IN VITRO ANTIFUNGAL ACTIVITY OF SELECTED INDIGENOUS MEDICINAL PLANT EXTRACTS ON HUMAN SKIN FUNGAL INFECTIONS.

D.G .H Madushan**, A.G.B Aruggoda

Department of Agricultural and Plantation Engineering, The Open University of Sri Lanka.

INTRODUCTION

Skin fungal infections are common in developing countries. Fungi are the major cause of infections on the skin, scalp, hair and nails (Senguttuvan et al., 2013). With regard to skin fungal infections, there are three groups of fungi that are prominent, namely, Dermatophytes, *Candida* and *Malassezia*. The last few decades have recorded a significant increase in the number of patients with skin-related fungal infections (Pento et al., 2013). According to a recent study on dermatology in the United States, between 1997 and 2000, the number of cases of skin fungal infections has increased by 207% (Vila et al., 2013). The World Health Organization has reported a 20% of global prevalence of dermatomycoses (Aneja et al., 2012). Poor hygiene, limited availability of different types of antifungal drugs, and the organisms' resistance increased due to indiscriminate use of commercial antifungal drugs commonly used for the treatment of skin fungal infections can be cited as reasons for the increase in skin fungal infections. Therefore, it is a timely need to conduct research studies to find new antifungal drugs from various sources including medicinal plants (Bansod and Rai. 2008). Many plants contain medicinal compounds that can be used to treat microbial infections. There are many herbal antifungals and antibacterial remedies available in the field of Indigenous Medicine and the Ayurveda Medicine system such as “Wishaharnadi thilaya, Kushta thailaya, Jiwaka thilaya, Shwetha karawiradi thilaya, Sarwawishadi thilaya” etc. This research identifies the effective plant extracts from five selected plant species for inhibiting three common fungal species causing human skin infections and determines the minimal inhibition concentration of crude plant extracts.

METHODOLOGY

The experiment was conducted at the Research Lab, Department of Agricultural and Plantation Engineering, The Open University of Sri Lanka, Nawala.

Collection of Plant Materials and Fungal Species

The plant materials were collected from rural areas and herbal gardens. Aththora and Kumburu leaves were collected from Hikkaduwa, Nalagasdeniya village, Rankihiriya was collected from Nature’s Beauty Creations Ltd, Horana and Dumalla was collected from a registered supplier in Ayurveda Research Institute, Nawinna. The fungal cultures were collected from the Department of Microbiology, Faculty of Medical Sciences, and University of Sri Jayawardanapura, Sri Lanka.

Preparation plant extracts

Plant extract preparations were done as adapted by T. Somarathna (2018) with some modifications. The extracts of five plant species: *Senna alata* (Aththora) leaves, *Caesalpinia bonducella* (Kumburu) leaves, *Trichosanthes cucumerina* (Dummala) whole plant, *Alpinia malaccensis* (Rankihiriya) rhizome, *Nerium oleander* (Kaneru) leaves were used for antimicrobial susceptibility assay against selected fungi. Four solvents including Methyl alcohol, Hexane, Petroleum Ether and Isopropyl alcohol were used as extracting media (solvents). Among the selected solvents, Petroleum Ether and Hexane were non-polar solvents than Methanol and Isopropyl Alcohol. The cleaned plant materials were dehydrated under 40-45 ^{0}C temperature and for 24-48 hours in a dehydrator.

The dried plant material of each plant species was grounded into fine powder and 25 g was soaked in 250 ml of Methyl Alcohol, Hexane, Petroleum Ether and Isopropyl alcohol separately, followed by orbital shaking for 48 hours in 120 rpm. To attain a clear filtrate, the solutions were filtered through Whatman's No. 1 filter paper. The filtrates were evaporated and dried at 60 ^{0}C under reduced pressure using a rotary vacuum evaporator. The extract yields were stored in small sterile bottles and placed in a refrigerator at 4 ^{0}C for future studies.

Determination of antifungal activity of the plant extracts

Three skin fungal species including *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Candida albicans* ATCC10231 were selected for the test. Those fungal strains were separately cultured in Sabouraud Dextrose Agar (SDA) plates and incubated at 37ºC for 4-10 days. The incubated plates were preserved in the refrigerator at 4ºC for future use. The Disc Diffusion assay with some modifications as adapted by Khan and Ahamad (2011) was performed to determine the sensitivity of fungal strains against the plant crude extracts. Hundred microlitres (100 µl) of spore suspension (1×10^{6} CFU ml^{-1}) was spread onto Sabouraud Dextrose Agar (SDA) plates. Sterile filter paper disc (6 mm) was mounted on the agar surface and impregnated with 10 µl of individual plant crude extracts. For the control test also, 100 µl of spore suspension (1×10^{6} CFU ml^{-1}) spread onto Sabouraud Dextrose Agar (SDA) plates. Sterile filter paper discs (6 mm diameter) were mounted on the agar surface and impregnated with 10 µl of solvents that use for extractions. The plates were incubated at 37°C for 4 days. Each experiment was conducted in triplicate and the average inhibition zone size was measured in millimetres. The highest average inhibition zone was given the highest antifungal activity or most effective plant crude extract that contained antifungal compounds comparatively.

Data were statistically analysed using ANOVA of the Minitab17 statistical software release for Windows. The level of significance was considered as 0.05. Means were compared using Turkey Test set at 95% confidence.

Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs)

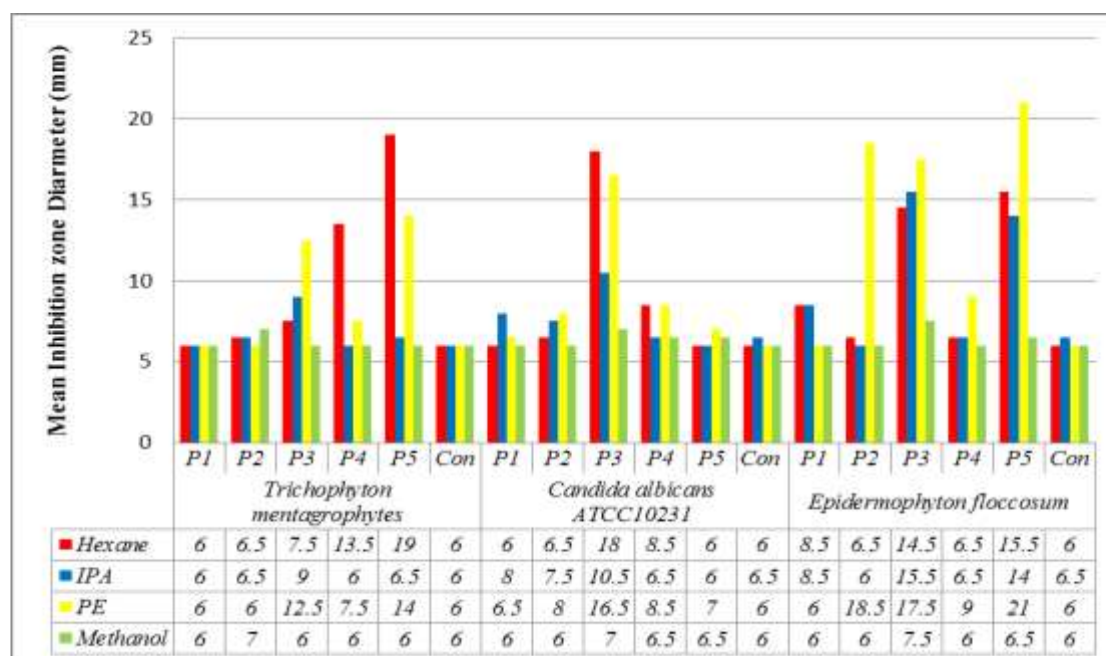
MIC was defined as the lowest concentration that inhibited visible fungal growth while MFC was the concentration at which no growth was observed. Each experiment was repeated three times and mean values was calculated for MICs and MFCs. The Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) of plant crude extracts were determined against the test strains by the Broth Micro Dilution Method using 96 microwell plates as adapted by Khan and Ahamad (2011) with some modifications. Among the plant extracts used for the Disc Diffusion assay, *Trichosanthes cucumerina* (Dummala), *Alpinia malaccensis* (Rankihiriya) and *Nerium oleander* (Kaneru) plant extracts, obtained separately with Petroleum Ether and Isopropyl alcohol, were tested for MIC. Each selected plant crude extract was serially diluted using a two-fold dilution method, ranging from 100µg/ml to 17.5µg/ml with a 1:10 Tween 20 and DMSO solvent mixture. Ten microliters (10 µl) of spore suspension (1×10^{6} CFU ml-1) and 100 µl Sabouraud Dextrose Broth were filled into each well containing 10 µl serially diluted plant crude extracts with 1:10 Tween 20, DMSO. The microplates were incubated at 37 °C for 3-10 days. Ten microliters (10 µl) of 1:10 Tween 20 and DMSO solvent mixture was used as the negative control and 1% Iitraconazole with 1:10 Tween 20 and DMSO was used as the positive control. All treatments were done in triplicate. The microplate was scanned on the first, second and tenth days using an ELISA microplate reader. The absorption values were obtained from spectrophotometrically with an ELISA microplate reader at 450nm. Absorption differences were calculated between the third and first day and between the tenth day and the first day respectively.

Data were statistically analysed using ANOVA of the General Linear Model of the Minitab17 statistical software at the 5% significance level. Means were separately compared with positive control and negative control using Dunnett's Test set at the 95% confidence.

RESULTS AND DISCUSSION

Antifungal activity of the plant extracts using Disk Diffusion Method

Twenty plant extracts were prepared from five selected plant species using four different solvents and investigated to evaluate their antifungal activities against three species of human skin pathogenic fungi using the Disk Diffusion Method. The five plant extracts possessed potential antifungal activity against the three pathogenic fungal species independently (Fig.01).



	P1	P2	P3	P4	P5	Con	P1	P2	P3	P4	P5	Con	P1	P2	P3	P4	P5	Con
	Trichophyton mentagrophytes						*Candida albicans ATCC10231*						*Epidermophyton floccosum*					
Hexane	6	6.5	7.5	13.5	19	6	6	6.5	18	8.5	6	6	8.5	6.5	14.5	6.5	15.5	6
IPA	6	6.5	9	6	6.5	6	8	7.5	10.5	6.5	6	6.5	8.5	6	15.5	6.5	14	6.5
PE	6	6	12.5	7.5	14	6	6.5	8	16.5	8.5	7	6	6	18.5	17.5	9	21	6
Methanol	6	7	6	6	6	6	6	6	7	6.5	6.5	6	6	6	7.5	6	6.5	6

Fig.01. Mean inhibition zone values obtained for plant extracts against three fungal species. P1: *Caesalpinia bonducella* (Kumburu); P2: *Nerium oleander* (Kaneru); P3: *Alpinia malaccensis* (Rankihiriya); P4: *Senna alata* (Aththora); P5: *Trichosanthes cucumerina* (Dummala); Con: Control

Alpinia malaccensis (Rankihiriya)-Petroleum Ether extract, *Senna alata* (Aththora)-Hexane extract and *Trichosanthes cucumerina* (Dummala)-Hexane extract gave significantly high mean zones of inhibition against *Trichophyton mentagrophytes*. However, *Guilandina bonduc* (Kumburu) and *Nerium oleander* (Kaneru) plant extracts with four different solvents did not show significant antifungal activity against *Trichophyton mentagrophytes*.

Alpinia malaccensis (Rankihiriya)-Hexane and -Petroleum Ether extracts gave significantly high mean zones of inhibition against *Candida albicans*. However, *Guilandina bonduc* (Kumburu), *Nerium oleander* (Kaneru), *Senna alata* (Aththora) and *Trichosanthes cucumerina* (Dummala) plants extracts with four solvents did not show any significant antifungal activity against *Candida albicans*.

Alpinia malaccensis (Rankihiriya)-Isopropyl Alcohol extract, *Nerium oleander* (Kaneru) and *Trichosanthes cucumerina* (Dummala)-Petroleum Ether extracts showed significantly high mean zones of inhibition against *Epidermophyton floccosum*. However, *Guilandina bonduc* (Kumburu) and *Senna alata* (Aththora) plant extracts done with four different solvents did not show any significant antifungal activity against *Epidermophyton floccosum*.

MIC and MFC of the plant extracts using Broth Micro Dilution method

The antifungal activities of different concentrations of the plant extracts were compared with the negative control and positive control. All treatments resulted in a significant difference compared to the negative control. No visible fungal growth was observed during treatment and negative control allowed the growth of the fungus. Any visible fungal growth was not observed in the treatments including the positive control test. Therefore, there was no significant difference between all the treatments and the positive control.

CONCLUSIONS/RECOMMENDATIONS

Comparing the overall activity of the selected plant extracts, *Alpinia malaccensis* (Rankihiriya) plant extracts showed the highest antifungal activity for all the selected skin fungi. *Trichosanthes cucumerina* (Dummala) plant extract gave a higher antifungal activity against *Trichophyton mentagrophytes* and *Epidermophyton floccosum. Caesalpinia bonducella* (Kumburu) and *Senna alata* (Aththora) plant extracts showed lower antifungal activities than other plant extracts. The MIC value could not be obtained as the selected concentration values were higher than the MIC value of the plant extracts tested. Therefore, it should be further tested by reducing the concentration of plant extracts. Further tests should be performed to isolate the active antifungal compounds from tested plant crude extracts.

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