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Antifungal and Phytochemical Screening of Wild Medicinal Plant against fungal Clinical Isolates from Dermatitis

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ABSTRACT

Dermatophytosis is currently treated with the commercially available topical and oral antifungal agents in spite of the existing side effects. Treatment of these cutaneous infections with secondary metabolites produced by wild plant is considered as an alternative approach. Exploring the unexplored aspect of the wild plants for developing antidermatophytic drugs is a novel attempt which needs further investigation. Study aims to screen eleven wild medicinal plants possessing antifungal activity against the clinical fungal isolates from dermatophytic patients. The methanolic plant extract were analyzed by well diffusion assay and phytochemical characterization of the active ingredient were determined possessing mycocidal activity. *Aspergillus* sp. was effectively controlled by the extracts of *C.roseus* , *R.communis*, *T. cordifolia*, *J. curcas*, *C. longa*; *Curvularia* sp. by *T. cordifolia*, *R. communis* , *T. erectus* , *C. longa*; *Cladosporium* sp. *C. roseus* , *R. communis* , *L. inermis* , *T. erectus*, *A. nilotica*; *Microsporium* sp. by *C. roseus*, *R. communis*, *J. curcas*, *L. inermis*, *A. nilotica* and *Penicillium* sp. by *A. nilotica*, *R. communis*, *C. longa*, *T. occidentalis* and *T. erectus* . Maximum Alkaloid was recovered from *T. peruviana*, Saponin in *R. communis* and *C. roseus*, Flavanoid from *R. communis*, Tannin in *T. erectus* and *C. roseus* and phenols from *L. inermis*. Methanolic plants extracts of *Catherenthus reseus*, *Riccinus communis*, *Tagetus erectus*, *Acacia nilotica*, *Lawsonia inermis* and *Thuja occidentalis* were found to be significantly controlling the test fungi. Data revealed that plants possessing higher phenol, tannin and saponin show antifungal activity.

Keywords: Antifungal and Phytochemical screening; Clinical fungal isolates; fungal dermatophytosis; Wild Medicinal plant

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INTRODUCTION

The main objective of the treatment is eradication of the dermatophyte fungal infection from various sites of the body as scalp, beard, skin, or nails, etc. These infections are typically treated based on clinical appearance, with use of potassium hydroxide (KOH) microscopy for confirmatory evidence in questionable cases, or the mandatory use of a confirmatory test (KOH microscopy, fungal culture) in the case of fungal dermatophytic infection.[1] Choice of topical or oral therapy depends on site of the infection. Currently, no topical antifungal class has been shown to be better than any other, but additional studies need to be performed.[2] Issues of safety of oral therapy should be considered, although there is low incidence of adverse events in the immuno competent population.[7] Length of time of treatment varies, depending on the agent chosen, from 2 to 8 weeks. Safety issues, cost, and differences in duration of treatment may influence choice of agent. Systemic antifungals are the mainstay of therapy, with limited efficacy information to choose one over another.[17] Griseofulvin,[13] the oldest available oral agent, and newer agents such as terbinafine and itraconazole, which can be effective when used for shorter periods, are all first-line options.[12] Both ketoconazole and fluconazole have been studied for tinea infection and are effective. Fluconazole, limited by adverse effects, and ketoconazole, which is hepatotoxic, are typically reserved as second-line agents.[17] Ketoconazole may cause severe liver injury and adrenal insufficiency. In July 2013, the US Food and Drug Administration (FDA) recommended that oral ketoconazole should only be used for life-threatening fungal infections where alternative treatments are not available or tolerated, and when the potential benefits of treatment outweigh the risks. Its use is contraindicated in patients with liver disease. If used, liver and adrenal function should be monitored before and during treatment.[31] This recommendation does not apply to topical formulations of ketoconazole.

Identification of some wild medicinal plants against clinically isolated fungi, carry out their phyto-chemical analysis, test the *in vitro* antifungal activities with respect to conventional antifungal drugs. Medicinal plants synthesize a vast array of secondary metabolites that are important for human life.[9] The particular medication and duration of treatment depends on the location of the infection. Scalp infections usually require treatment with an oral antifungal medication. Infections of other areas of the skin can be treated with topical antifungal medications. [CDC] http://www.cdc.gov/fungal/dermatophytes/treatment.html This gives us impetus to analyse the antifungal activity of the wild medicinal plants against the clinically isolated fungi from fungal dermatophytic infection. Wild medicinal plants are selected during the study conduct as for their

easy availability in abundance and no other significant economical benefit. The study also dealt with the photochemical screening of the plant extracts for the detection of the active ingredients responsible for antifungal property. The active ingredient can further be used as recommendation of the study conduct for pre-clinical trials.

MATERIALS AND METHODS

Test Plants:

Eleven plant were used for the analysis of their antifungal activity as *Cucurma longa* (Haldi), *Thuja occidentalis* (Morpankhi), *Murraya koenigii* (Meetha Neem), *Lawsonia inermis* (Mehndi), *Acacia nilotica (Babool), Tagetus erectus* (Gainda), *Thevatia peruviana* (Kaner), *Riccinus communis* (Arandi), *Catherenthus roseus* (Sadabahar), *Tinospora cordifolia* (Neemgiloya) and *Jatropha curcas* (Ratanjot). Fresh leaves were collected washed thoroughly 2-3 times with running tap water and once with sterile distilled water, air dried at room temperature on a sterile blotter and used for preparation of extracts.[19] (we have used wild plants that are commonly and easily available in Rajasthan)

Solvent extraction:

The dried powdered leaves were subjected to methanolic extraction by Soxhlet method. Plant extract were prepared by 15 grams fine powder of leaves was filled in the thimble and extracted successively with methanol for 48 hours at 55^{o}C. All the solvent extracts were concentrated using rotary flash evaporator under reduced pressure. The extracts were preserved in airtight brown bottle until further use.[10, 6] (Individual plant leaves were dried and the leaf powder was used for solvent extraction by soxhlet method using methanol. The plant extracts were then used for antifungal activity and phytochemical characterization)

Test fungi:

Fungal cultures were collected from Dr. B. Lal Clinical Laboratory, Jaipur reported to have fungal dermatophytic infection and were subjected to culture on Sabouraud Dextrose Agar medium (SDA). The plants extracts were used against clinically isolated five test fungi as *A. flavus, Curvularia* sp., *Microsporum* sp., *Cladosporum* sp. and *Penicillium* sp. for antifungal activity assay.

Antifungal activity assay by Well Diffusion technique

Antifungal activity were performed by well diffusion method on SDA medium with respect to positive control (Itracanezole) (R: reference) and negative control (C) as solvent (methanol). The sample (S) and positive control 5mg w/v per well. Samples showing activity index >1 represent

significant control of pathogens. The plates were incubated at 25±1^{o} C for seven days and ten replicates were maintained for each treatment. The zone of inhibition of mycelial growth was determined by antibiotic zone scale (Hi-media).[12, 30]

$$\text{Activity index} = \frac{\text{Zone of inhibition of sample}}{\text{Zone of inhibition of reference}}$$

Phytochemical Screening of Plant Extract

All the plant extract were subjected for phytochemical screening by quantitative analysis of alkaloids, flavonoids, saponins, phenols and tannins.

Alkaloid determination

5 g of the sample was weighed into a 250 ml beaker and 200 ml 20% acetic acid in ethanol was added and covered to stand for 4 h. this was filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitation was collected by filtration and weighed.[24, 15]

Flavanoid determination

10 g of the sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatmann filter paper No.1. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed.[6]

Saponin determination

20g of plant sample was dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55ºC. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of normal butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample were dried in the oven into a constant weight. The saponin content was calculated in percentage.[21]

Determination of total phenol content

The total phenolic content of the *plant* extract was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth.[28] Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of the plant extract (100 µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized

with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using double beam UV-VIS spectrophotometer (Systronics 119). The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

Determination of tannins

500 mg of the sample was weighed into 100 ml bottle; 50 ml of distilled water was added and shaken for 1 h in a shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipette out into a tube and mixed with 3 ml of 0.1 M FeCl3 in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured in a spectrophotometer at 120 nm wavelength within 10 min. A blank sample was prepared and read at the same wavelength. A standard was prepared using tannin acid to get 100 ppm and measured.[32]

RESULTS AND DISCUSSION

Development of synthetic products to control plant diseases has become difficult because of strict requirements of their efficacy, selectivity, toxicology and general impact on the environment. Consequently, there is an increasing interest in evaluating other mechanisms of control including the effects of plant metabolites on plant pathogens.[26] Several higher plants and their constituents have shown success in plant disease control and proved to be harmless and non phytotoxic unlike chemical fungicides.[30] Indiscriminate use of chemical not only hazardous to living beings but adversely affects the microbial population present in the ecosystem.[8]

Alternative to this effect, to control plant diseases, plant products are gaining prominence as fungicides and bactericides.[8] Antifungal compounds from higher plants are advantageous over synthetic fungicides due to their easily biodegradable nature.[30] The clinical samples (150) suspected for fungal dermatophytic infection were collected from Dr. B. Lal clinical Laboratory. During the study conduct the relative percent occurrence (RPO) of among various fungi maximum incidence was shown by *Aspergillus* sp. followed by *Trichophyton* sp., *Fusarium* sp., *Microsporum* sp. and *Cladosporium* sp.[1]

Antifungal Activity of Methanolic Plant Extracts (During the study conduct methanolic plant extract were used)

In the present investigation, eleven methanolic plant extracts were used for their antifungal activity against 5 test fungi clinically isolated from dermatophytic infected sites. The activity index was

evaluated with respect to the standard drug itracanezole. *Aspergillus* was effectively controlled by all the extract of but maximum activity was reported by *Catherenthus roseus* , *Riccinus communis* , neemgiloye, *Jatropha curcas* and *Cucurma longa* (A.I.= 3.1, 2.3, 1.43, 1.41, 1.4) except *Murraya koenigii*. *Curvularia* was found to be significantly contolled by all the extracts except *Murraya koenigii* and *Jatropha curcas* whereas the extracts of neemgiloye, *Riccinus communis* , *Tagetus erectus* , *Cucurma longa* with A.I. 2.2, 1.53, 1.44, 1.4 respectively. *Cladosporium* was notably controlled by extract of *Catherenthus roseus* , *Riccinus communis* , *Lawsonia inermis* , *Tagetus erectus* , *Acacia nilotica* with the activity 2.4, 1.4, 1.25, 1.3, 1.21. *Microsporium* was significantly controlled by *Catherenthus roseus* , *Riccinus communis* , *Jatropha curcas* , *Lawsonia inermis* , and *Acacia nilotica* with A.I. 2.0, 1.33, 1.36, 1.25, 1.26 but the extracys of *Cucurma longa*, *Murraya koenigii* and neemgiloye did not showed any activity. *Penicillium* was significantly controlled by all the extracts except neemgiloye and *Jatropha curcas* . Best results were encountered by *Acacia nilotica* , *Riccinus communis* , *Cucurma longa*, thuja and *Tagetus erectus* (1.38, 1.26, 1.2, 1.15, 1.13). (Table 1, Figure 1)

Table 1 : Zone of inhibition and activity index of the eleven plant extract (methanolic) against various test fungus.

Plant	**Well**	***Zone of Inhibition (in cm)***				
		A.flavus	***Curvularia***	***Microsporum***	***Cladosporum***	***Penicillium***
Cucurma longa	S	1.4	Nil	1.2	1.1	1.1
	C	1.2	Nil	1.3	1.5	1
	R	1	Nil	1	1.2	0.9
Activity index:		1.4	1.4	0	1.2	1.2
Thuja occidentals	S	1.2	1.6	2.1	1.5	2.3
	C	1.1	1.2	1.8	1.3	1.9
	R	1.6	1.5	1.9	1.6	2.0
Activity index:		0.75	1.06	1.10	0.93	1.15
Lawsonia inermis	S	1.3	1.5	1.3	1.5	1.2
	C	1.5	1.2	1.4	1.6	1.2
	R	1.2	1.2	1.3	1.4	1.1
Activity index:		1.1	1.1	1.25	1.25	1.1
Acacia nilotica	S	1.6	2.8	2.9	2.3	2.5
	C	1.1	1.7	1.6	1.7	1.5
	R	1.6	2.3	2.3	1.9	1.8
Activity index:		1.0	1.21	1.26	1.21	1.38
Murraya koenigii	S	1.3	1.3	1.2	1.7	1.1
	C	1.6	1.4	1.5	0.8	Nil
	R	1.5	1.4	1.4	1.7	Nil
Activity index:		0.87	0.87	0.93	0.86	1
Tagetus erecta	S	1.1	1.3	1.8	1.3	2.6
	C	2.4	1.7	2.2	1.7	2.0

	R	1.00	0.9	1.6	Nil	2.3
Activity index:		1.1	1.44	1.12	1.3	1.13
Riccinus communis	S	2.3	2.00	1.2	1.4	2.4
	C	2.4	1.7	2.2	1.7	1.6
	R	Nil	1.3	0.90	Nil	1.9
Activity index:		2.3	1.53	1.33	1.4	1.26
Catherenthus roseus	S	3.1	2.1	2.00	2.4	1.0
	C	2.4	1.7	2.2	1.7	1.0
	R	Nil	1.7	Nil	Nil	1.1
Activity index:		3.1	1.23	2.00	2.4	0.91
Tinospora cordifolia	S	2.3	2.2	1.4	1.2	1.4
	C	2.4	1.7	2.2	1.7	Nil
	R	1.6	Nil	2.5	1.5	Nil
Activity index:		1.43	2.2	0.56	0.80	Nil
Jatropha curcas	S	2.4	1.7	2.6	1.4	1.1
	C	2.4	1.7	2.2	1.7	1.5
	R	1.7	2.00	1.9	1.4	1.3
Activity index:		1.41	0.85	1.36	0.00	0.85

Note: Sample: 5 mg (w/v) test methanolic plant extract (50 µl loaded per well) (S); Control Positive Control itraconazole 5 mg (v/v) per well (C); Reference: Solvent used for solvent extraction (methanol) 50 µl loaded per well (R): Diameter of well: 8 mm

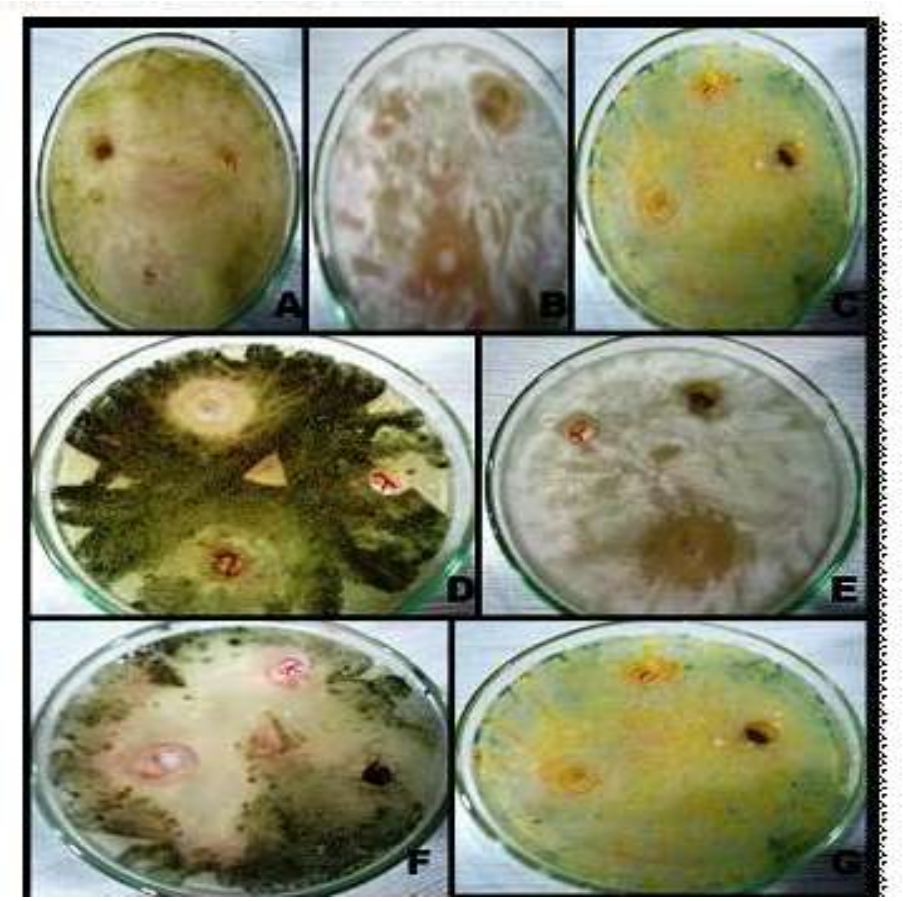

Figure 1: Photoplate showing antifungal activity of plant extract:

Comparative results of methanolic plant extracts of *Pimpinella anisum* L. (*Apiaceae*) and Illicium *verum* Hook. f. (*Illiciaceae*), were reported for effective control the dermatophytic fungal infections as compared to the standard drugs. This was tested for their potential antifungal activities.[33] Antidermatophytic activities of five selected medicinal plants (leaves) viz. *Euphorbia balsamifera* Ait, *Mitracarpus scaber Zucc*, *Pergularia tomentosa* L, *Streospermum kunthianum* Cham and *Holarrhena floribunda* was screened for treatment of dermatophytoses[29] Antidermatophytic activity of dichloromethane and methanol extracts of whole plant of *Allamanda cathertica* was evaluated.[22] Whereas in a study conduct extract of *quercifolia* with four different solvents such as ethanol, methanol, acetone, di-ethyl ether and water, were used to extract the bioactive compounds from the rhizome of *D.quercifolia*[23] *Anogeissus leiocarpus* and *Terminalia avicennioides.*[20]

The efficacy of ethanol and distilled water extracts of *Azadirachta indica, Jatropha curcas, Jatropha gossypifolia, Cassia alata, Anacardium occidentale* and *Aloe vera* was determined.[1] The activity of the methanolic extracts of the 5 selected plants was determined against different pathogenic fungus.[11] Crude methanol extracts from leaves of *Cassia alata, Cassia fistula* and *Cassia tora* were investigated for their antifungal activities.[25] These results support the plant oils can be used to cure mycotic infections and plant oils may have role as pharmaceutical and preservatives.[3] Compared to ketoconazole used as standard antifungal the compound isolate could be considered as a promising antidermatophytic agent.[26]

Phytochemical Screening of Plant Extract

Plants are reservoir of biological active compounds to combat various pathogens containing alkaloids, flavonoids, saponins, phenols and tannins. The mode of action of extracts was determined on cell wall and enzyme production of fungi.[34] In the present study the plant extracts were prepared and used for their antifungal property to detect their bioefficacy against clinically isolated fungi. Plant extracts (methanolic) were subjected to phytochemical screening as the quantitative analysis of Alkaloid, Saponin, Flavanoid, Tannin and Phenol through spectrophotometric analysis. Maximum Alkaloid was found in Methanolic extract of *Thevatia peruviana* (1.27 gm) followed by *Tagetus erectus* (0.98 gm) and Neem giloye (0.36gm). Saponin was found highest in extract of *Riccinus communis* and *Catherenthus roseus* (0.68gm) subsequently by Thuja (0.36 gm). Maximum amount of Flavanoid was recovered in extract of *Riccinus communis* (0.72 gm), Jatropha (0.51 gm) and Neemgiloye (0.37 gm). Maximum Tannin was found in methanolic extract of *Tagetus erectus* and *Catherenthus roseus* (0.71 gm) followed by *Jatropha curcas* (0.64 gm) and Neemgiloye (0.58 gm). Amount of phenols was recovered

utmost from *Lawsonia inermis* (0.479 gm), *Riccinus communis* (0.465 gm), *Murraya koenigii* (0.474 gm) and Neem giloye (0.343 gm). (Table 2) A study indicated by *Lawsonia inermis* inhibiting the production of catalase in *Aspergillus niger* and *Fusarium oxysporum*.

The active compounds were proteinaceous in nature or proteins and they are effective against plant pathogens.[5] The water base extraction technique was the most effective in inducing antifungal properties of lime whiles ethanol base extraction technique was the best for ginger. Garlic on the other hand exhibited a good antimicrobial (antifungal) property in both ethanol and water extraction media. The study thus confirms the antifungal properties of these medicinal plants and suggests the type of extraction to yield the best results.[4] The methanol extract of *Sida cordifolia* exhibited significant antifungal activity against *F. verticillioides*.[8]

Table 2: Phytochemical Characterization of methanolic Plant Extract

Plant Extracts	Alkaloid (gm/ml)	Saponins (gm/ml)	Flavanoid (gm/ml)	Tannin (gm/ml)	Phenol (gm/ml)
Curcuma longa (Haldi)	0.03	0.04	0.04	0.00	0.339
Thuja occidentalis (Morpankhi)	0.15	0.36	0.12	0.07	0.251
Murraya coenigii (Meetha Neem)	0.17	0.24	0.05	0.17	0.474
Lawsonia inermis (Mehendi)	0.03	0.17	0.05	0.20	0.479
Acacia nilotica (Babool)	0.01	0.17	0.12	0.38	0.212
Tagetus erecta (Gainda)	0.98	0.25	0.03	0.71	0.198
Thevatia peruviana (Kaner)	1.27	0.29	0.07	0.38	0.200
Riccinus communis (Arandi)	0.17	0.68	0.72	0.29	0.465
Catharanthus roseus (Sadabahar)	0.18	0.68	0.14	0.71	0.266
Tinospora cordifolia (Neem- giloye)	0.36	0.16	0.37	0.58	0.343
Jatropha curcas (Ratanjot)	0.13	0.09	0.51	0.64	0.234

In the present study, data revealed that higher amounts of phenol, tannin and saponin show significant antifungal activity against the test fungus. The study was supported by the qqualitative phytochemical tests, thin layer chromatography and TLC-bioautography of certain active extracts demonstrated the presence of common phytocompounds in the plant extracts including phenols, tannins and flavonoids as major active constituents.[19]

Volatile oil from the rhizome of *Curcuma longa* always isolated, characterized by Gas Chromatography- Mass Spectroscopy. The antimicrobial activity of oil was tested against the human and plant pathogenic bacteria and fungi.[16] Phytochemical screening revealed the presence of saponin, steroids, tannin, glycosides, alkaloids and flavonoids in the crude stem extracts of *J. curcas* to inhibit the growth of bacteria and fungi is an indication of its broad spectrum antimicrobial potential which may be employed in the management of microbial infections.[18] Another study indicates the comparative analysis of the phytochemical profile and mycostatic

activity of leaf and flower extracts of *Tagetes erecta* Linn and *Tagetes patula* Linn in ethanol. Phytochemical screening has indicated the presence of alkaloids, flavonoids, steroids, tannins and phenolic compounds as the major secondary metabolites in extracts of both species has revealed the inhibitory effect of all the extracts on growth of *Candida albicans, Aspergillus niger, Saccharomyces cerevisiae* and *Aspergillus flavus. T. erecta* leaf extract showed highest anti-fungal activity among all the four extracts tested.[27] Modern analytical spectroscopies of high intrinsic dimensionality can provide rapid accurate microbial characterization techniques, but only when combined with appropriate chemometrics.

Leaf extract (methanolic) of *Catherenthus roseus* (Sadabahar), *Riccinus communis* (Arandi), *Tagetus erectus* (Gainda), *Acacia nilotica (Babool), Lawsonia inermis* (Mehndi) and *Thuja occidentalis* (Morpankhi) serves as a potent plant against clinically isolated fungi from mycotic dermatitis. It was interpreted that the plants possessing higher amounts of phenol, tannin and saponin shows significant antifungal activity against the test fungus.

CONCLUSION

The management of dermatophytic infections needs personal hygiene, awareness of infection, proper diagnosis and medication. At present there are a large number of antidermatophytic drugs available commercially. With increasing incidence of fungal infection, microbial resistance to the existing drugs, cost and side effects, there is a need for an antifungal drug that can overcome all these limitations. out of eleven plants *Catherenthus roseus* (Sadabahar), *Riccinus communis* (Arandi), *Tagetus erectus* (Gainda), *Acacia nilotica (Babool), Lawsonia inermis* (Mehndi) and *Thuja occidentalis* (Morpankhi) remains to be an unexhausted source of bioactive compounds and a boon to the medical field. It was interpreted that the plants possessing higher amounts of phenol, tannin and saponin shows significant antifungal activity against the test fungus. Screening of plants of wild nature can be a novel approach for obtaining potential lead molecules for clinical trials and later treatment of dermatomycosis as compared to the standard drugs.

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