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Original Article

In *vitro* antimycotic activity of extracts of some medicinal plants against Piedra hair infection

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ABSTRACT

Piedra hair infection is a fungal infection of the hair characterized by the presence of nodules of different hardness on the surface of hair shafts. Two types of Piedras have been found, White Piedra and Black Piedra. White Piedra caused by Basidiomycetous yeast Trichosporon spp. and Black Piedra caused by the ascomycete Piedraia hortae and Trichosporon ovoides. The low spread of these infections and the harmlessness of the disease probably explain why so few studies have been made on these infections. The present work is aimed at studying the antimycotic activity of ten medicinal plants in comparison to known antifungal agents against five Trichosporon species involved in Piedra hair infection. These plants selected for the present study have been reported to be used for the treatment of various other diseases. Methanol, Ethanol, Acetone and Chloroform extracts of the leaves, bark and roots of these plants were examined for antimycotic activity. The results showed that methanol and ethanol extracts were effective against all the species of the pathogens tested, with methanolic extracts exhibiting more activity. The average diameter of zone of inhibition observed against these fungi ranged from 10-35 mm. The most effective plant was found to be Plumbago zeylanica with zone of inhibition 35 mm. The MIC and MFC of 6.2-500 mg/ml of methanolic extracts were recorded. Ketoconazole and Nystatin B were used as positive controls. DMSO was used as a negative control.

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1. Introduction

Piedra meaning stone in Spanish is a hair infection limited to the hair shaft without involvement of the adjacent skin [1]. Two varieties of Piedra may be seen: Black Piedra and White Piedra. The causative agents of Black Piedra are Piedraia hortae and Trichosporon ovoides and of White Piedra are Trichosporon asahii, Trichosporon cutaneum, Trichosporon inkin, Trichosporon ovoides and Trichosporon mucoides.

Black Piedra is a condition that presents as stone hard, black nodules on the scalp, beard, moustache and pubic hair shaft [2]. Brown-black hard nodules along the hair shaft characterize Black Piedra, with the fungal activity limited to the cuticle and with no penetration of the hair shaft. Black Piedra is more frequent and less sporadic than White Piedra. White Piedra is characterized by white to tan nodules along the shafts of hair in the scalp, beard, eyebrows, eyelashes and groin, genital and perigenital areas [3].

Numerous discrete soft nodules that are barely visible to the naked eyes are attached to the hair shaft and produce a gritty sensation when palpated [4]. The nodules may be detached easily, and the affected hairs may be split or broken [1]. T. asahii and T. inkin can behave as opportunistic pathogens, particularly in immunosuppressed patients, where they can cause serious and life threatening symptoms [5].

Clinically many hair disorders can be confused with Piedra [1]. Infections can co-exist with dermatophytes or candida infection and erythrasma [6].

Black Piedra occurs frequently in wet tropical areas and is common in certain tropical areas of central South America and South East Asia, whereas White Piedra occurs in semitropical and temperate countries [3]. The natural habitats of Trichosporon species are soil, lake water and plants but they are occasionally

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The classical and most effective therapy for White and Black Piedra has been and still is the cutting and shaving of the hairs, a treatment recommended by the American Academy of Dermatology [3] but this is often not considered acceptable, particularly by women. Topical antifungals are also recommended and the application of Clotrimazole cream alone [10] or after shampooing with Ketoconazole proved to be good therapeutic treatment for White Piedra [11].

However these antifungal agents are expensive and have varying degrees of toxicity. Hence, there is a need for new antifungal companions with broad spectrum activity which are cheaper and with less toxicity. So the present report describes the antifungal potential of different solvent extracts of some medicinal plants against the fungi causing Piedra hair infection and whether these can be used as a substitute for drugs of chemical origin.

2. Materials and Methods

2.1. Collection, identification and extraction methods

Fresh plants/ plant parts were collected from the various regions of Dehradun city. The plants screened along with their families, vernacular names, parts used and their traditional therapeutic uses are listed in Table I. The taxonomic identities of these plants were confirmed by Department of Botany, Forest Research Institute, Dehradun. Fresh plant materials were washed under running tap water, air dried and then homogenized to fine powder and stored in air tight bottles. The air dried and powdered plant material (100 g of each) was extracted with 200 ml of each solvent (Methanol, ethanol, acetone and chloroform), kept on a rotary shaker for 24 hrs. Thereafter it was filtered and centrifuged at 5000 x g for 15 min. The supernatant was collected and evaporated to dryness to give the crude dried extract.

2.2. Fungal cultures

The test fungal strains investigated include five Trichosporon species which were procured from Microbial Type Culture Collection Centre (MTCC), Chandigarh and National Collection of Yeast Cultures (NCYC) Norwich, United Kingdom. They were maintained on selective media Yeast Malt Agar slants and plates at an optimum temperature of 250C and experiments were carried out on Sabouraud Dextrose Agar. The yeast cultures used were as follows:

1. *Trichosporon asahii* (MTCC No. 6179) 2. *Trichosporon cutaneum (MTCC No. 255)* 3. *Trichosporon inkin* (NCYC No. 2515) 4. *Trichosporon ovoides* (NCYC No. 2796) 5. *Trichosporon mucoides (NCYC No. 2474)*

2.3. Antifungal assays

2.3.1. Agar well diffusion assay

Preliminary analysis of antifungal activity was conducted using Agar Well Diffusion Assay [12]. Fungal inoculum was prepared in Tween 80 saline solution and incubated for 1 hour. 1 ml of this solution was homogeneously inoculated into presterilized petriplates. After that molten Sabouraud Dextrose Agar (SDA) was added and kept for solidification. After solidification, wells of 6 mm diameter were punctured in the culture medium using sterile cork borer. A fixed volume (100 µl /ml) of respective crude extract in 20% Dimethyl Sulphoxide (DMSO) was loaded in the well using sterilized micropipettes. Plates were incubated for 2 days at 250C and zone of inhibition (in mm) of different extracts was determined after 48 hrs. Sterile 5% aqueous DMSO was used as negative control while Ketoconazole (50 µg/disc) and Nystatin (100 µg/disc) were used as the positive control. All experiments were carried out in triplicates.

2.3.2. Minimum inhibitory concentration and minimum fungicidal concentration by broth dilution assay

MIC of the extracts against the test fungi was determined using the Broth Dilution Method [13]. Various concentrations (1.55-500 mg/ml) of the extracts were prepared by dissolving extracts in 5 % DMSO. 1 ml of the plant extract (100 mg/ml) was added to 1 ml of Sabouraud Dextrose broth in test tubes and subsequent concentrations were prepared by using serial dilution technique. 1 ml fungal culture prepared in saline water was inoculated into each test tube and mixed thoroughly on a vortex mixer. The test tubes were then incubated at 25°C for 2 days. Nystatin and Ketoconazole were used as a positive control. DMSO was used as a negative control. The MIC values were determined macroscopically after 48 hrs of incubation in comparison with the growth and sterility controls [14]. SDA plates were divided into six different sections and labelled with the different concentrations on the plates. The plates were incubated for 48 hrs at 25°C after which the MFC were recorded [15].

2.4. Statistical analysis

The inhibitory zones of plant extracts were expressed as the mean + Standard deviation and compared using Student Waller Ducan test at $P < 0.05$.

3.RESULTS

The results illustrated in Table II and Table III indicated that 6 plants out of 10 were effective against the Trichosporon species. Four plants showed less inhibition or no inhibition at all. Out of four extracts Methanolic extract and ethanolic extract were more effective as compared to Acetone and Chloroform extracts. Methanolic extracts gave the maximum inhibition zones. Five Trichosporon species were used in the study out of which Trichosporon ovoides was found to be more sensitive towards the extracts used.

The antifungal sensitivity of the crude extracts and their potency were assessed quantitatively by determining zone of inhibition by well diffusion assay shown in Table II and Table III and by determining MIC and MFC by dilution assay respectively shown in Fig (1-5).

In Agar Well Diffusion Assay effect of four crude extracts of 10 plants each belonging to different families was tested against Trichosporon species. All the 40 crude extracts types have significant antifungal activities against all the test fungi. The inhibition zone ranged from 10-35 mm by different crude extracts

against the Trichosporon species. The antifungal discs of Ketoconazole (50 µg/disc) , Nystatin B (100 µg/disc) were used as positive control. Some of the plant extracts showed good inhibition activity as compared to the antifungal discs.

3.1.Agar well diffusion assay

3.1.1. Methanol extract

Out of 10 plants, methanolic extract of 5 plants were found to be effective against all the Trichosporon species. Other five plants showed inhibition only in some cases of the yeast species. Maximum activity was exhibited by P. zeylanica showing inhibition zone of 35 mm against T. cutaneum, followed by inhibition zone of 25 mm against T. inkin, 21 mm against T. ovoides, 20 mm against T. mucoides & 18 mm against T. asahii. W. somnifera was also found to be active showing high inhibition zones against all the Trichosporon species. Methanolic extracts of W. somnifera showed inhibition zone of 25 mm against T. cutaneum, 23 mm of inhibition zone against T. asahii and T. mucoides both, 22 mm inhibition zone against T. ovoides and inhibition zone of 20 mm against T. inkin. Methanolic extracts of I. ensata was next to W. somnifera in showing inhibition zone. It showed maximum inhibition zone of 35 mm against T. cutaneum and minimum inhibition zone against T. asahii and T. inkin with 18 mm inhibition zone. Methanolic extracts of H. isora showed minimum activity of all the plants. It only showed inhibition zone of 12 mm against T. ovoides and no inhibition against other Trichosporon species.

3.1.2. Ethanol extract

Ethanolic extract of *P. zeylanica* showed maximum activity showing high inhibition zones against Trichosporon species. It showed inhibition zone of 30 mm against *T. cutaneum* followed by 25 mm against *T. inkin*, 24 mm against **T. mucoides**, 22 mm against *T. ovoides* and 18 mm against T. asahii. I. ensata was the next plant showing maximum inhibitions followed by *B. ciliata*, *C. paniculatus*, *A. calamus*, *W. somnifera*, *A. aspera*, *H. isora* and *C. asiatica* which did not show any activity against any of the species tested.

3.1.3. Chloroform extract

Chloroform extract of three plants were active towards the five Trichosporon species. Maximum activity was shown by *P. zeylanica* against *T. cutaneum* exhibiting 30 mm of inhibition zone followed by 25 mm against *T. inkin. H. isora* and *C. asiatica* were not active at all showing no inhibitions.

3.1.4. Acetone extract

4 plants exhibited high activity against all the Trichosporon species. Three plants were inactive towards all species. Maximum activity was shown by *P. zeylanica* against *T. cutaneum* exhibiting 26 mm of inhibition zone followed by 25 mm inhibition zone against T. ovoides.

From the above results it was noted that P. zeylanica was the most active plant against the Trichosporon species in all the solvents giving maximum inhibition zones followed by I. ensata, W. somnifera and B. ciliata. In case of positive controls Ketoconazole (50 µg/disc) and Nystatin B (100 µg/disc), the zone of inhibition ranged from 11-25 mm. Maximum inhibition (25 mm) was observed against T. ovoides and T. mucoides by Ketoconazole.

3.2. MIC and MFC of methanolic extracts

Another result of the Antifungal activities of the extracts as determined by measuring the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal concentration (MFC) is given in Fig (1-5). The MIC data for the organisms are also variable and concentration dependent. The methanol extracts of all the screened plants showed moderate antifungal activity against all the strains of yeast species. The lower concentration of all the screened plants (1.55-3.1 mg/ml) did not show any activity against the Trichosporon species while the higher concentrations (100-500 mg/ml) showed good antifungal activity. The effect of different plant extracts was different with different species of Trichosporon. The methanolic extract of B. ciliata, P. zeylanica and I. ensata showed best antifungal activity against all the species of Trichosporon (6.2 mg/ml) followed by W. somnifera which showed MIC value of 12.5 mg/ml against T. asahii, T. ovoides and T. mucoides. Most effective methanolic extract which showed highest inhibition at lowest conc. was P. zeylanica (6.2 mg/ml), W. somnifera (12.5 mg/ml) and I. ensata (6.2 mg/ml).

Lowest MIC and MFC was determined by the methanolic extract of B. ciliata against T. asahii at 6.2 mg/ml and MFC at 12.5 mg/ml followed by I. ensata and P. zeylanica against T. cutaneum with MIC value of 6.2 mg/ml and MFC value of 12.5 mg/ml. W. somnifera inhibited the growth of T. asahii, T. ovoides and T. mucoides at 12.5 mg/ml with MFC at 25 mg/ml. P. zeylanica and I. ensata showed inhibition of T. mucoides at 12.5 mg/ml and MFC value of 25 mg/ml. C. paniculatus inhibited the visible growth of T. asahii, T. cutaneum and T. ovoides at 6.2 mg/ml concentration and showed MFC value of 50 mg/ml. B. ciliata showed MIC value at 25 mg/ml against T. cutaneum and T. mucoides. E. alba showed MIC value at 50 mg/ml concentration against T. cutaneum and T. ovoides and MFC value of 100 mg/ml for the same. C. paniculatus inhibited the growth of T. inkin and T. mucoides at the concentration of 50 mg/ml with MFC at 100 mg/ml concentration.

The overall results suggest that T. cutaneum is the most susceptible fungal species and the most resistant was T. inkin. The antifungal activity of the studied plant extracts was compared with standard antifungals Ketoconazole (50 µg/disc) and Nystatin B (100 µg/disc).

The Minimum fungicidal concentration (MFC) of the crude methanolic extracts on the tested fungal strains is presented in Fig 1-5 with MIC. The results obtained in this study shows that the crude methanolic extract of plants used was fungicidal for all the tested yeast species.

Table I. Ethnomedical information of the Screened Plants.

Plant Name	Local name	Family	Parts Used	Traditional uses
Achyranthes aspera	Apamarg	Amaranthaceae	whole plant	Piles, cough, boils, skin eruption, colic, snake bite, anti-inflammatory and rheumatism.
Acorus calamus	Buch	Acoraceae	Rhizome	Anthelmintic, diuretic expectorant, astringent, antispasmodic, aphrodisiac, anti- inflammatory, antipyretic, insecticidal, nervinetonic, sedative and tonic.
Berginia ciliata	Pashanved	Saxifragaceae	Root	Fever, diarrhoea, pulmonary nfections, cough, colds, asthma and urinary problems
Celastrus paniculatus	Malkangni	Celastraceae	Seeds	Epilepsy, insomnia, rheumatism, gout, dyspepsia, abdominal disorders, leprosy, pruritis, skin diseases, paralysis, asthma, leucoderma and inflammation.
Centella asiatica	Brahmi	Apiaceae	Whole plant	Antibacterial, anti inflammatory, anti-febrile, diuretic, brain tonic, cardiotonic, carminative, expectorant, fever, measles, diarrhoea and dysentery.
Eclipta alba	Bhringraaj	Asteraceae	Whole plant	Dysentery, anaemia, eye diseases, asthma, liver cirrhosis, bleeding haematuria, itching, hepatitis, diphtheria, expectorant, antipyretic and antispasmodic.
Helicteres isora	Marodfali	Sterculiaceae	Root, Leaves Bark, Pod	Cough, asthma, eczema expectorant, astringent, diarrhoea, dysentery, scabies, colic and flatulence
Iris ensata	Anarjal	Iridaceae	Roots	Anthelmintic, antidote, appetizer, diuretic, hepatic, vermifuge, liver complaints and dropsy.
Plumbago zeylanica	Chitrak	Plumbaginaceae	Leaves, Roots	Abortifacient, diaphoretic emollient, dysentery, leucoderma, inflammation, piles, bronchitis, itching, ringworm and scabies.
Withania somnifera	Ashwagandha	Solanaceae	whole plant	Aphrodisiac, Anthelmintic, Narcotic, diuretic, Psoriasis, asthma, ulcer, scabies, insomnia and arthritis.

Table II . Antifungal activity of the extracts of the medicinal plants using Agar well Diffusion Assay

Plant Name	Average Zone of Inhibition of different Plant extracts (In mm)									
	Methanol (mg/ml)					Ethanol (mg/ml)				
	Ta	Tc	Ti	To	Tm	Ta	Tc	Ti	To	Tm
Achyranthes aspera	11+1.5	-	-	13+1.1	-	12+1.1	-	20+1.1	-	-
Acorus calamus	s -	-	20 +1.1	21+1.5	13+1.2	-	-	21+1.5	18+2.0	12+1.0
Berginia ciliata	28+1.0	18+1.1	18+1.1	18+1.1	20+1.5	20+1.1	15+1.5	20+1.1	15+1.0	16+1.5
Celastrus paniculatus	10+1.0	10+1.0	12 +1.1	15+1.5	12+1.2	12+1.0	14+1.5	15+1.1	14+1.0	18+1.5
Centella asiatica	-	15+1.1	-	-	-	-	-	-	-	-
Eclipta alba	-	15+1.0	-	11+1.0	-	-	-	-	10+0.5	-
Helicteres isora	-	-	-	12+1.5	-	-	-	-	18+1.1	-
Iris ensata	18+1.5	35+1.5	18+1.1	15+1.5	21+1.1	20+1.0	25+1.0	22+1.2	15+1.5	13+1.0
Plumbago zeylanica	18+1.0	35+1.5	25+1.5	21+1.2	20+1.5	18+1.1	30+1.5	25+1.5	22+1.5	24+1.5
Withania somnifera	23+1.1	25+1.5	20+1.1	22+1.5	23+1.5	12+1.1	13+1.1	-	-	12+1.5

Ta= Trichosporon asahii, Tc= Trichosporon cutaneum, Ti= Trichosporon inkin, To= Trichosporon ovoides,
Tm= Trichosporon mucoides, (-)= no inhibition

Table. III. Antifungal activity of the extracts of the medicinal plants using Agar well Diffusion Assay

Plant Name	Average Zone of Inhibition of different Plant extracts (In mm)									
	Chloroform (mg/ml)					Acetone (mg/ml)				
	Ta	Tc	Ti	To	Tm	Ta	Tc	Ti	To	Tm
Achyranthes aspera	-	-	-	-	-	13+1.0	-	-	-	-
Acorus calamus	-	-	-	12+1.1	-	-	-	-	18+1.1	13+1.0
Berginia ciliata	16+1.5	10+1.1	20+1.5	15+1.5	14+1.2	18+1.1	18+1.1	25+1.1	20+1.5	20+1.5
Celastrus paniculatus	18+1.5	14+1.5	10+1.1	17+1.5	18+1.0	10+0.5	11+1.0	12+1.0	12+1.1	15+1.5
Centella asiatica	-	-	-	-	-	-	-	-	-	-
Eclipta alba	-	15+1.5	-	-	-	-	-	-	-	-
Helicteres isora	-	-	-	-	-	-	-	-	-	-
Iris ensata	-	11+1.0	-	-	-	20+1.1	22+1.0	21+1.5	25+1.0	13+1.5
Plumbago zeylanica	20+1.5	30+1.1	25+1.5	22+1.5	22+1.5	15+1.0	26+1.5	23+1.5	25+1.1	21+1.5
Withania somnifera	15+1.5	16+1.0	-	-	-	13+1.0	12+1.5	-	13+1.0 -	-

Ta= Trichosporon asahii, Tc= Trichosporon cutaneum, Ti= Trichosporon inkin, To= Trichosporon ovoides, Tm= Trichosporon mucoides, (-)= no inhibition

Fig.1. MIC and MFC of methanolic plant extracts against T. asahii (in mg/ml)

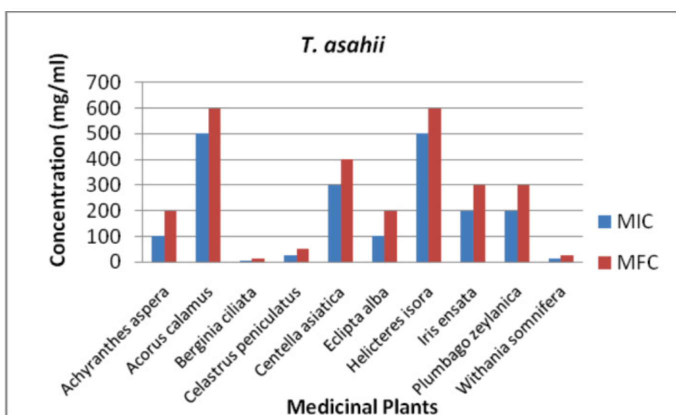


Fig.2 MIC and MFC of methanolic plant extracts against T. cutaneum (in mg/ml)

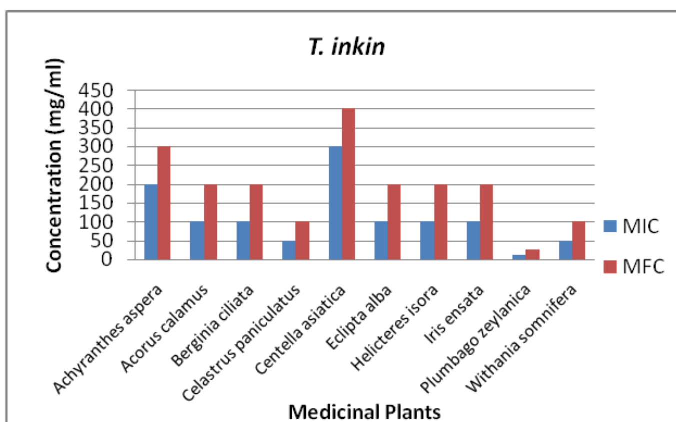


Fig.3 MIC and MFC of methanolic plant extracts against T. inkin (in mg/ml)

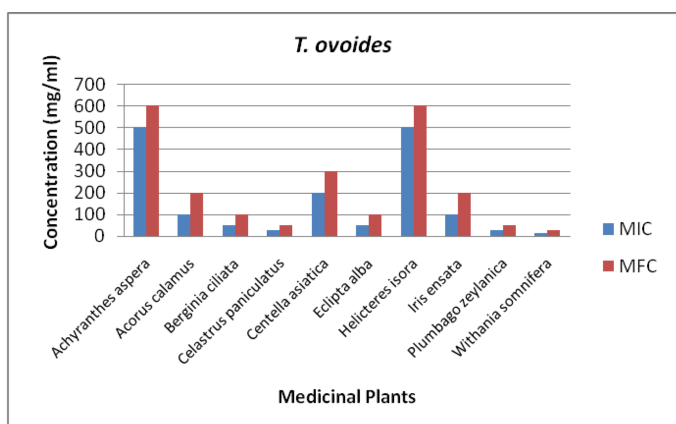


Fig.4. MIC and MFC of methanolic plant extracts against T. ovoides (in mg/ml)

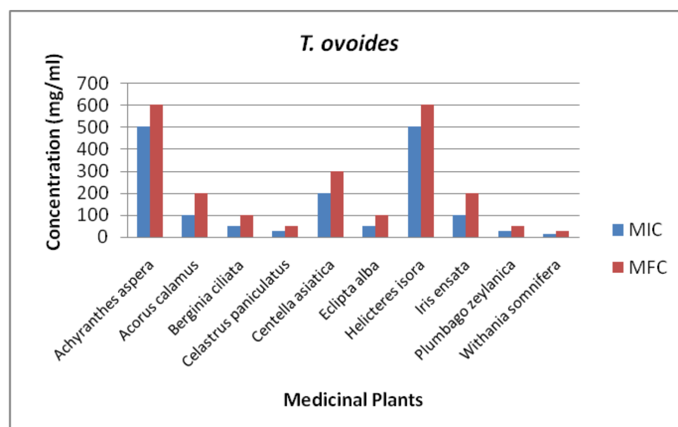
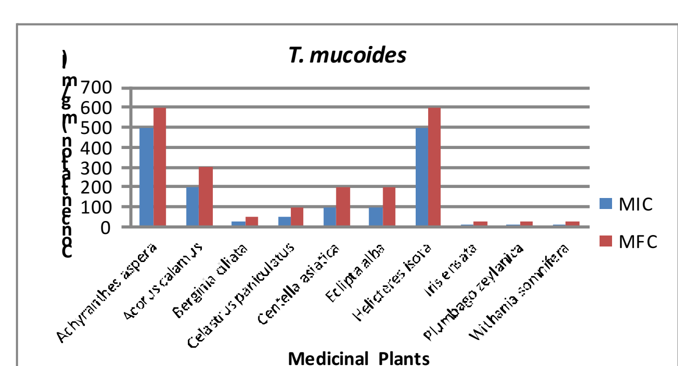


Fig.5 MIC and MFC of methanolic plant extracts against T. mucoides (in mg/ml)



4. Discussion

Higher plants have been shown to be a potential source for the new antifungal agents. The screening of plant extracts has been of great interest to scientists for the discovery of new drugs effective in the treatment of several diseases [16]. This study in line with other studies showed that many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. Plants still continue to be almost the exclusive source of drugs for the majority of the world's population [16]. This study indicated that among all the four extracts (methanol, ethanol, acetone and chloroform) of ten plant methanol extracts was most active followed by ethanol, acetone and chloroform extracts. This study emphasizes that few medicinal plants commonly used in Garhwal region are also active against Piedra hair infection caused by Trichosporon sp. Some plants have been seen to be more effective than the synthetic drugs showing higher inhibition zones. The MFC values were in most cases higher than the MIC values suggesting that the plants crude extracts were fungicidal at high concentration and fungistatic at low concentrations.

Overall the test pathogens were more found to be sensitive to methanol and ethanol extracts than to acetone and chloroform extracts which suggests that some of the active compounds in the crude extracts are polar and thus dissolved readily in the methanol and ethanol, while the acetone and chloroform may have dissolved out non-polar compounds that possess less antifungal activity.

Previous studies have noted alcohols to be reliable and consistent solvents for the extraction of antimicrobial substances from medicinal plants [17]. The antifungal effectiveness of the plant parts on the tested fungal species resulted within 48 hrs. of incubation in all the crude extract screening and MIC values. Further in vitro and in vivo studies are required in order to prove the bio-efficacy of the extracts. The encouraging results indicate that these extracts might be exploited as natural drugs for the treatment of several diseases caused by these organisms and could be useful in understanding the relation between traditional cures and current medications.

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