized seitz filtered aqueous extract of Pongamia pinnata extract and the efficacy was similar to Fluconazole & MIC. However the ethanolic extract of Pongamia seed extract did have Inhibitory activity on Candida. Another inhibitory observation was the antifungal activity of Azadirachta indica which exhibited inhibition action on 2-3 of the Fungi. However, ethanolic seitz filtered extract had activity on Candida albicans . Most interesting was the activity of lyophilized aqueous extract of Terminalia chebula which has activity superior to Fluconazole & Pongamia pinnata because MIC's values on 100µg to extract compared to the of Fluconazole. Also the aqueous extract from to be superior to70% of ethanolic extracts

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