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In vitro antimycotic and antioxidant activity of Eclipta alba leaf extract against Malassezia yeast like fungi

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ABSTRACT

Mycotic infections are one of the major health problems in tropical countries, and Malassezia is one of the most common fungi associated with skin diseases. In immunocompromised hosts Malassezia can also cause entire systemic infections. Malassezia is a monophyletic genus of fungi found on the skin of 7 billion humans and associated with a variety of conditions, including dandruff, dermatitis, pityriasis versicolor, seborrheic dermatitis, and folliculitis. Eclipta alba leaf was tested against four common species of Malassezia. In this present investigation In-Vitro antifungal activity of Eclipta alba extract has been evaluated against Malassezia using broth micro dilution method recommended by CLSI. The minimum inhibitory concentration (MIC) of Eclipta alba leaf extract against Malassezia has been determined and their comparison have also done with – placebo –, a commercially available synthetic antifungal drugs Fluconazole and Ketoconazole. The results showed that all the test pathogens i.e. Malassezia globosa, Malassezia furfur, Malassezia sympodialis, and Malassezia restricta could be inhibited by Eclipta alba leaf extract. MIC (MIC range 0.625 to 1.25 mg/ml) of Eclipta alba leaf extract, against Malassezia was found very close to popular synthetic antifungal drugs Fluconazole and Ketoconazole and have less side effects. The antioxidant activity was also determined by means of the DPPH free radical scavenging test. Further, a modified DPPH approach is presented in this study.Based on these findings, now we can say that the formulations based on Leaf extracts of Eclipta alba and its constituents can be an alternative source for the treatment of Dandruff and Malassezia associated skin diseases.

Keywords: Antidandruff activity; Antimycotic; Fluconazole; In Vitro; Ketoconazole; Malassezia

INTRODUCTION

Mycosis infections have significant impact on healthcare, costs of hospitals and communities, especially in the poor developing countries, where mycoses appear endemically. Malassezia (earlier known as Pityrosporum) species form the cutaneous commensal flora, which are associated with varied clinical manifestations ranging from benign skin conditions, such as tinea versicolor, to fungemia in the immunocompromised [1].In the last decade the genus Malassezia has been a topic of intense basic research on taxonomy, physiology, biochemistry, ecology, immunology, and metabolomics [2].Malassezia, a lipophilic, dimorphic, fastidious, and yeast-like fungus, occurring in human skin as an opportunistic pathogen, causes diseases such as dandruff, Pityriasis versicolar and Seborrheic dermatitis. In April 2021, Species Fungorum accepted 22 species of Malassezia[3].New pathogenic roles of Malassezia beyond the skin has been reported, and is associated with Crohn's disease and pancreatic ductal carcinoma[4,5-8].The genus Malassezia

includes 18 species of basidiomycetous yeast as per study conducted in 2021 by Leibund Gut-Landmann Sand Dawson TL Jr[8]. In a recent study published in Nature by Hindson, Malassezia was linked to promote pancreatic cancer in special cases [5].Mycotic infection of the skin by the dermatophytes may be categorized into superficial and deep fungal infections.Dandruff (D) is a common type of scalp irritation, usually referring to the state of the skin in which shiny, silvery scales tear off from the scalp and gather amidst the hair. According to health experts, the condition appears to be caused by a yeast-like fungus called Malassezia [9]. The Malassezia fungus is normally found on the scalp without causing any problems.

The infection of the scalp clinically is represented as Dandruff. Dandruff is occurring in at least 40-50% and in Seborrheic dermatitis (SD) 1-3% of the general population [1]. Pityriasis versicolor (PV) is a mild, chronic infection of the skin caused by Malassezia yeasts, characterized by discrete, scaly, dark or hypo -pigmented patches, mainly on the upper trunk patches extend up to the neck, abdomen and other parts. The age group that commonly gets affected with Pityriasis versicolor (PV) is from 20 to 40 years old. However, in India, it has been commonly observed between the age groups of 10 and 30 years old. Noteworthy, PV is uncommon in children and is rarely found in the elder population [10-13]. Seborrhoeic dermatitis is a frequent relapsing skin disorder characterized by greasy scaly reddish patches with predilection of sebum-rich areas and it occurs in around 2–5% of the healthy population; however, its incidence is much higher in immunocompromised individuals, especially those with AIDS, ranging from 30% to 80% (Table 1) [14.15].

The vast majority of recent data support a direct causal link between Malassezia and D/SD. The factors that supports are:
(1)The effectiveness in treating disease by antifungal drugs (Table 1)[7, 9, 15-17] and
(2) Improvement in SD/D accompanied by a reduction in Malassezia levels on the scalp [15, 24-25].
A novel method of Antifungal susceptibility for Malassezia prompts for integrated therapy of naturpaopathy and synthetics [18-24].

Table 1. Clinical feature, causal organism and treatment for Dandruff, SD,PV,MF,IMI

Disorders	Scaling	Pruritus	Inflammation	Organisms	Treatment Strategies	References
Dandruff	Yes, flakes are losely adherent, oily, white or gray	Possible, generally mild	No	*Malassezia*	Topical treatments: antifungal, keratolytic, antiproliferatives	14,15,19, 29,30,31,
	Yes,flakes large, greasy, yellowish	Yes, varies	Yes	*Malassezia*	Topical treatments: antifungal, keratolytic, antiproliferatives	14,15,32, 33
Seborrheic Dermatitis (MF)	lesions back, chest and upper arms small, uniform, itchy papules	yes	Yes	*Malasseziapachydermatis*, *M. globosa* and *M. furfur*	Topical treatments such as selenium sulfide shampoo, econazole solution and systematic treatment Oral triazoles	16,29

PV	Fine white scaly,hypo or hyperpigmented macules that are irregular and most most often occurring on the oily parts of the body, trunk and extremitie	Yes	Yes	*M. globosa* and *M. furfur*	Topical treatments:antifungal, systematic treatment Oral triazoles (fluconazole, itraconazole)	16,17,35-40
Invasive *Malassezia* infections	Mixed symptoms	yes	Yes	*Malassezia*	fluconazole or voriconazole), amphotericin B	17,35,40

In most of the synthetic shampoos for controlling Malassezia and dandruff, SD, and PV, the antifungal ingredients used are Zinc pyrithione, selenium sulfide, salicylic acid, ketoconazole, miconazole and coltar(Table2)[26-32].Ketoconazole is a broad spectrum, antifungal agent that is active against Malassezia and other yeasts. In azole drugs, ketoconazole has become a leading antifungal among treatment options because of its effectiveness in treating D/SD/PV/IMI [33-34]. Dandruff is the most commercially exploited skin disease [35].Every population in any geographical region is affected by dandruff at some stage in life [36].Among these treatments, most frequent and effective chemical used is Ketoconazole, which is very dangerous not only for hair but also for body health causing frequent strokes of nausea, vomiting, rashes on skin, etc if used for longer period. Ketoconazole shampoo effect of long-term use result in androgenic alopecia [40]. Currently used antifungal drugs [polyenes like AmpB, triazoles like itraconazole, Fluconazole, and imidazoles such as Miconazole] are limited in use because of their side effects [37-40]. The usefulness of azoles has diminished in recent years as a result of increased incidence of resistance [38]. The increase in infection, combined with the reduced efficacy of currently available drugs, highlights the need for new antifungal drugs with distinct mode of action. Amp B, used alone or in combination with 5-FC is a polyene antifungal that has a broad-spectrum activity, but its utility is limited by nephrotoxicity[41-42]. The newer azoles such as Fluconazole are fungistatic agents that are relatively safe and free of side effects no matter how much, resistance is emerging [38].

Table 2.Synthetic products used in the treatment of dandruff/SD/PV and their drawbacks [19,21,29-42,56]

S.No	Drug	Infection type	Products	Drawbacks
1.	Zinc Pyrithione	Dermatophytes/ Yeast	Head and shoulders	Increased scaling if in continuous use.
2.	Coal Tar	Dermatophytes	Neutrogena T/Gel, Tegrin	Has an earthy smell. Can given light coloured hair, an orange tint and treated skin may become more sentitive to sunlight.
3.	Selenium Sulfide	Dermatophytes/ Yeast	Selsun, Excel	Can discolor hair blonde, gray or chemically colored hair.
4.	Salicylic acid	Dermatophytes	Lonil T	Leaves scalp dry leading to more flaking.
5.	Ketoconazole	Deep Mycoses/ Dermatophytes/ Yeast	Nizoral	Nausea, Vomiting, Hepatitis, Loss of hair, rashes, doses higher than required for most fungi.
6.	Miconazole	Deep Mycoses/ Dermatophytes/ Yeast	-	Frequent hypersensitivity fever and chills, skin rash or itching.

In tropical countries like India, fungal infections are of common occurrence. Identifying yeast as the causative agent creates the potential for being able to control this condition with antifungal agents. Dandruff and seborrheic dermatitis patients may require regular, long-term use of therapeutic agents. It is important that the treatments be formulated so as to be aesthetically and cosmetically acceptable to the patient. As such, the study was done mainly to discover the potential active ingredients from the selected plants active against the test pathogen. Interest in the organism of *Malassezia* has increased considerably in recent years, as this yeast has been implicated as the primary cause of the scalp disease known as seborrheic dermatitis or dandruff [18-19, 56].

Regular use of unhealthy synthetic cosmetics adversely affects and leads to cosmetic embarrassments. Therefore, people are switching over to alternative options of botanicals which are safer and have less side-effect. Widespread, unscientific use of antifungal drugs has resulted in the development of resistance, and serious health problems due to their carcinogenicity, making it necessary to discover new therapeutic alternatives. This has prompted the identification and development of eco-friendly naturopathy alternative.

Eclipta alba (L.) is an annual herbaceous plant, commonly known as 'False daisy' or Bhringaraja, belongs to the family of Asteraceae and used in different medicinal systems. *E. alba* is extensively used in India's traditional ayurvedic system of medicine. *E. alba* has been used for the treatment of skin diseases, liver diseases, hair treatment and also as an antimicrobial, analgesic, anti-haemorrhagic, anti-hyperglycemic, and antioxidant agent [43-44]. In China, Korea, Philippines and Nepal the plant is also used to treat snake bite, dysentery, heamaturia urine, sprains, furuncles, dermatitis, catarrhal problems, jaundice and scorpion stings[43-45]. *E. alba* contains a wide range of active metabolites, such as coumestans, triterpenoid saponins, flavonoids, alkaloids, and a glucoside of a triterpenic acid [44].The main aim of our study is to evaluate antifungal activity of *Eclipta alba*. Further, the antioxidant activity of this plant was also tested by the DPPH free radical.

MATERIALS AND METHODS

This study was conducted at the Biological Product Laboratory, Department of Botany, University of Allahabad, and dermatology clinic of Motilal Nehru Medical College, Prayagraj(Allahabad). During sample collection and isolation of *Malassezia* from patient, it was found that most predominant species associated with Dandruff and PV is *Malasseziaglobosa* as previously reported clinico-mycologic study in India [12]

Test causal organism

Malassezia furfur MTCC 1374, MTCC 1765 and *Malasseziapachydermis* MTCC 1369 were obtained from Institute of Microbial technology Chandigarh, India. Ten cultures of unicellular yeast like fungus *Malassezia* spp., namely, *M. furfur*, *M. globosa*, *M. restricta*,*M. sympodialis*, *M. obtusa*,*M. sloffiae*, *M. dermatis*,*M. yamatoensis*, *M. nana*, and *M. japonica*, were also obtained from Central bureau voorSchimmelcultures (CBS) Fungal Biodiversity Centre, Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW).Sub culture of *Malassezia* species was done on Lemming and Notman Agar modified (MLNA) medium according to CBS, and patented BPLA2R medium (Indian Patent, 290771)[20,22]. These strains were routinely cultured on slants and modified culture medium at 35^0C. The pH was adjusted to 5.8 prior to autoclaving at 15 lbs- 121^0C for 15 min. All the cultures were maintained at 34±2 ^{0}C in incubator for 4 days.

Standardization of protocol forbroth micro dilution method for Malassezia species

The antifungal activity of sample extract / oil was determined by broth micro dilution method recommended by Clinical Laboratory Standards Institute (CLSI) formerly known as NCCLS with slight modification using BPL modified medium broth or RPMI supplemented with fatty acid. RPMI 1640 supplemented with fatty acid and BPL modified medium broth was used. *Malassezia* culture was maintained on BPL modified medium and Modified Leeming Notman agar medium (MLNA). The 96-well microtitre plates were used for two-fold serial dilution. The proper growth control, drug control and the blank were adjusted. Stock solution was prepared in DMSO at a concentration of 50mg/ml, 20 µl of stock solution of sample extract was added into 4th well of 96-well microtitre plate horizontally having 180µl BPLbroth. So, the maximum concentration of the extract will become 2.5mg/ml. Then the solution was serially diluted up to 4th well to 11th well resulting into the half of the concentration of the test essential oil. The yeast inoculum was prepared at 0.5 McFarland standards; the absorbance was equal to the inocula suspension containing $1x10^6$. Then standard yeast inocula was added and kept for incubation at 32 ^{0}C in a moist chamber. The Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration at 50% (IC_{50}) was recorded spectrophotometrically at 530 nm using SpectraMaxplus384 after 48 or 72 h of incubation.

Organism used and culture conditions

Four common strains and 2 patients' isolate of *Malassezia*, were used throughout the study of antifungal susceptibility testing.

Malasseziaglobosa 7966, *Malasseziarestricta*7877, *Malassezia furfur* CBS 1878, and *Malasseziasympodialis* CBS 9974, 9968 were selected for this study. All these cultures were maintained in solid media BPL5M supplemented with

powdered milk (Figure 1) [20, 22].These strains were routinely cultured on MLNA, and patented BPLA2R medium along with Dutta and Dikshit modified medium-slants at 35^0C on daily basis. Since the *Malassezia*species are very fastidious, short lived and very difficult to maintain in its pure form, its culture was collected by scraping the affected areas of the patients from a specialty clinic at Prayagraj (Allahabad) under the supervision of a senior Dermatologist Prof.A.K.Bajaj, who lost his life during recent Corona pandemic.

In vitro investigation of antifungal activity of synthetic drugs which are available in the market was also determined and comparative study was conducted. Two synthetic drugs Ketoconazole and Fluconazole were used as standard control.

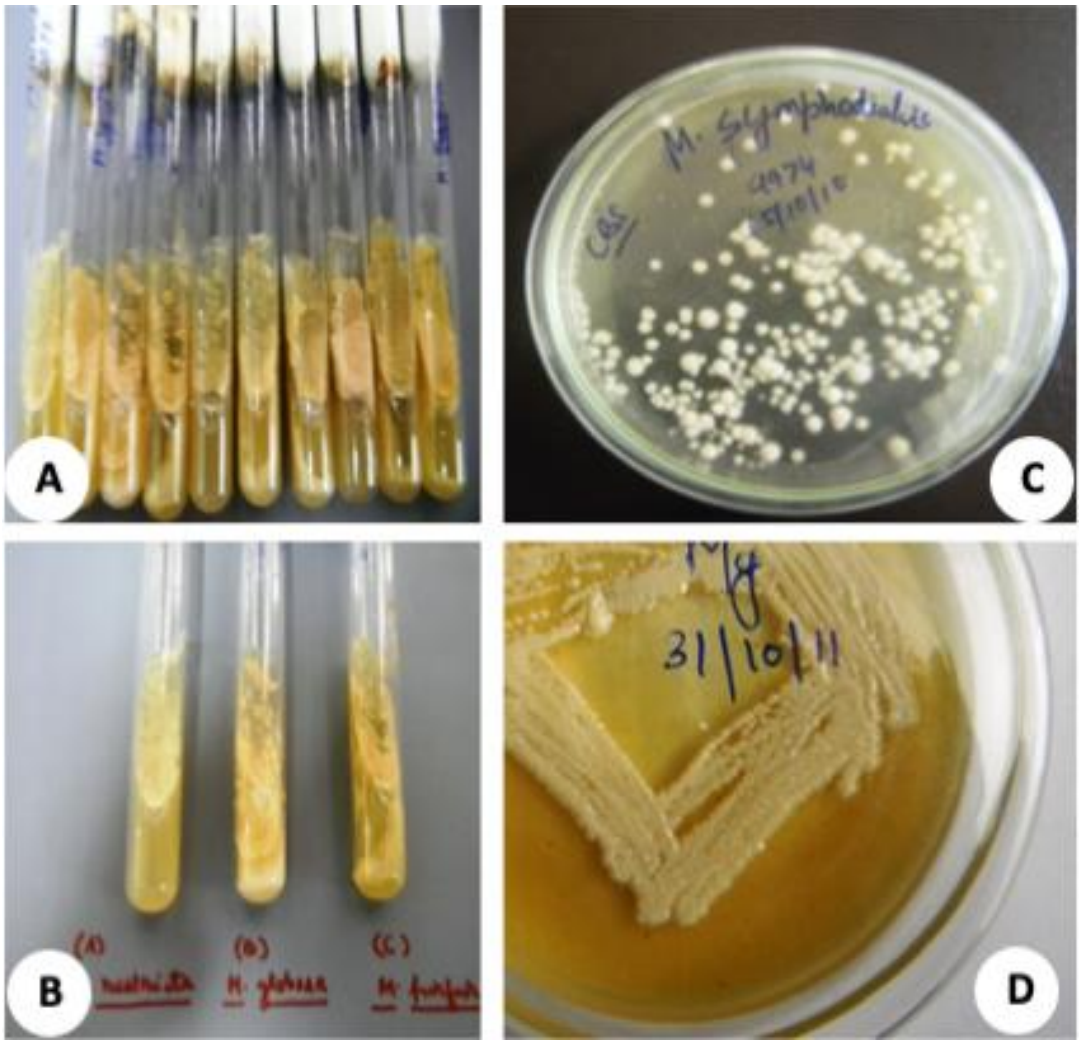


Figure 1. (A) 10 *Malassezia* species on Slant (B) Main dandruff /PV causing species on Slant (C) *Malasseziasympodialis* CBS 9974 (D) *Malasseziaglobosa* CBS 7966

Plant material and preparation of ethanol extract

The fresh healthy leaves of *Eclipta alba* (L.) were collected from Roxburgh garden, Science faculty, University of Allahabad, Prayagraj India. The plant was identified and authenticated from Botanical Survey of India (BSI), Prayagraj and voucher specimen was kept in Duthie Herbarium, Dept. of Botany, and University of Allahabad with reference number BPL/EA/1001. 50% alcoholic-aqueous solution was used for extraction of plant secondary metabolites. 5 gm of each Plant parts soaked in 50 ml of 50% EtOH solution (50% v/v) and then incubated for the period of 24 hours at room temperature. Freshly collected leaves of *Eclipta alba* (L.) were cut into small pieces and shade dried at room temperature. They were then minced using mortar. The processed sample was mixed in 50 % ethanol for 24 h. The extracts were finally filtered and concentrated using Rotatory evaporator and stored at 5 ℃.

Antifungal susceptibility testing

The antifungal susceptibility testing of *Eclipta alba* extract was evaluated against *Malassezia* spp. using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) with slight modification [47].Freshly prepared broth medium BPL5O supplemented with cottonseed oil was used for the assay [21-23]. Stock

solution (50mg/mL) of extract was prepared in DMSO. In brief, the initial fungal inocula suspension, prepared as per 0.5 McFarland standard (corresponding to a CFU of 1.5 ×107 cell/mL), was inoculated in two-fold serially diluted candidate extract to be tested. Ketoconazole and Fluconazole, as a synthetic standard, were also subjected to the antifungal assay. The MICs and IC_{50} were obtained by measuring absorbance using spectrophotometer (SpectraMax Plus384, Molecular Device Corporation, USA) at 530 nm, after an incubation of 72 h at35 ± 2°C and experiments were conducted in triplicate. The comparative method used for antifungal testing is economical, accurate, rapid, cheaper and comparisons for four drugs, one organism and vice-versa was performed in single plate [18]. The modified susceptibility method can be also used for unicellular yeast and other *Malassezia* species.

Antioxidant activity (DPPH ASSAY)

Traditional DPPH assay as described by Brad-Willam et al, (1995) was modified for this study [48]. 25 ml of 400 µM DPPH was added in 25 ml of 0.2 M MES buffer (pH 6 adjusted with NaOH) and 25 ml 20% (v/v) ethanol. Serial dilution of the extract was done from stock solution of 50 mg/ml and 200mg/ml accordingly, starting concentration at 2.5 mg/ml and 10 mg /ml respectively. DPPH cation solution 150 µl was mixed with 50 µl Sample extract (3:1) and kept in dark at room temperature for 20 minutes. Reduction in the absorbance at 517 nm was recorded by SpectraMax Plus384 using 96 well Microtitre plate. Results were expressed in IC_{50} (mg/ml) of the extract, which reduces 50 % of free-radicals in the solution. Ascorbic acid was taken as standard anti-oxidant with IC_{50} 0.5 mg/ml. The per cent inhibition was taken into account by its FRSA count and calculated by following formula:

$$\% \text{ inhibition} = \frac{O.D_{blank} - O.D_{sample}}{O.D_{blank}} \times 100$$

In-silico study

For studying the phylogeny, phylogenetic tree was constructed using the ITS1, 5.8 S and ITS2 region of ribosomal DNA of *Malssezia*. Gene sequences obtained from database of Gene Bank NCBI were blasted in the blastxprogramme of NCBI, and amino acid sequences were obtained for the strains (CBS 1878, CBS 7966, CBS 7877, and CBS 9968) used for study.The alignment of the gene sequence was done by ClustalW analysis,and further phylogeny was constructed. The sequences were collected in fasta format and Cladogram and Phylogram were prepared using ClustalW (EBI) for prediction of phylogentic relationship [49-50].

RESULTS AND DISCUSSION

In-vitro antifungal susceptibility testing

The ethanolic extract of*Eclipta alba* (L.)(EA)displayed an IC_{50} (mg/mL) of 0.355, 0.612, 0.903 and1.23, and MIC (mg/mL) of 0.625, 0.626, 1.48 and 1.42 against *M.globosa, M. furfur*,*M. restricta* and *M. sympodialis* (Figure 2, Table 3).Ketconazole and Fluconazole synthetic drugs were used as standard in our study. Ketoconazole showed theIC_{50} (mg/mL) values of 0.02286, 0.02289 and002845 against *M. furfur, M. restricta* and *M. sympodialis* respectively, while no activity in range was found for*M.globosa*(Figure 3a); and MIC (mg/mL) values were 0.009, 0.0.0325, 0.044 and 0.0581against *M.globosa*, *M. furfur*, *M. restricta*,and *M. sympodialis* respectively.Fluconazole exhibited MIC (mg/mL) values of 0.0061and 0.080against *M.globosa* and *M. furfur*,while no activity was recorded in concentration range for *M. restricta* and *M. sympodialis* (Figure 3b).

Nyctanthes arbor-tristis L. leaves ethanolic extract evaluated for antifungal testing of *Malassezia* spp.and MIC values of the ethanolic extract for *M. globosa*, 7966, *M. furfur* 1878, *M. restricta* 7877, and *M.sympodialis* 9974 ranged from 1.05 to 1.47 mg/mL(MFC= 3.12 mg/mL) and its effect influenced cell membrane integrity[51,55]. Ethanolic extracts of lichen-*Cladonia aggregata* exhibited antifungal activity against *Malassezia* atMIC (mg/mL) values of 2.72, 0.63, and 1.28 against *M.furfur*, *M. globosa* and *M. sympodialis*, respectively, while no activity was recorded against *M. restricta*.Fluconazole was used as the reference standard (MICvalues ranging from 0.006 to 0.051 mg/mL)[24,55].

Nineteen plant extracts for the antimycotic activity against *M. furfur* 1374 were done by disc diffusion method and found *Aloe vera*, *Eucalyptus globulus*, *Phyllanthus emblica* and *Wrightia tinctoria* leaf extracts and oil showed antifungal property as they progressively inhibited the growth of *M. furfur* on Sabouraud's destrose agar(SDA) medium. *E. globulus* (30 ± 1.63) and *A. vera* (29± 1.14) were more effective than other species and tested antibiotic Clotrimazole (24.6 ± 0.94 [57,58].

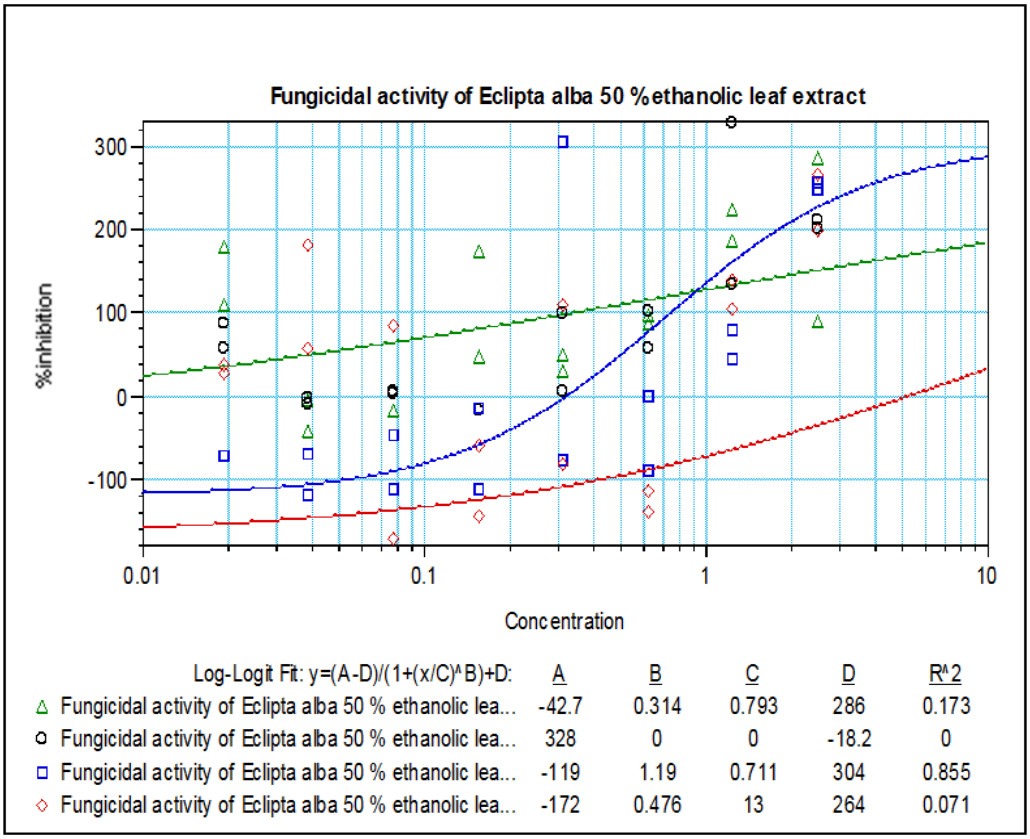
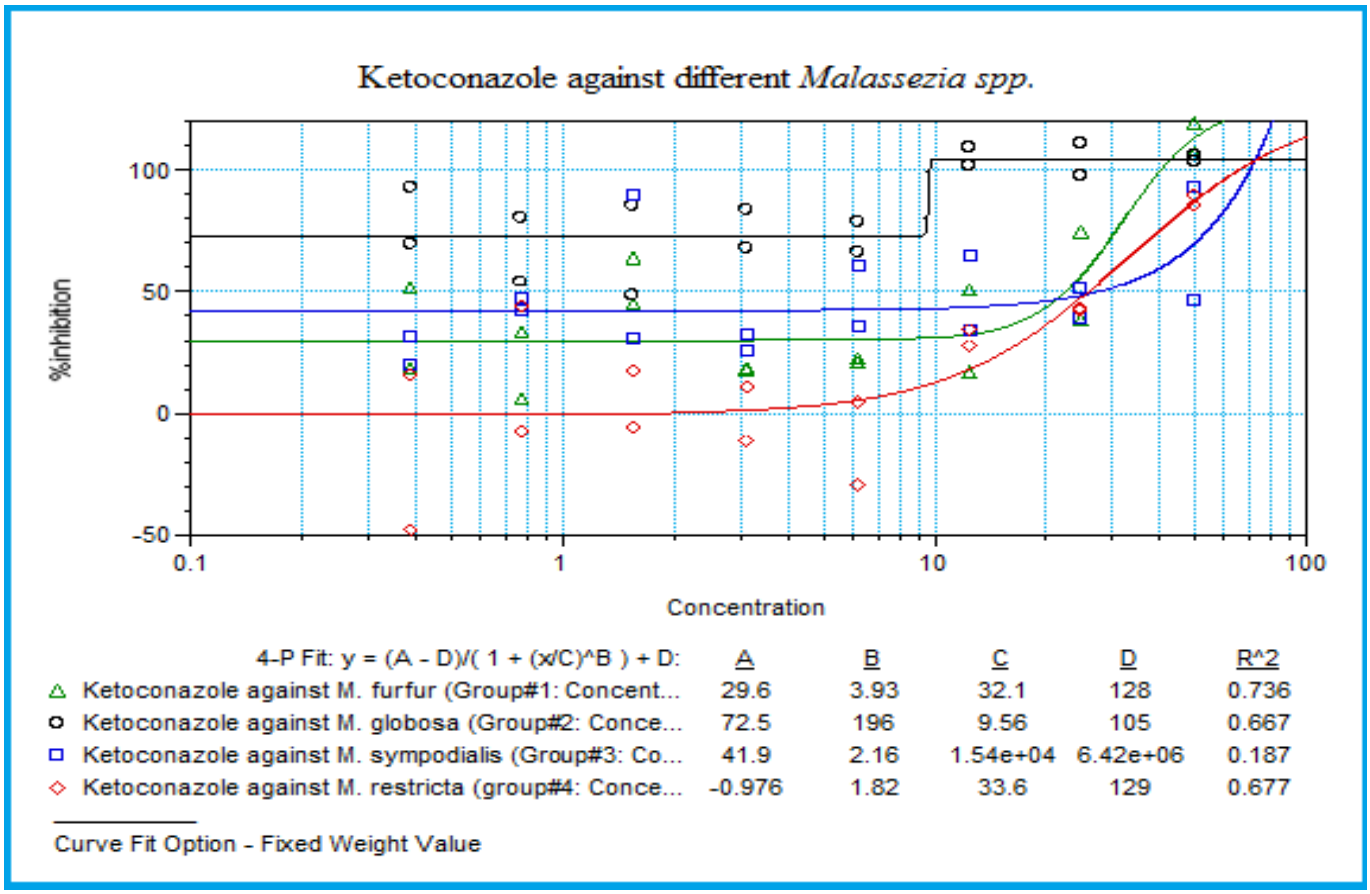


Figure 2.% inhibition curve of *Eclipta alba*against *Malassezia* spp. (generated by SoftMax Pro using model SpectraMax Plus384).

Table 3.*In-vitro* activity of synthetic and of *Eclipta alba*leaf ethanolic extract against *Malassezia*(IC_{50} and MICare given in mg/ml). Mg =*M. globosa*, Mf = *M. furfur*,Mr = *M. restricta*,and Msy = *M. sympodialis*

S.N.	Pathogen / Sample	Mg		Mf		Mr		Msy	
	Synthetic	IC 50	MIC	IC50	MIC	IC50	MIC	IC 50	MIC
1	**Ketoconazole**	NR	0.009498	0.02286	0.032593	0.02289	0.04421	0.02845	0.0581
2.	**Fluconazole**	NR	0.006105	0.06645	0.080719	NR	NR	NR	NR
	Herbal								
3	***Ecliptaalba (L.)* EA leafethanolextract**	0.355	0.625	0.612	0.626	0.903	1.148	1.23	1.42

% inhibition curve of Ketoconazole against Malassezia *spp. (generated by SoftMax Pro using model SpectraMax Plus384*



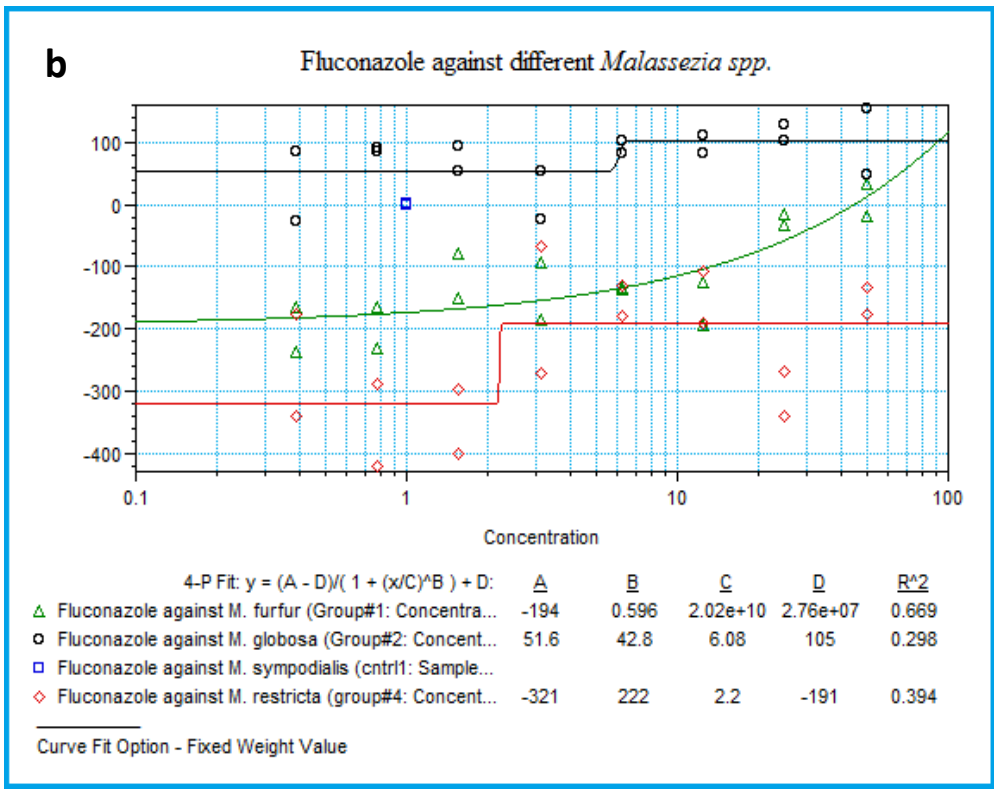


Figure 3b.% inhibition curve of Fluconazole against *Malassezia* spp. (Generated by SoftMax Pro using model SpectraMax Plus384).

Synthetic Drug and their Mechanism of action

The three major groups of drugs in clinical use are polyenes, azoles and pyrimidines with the exception of 5-FC, the azoles and polyene antifungal drugs in common usage are directed in same way against Ergosterol, the major sterol in fungal plasma membrane. Ergosterol in fungal membrane contributes to a variety of cellularfunctions. It is important for the fluidity and integrity of the membrane and for the proper function of membrane bound enzymes including chitin synthetase, which is important for proper cell growth and division. For azole drugs mode of action, several lines of evidences suggests that the primary target of azoles is heme protein which co-catalyses cytochrome P450 14α-demethymation of lanosterol. Inhibition of 14α-demethylase leads to depletion of Ergosterol and accumulation of sterol precursors, including 14α-methylated sterols resulting in the formation of a plasma membrane with altered structure and function. However, a large number of patients have experienced azole treatment as a failure due to the development of drug resistance in *Malassezia* species. Ketoconazole is an imidazole derivative, broad spectrum antimycotic agent that is active against Pityrosporum ovale and is effective against many fungi both in-vivo and in-vitro. It is also effective in many dermatomycoses, including Pityriasis versicolor. However, in very severe cases of dandruff, Ketoconazole based shampoos are preferred despite their relatively higher costs. Herbal ingredients like tea tree oil, rosemary oil, coleus oil, clove oil, pepper extract, neem extract, and basil extract are also recorded anti-pityrosporum activity, but their MICs are much higher than the synthetic ingredients[18-19,56].

One study reported among synthetic drugs MICs ranges were <0.03-4 µg/ml for Ketoconazole and <0.125 to >64 µg/ml for Fluconazole against *Malassezia*[18,59] . Another integrated study showed MIC of Ketoconazole 2.5 µg/ml, Fluconazole 2.5 µg/ml, Clove oil 1000 µg/ml, Coleus oil 25 mg/ml, and Basil oil 10 mg/ml was effective against *Malassezia furfur* by disc diffusion method [18,60].However,in the present investigation into the synthetic drugs the most effective was Ketoconazole, MICs ranges were 0.009 to 0.0325mg/ml fungicidal against *M.furfur, M.globosa, M.sympodialis* and *M.restricta*(see Tables 4a-d, and Tables 5a-b).

The slow growth of yeast like fungi *Malassezia*, its fastidious nature, difficulty in sub culturing and maintenance of standard culture, instance lack of standard protocol for antifungal susceptibility testing,generally used disc diffusion method, and developing resistance to synthetic drugs discourage so many researchers to work in this field.Modification of protocol and patented medium will open doors for new researches working in this area and producing reliable result and also very helpful diagnosis of disease in pathological lab [20].*Eclipta alba* was used in traditional medicine for hair care but no report was found to in vitro antimalassezia activity and that prompted us to explore it against *Malassezia*.

The minimum inhibitory concentration (MIC) of the *Eclipta alba* 50 % ethanolic leaf extract against *M. restricta* was found to be 1.148mg/ml;however, it was fungicidal at 2.5 mg/ml(Table 6a). The IC_{50} value was determined as 0.903mg/ml. Moreover, the minimum inhibitory concentration (MIC) of the *Eclipta alba* 50 % ethanolic leaf extract against *M. globosa*was found to be 0.625 mg/ml but it was fungicidal at 1.25 mg/ml.

Phylogenetic analysis

Phylogenetic analysis of four common pathogenic *Malassezia* spp. (tested in this study) revealed that *M. globosa* and *M. restricta* are closely related to each other and form sister group to *M. furfur*. Further, it was found that *M. sympodialis*is another branch and it was not much close as *M. globosa* and *M. restricta* (Figure 4). Phylogenetic relationship of most common species of *Malassezia*(which is responsible for causing dandruff in human)has been studied on the basis of phylogenetic tree of concentrated ITS1, 5.8 S and ITS2 region of ribosomal DNA. The present in silico prediction study was conducted for determination of evolutionary relationship among most common species of dandruff causing microorganisms for which gene sequences were collected from National centre for Biotechnology information (NCBI) database on the basis dandruff causing activity of *Malassezia* species.

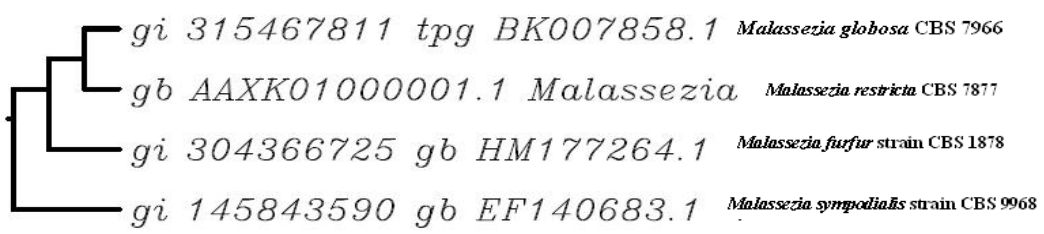


Figure 4. Phylogentic tree based on the ITS1 5.8 S and ITS2 region of ribosomal DNA of dandruff causing pathogens

Antioxidant activity

DPPH assay, violet color DPPH solution was reduced to yellow colored product, (diphenylpicryl hydrazine) by the addition of the extract in a concentration dependent manner. The scavenging effects of extract increased with their

concentrations to similar extents, *Eclipta alba* (80 %) showed potent DPPH radical scavenging activity while standard ascrobic acid had 84% inhibition with same concentration of 100µg/ml (Table 4, Figure 5). The leaf extract of *Eclipta alba* showed good antioxidant activity using DPPH radical scavenging capacity, which was clearly reflected in the results showingIC_{50} of 50µg/µl. The reference standard used was ascorbic acid with IC_{50}, 40µg/ml. The efficiency of plant leaf extract as potential antioxidant is a value-added trait that can be used in prevention of human diseases caused by superficial mycosis.

Table 4.Antioxidant activity of 50 % ethanolic leaf extracts of *Eclipta alba* and standard ascorbic acid.

Sample conc.(µg/ml)	% inhibition *Eclipta alba*	Vitamin C (Ascorbic acid)
3.125	6.2	24
6.25	12	28
12.5	22	32
25	35	42
50	53	65
100	80	84
200	82	88
400	86	94

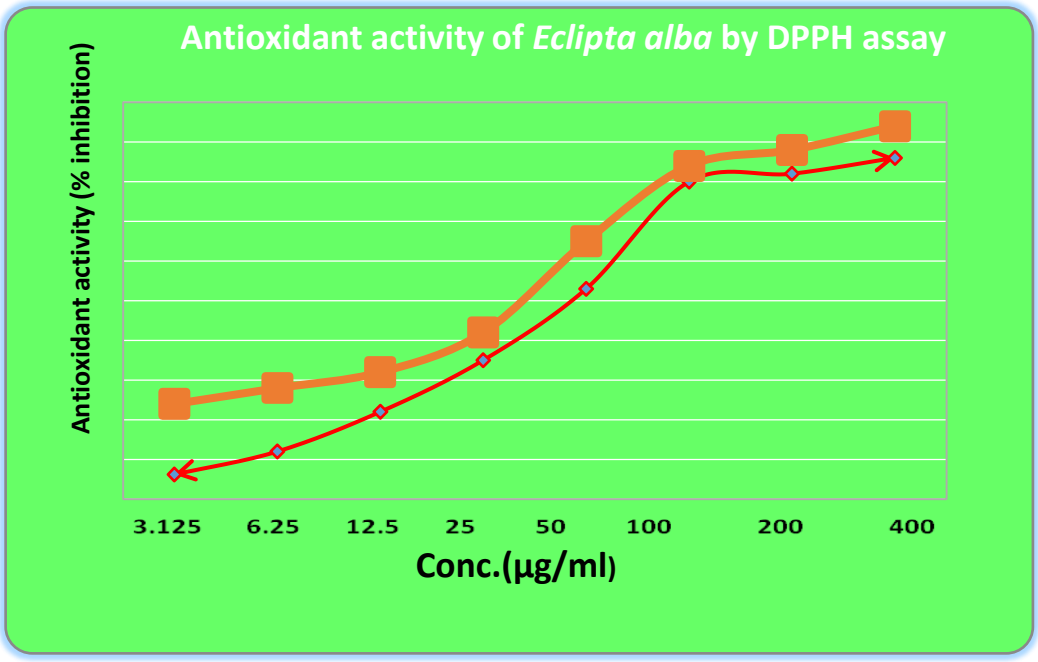


Figure 5.Graph showing Antioxidant activity (%inhibition) of 50 % ethanolic leaf extracts of *Eclipta alba (EA)* and standard ascorbic acid

CONCLUSIONS

The management of *Malassezia* associated diseases are mainly based on old azole (Ketconazole and Fluconazole) drugs, and used frequently because easy availability in market and fast relief but have unpredictable clinical efficacy, emerging resistance and several side effects if repeated used by patient. These drawbacks of available synthetic drugs have prompted the research toward green conventional or integrated research. On the basis of the present investigation, it is concluded that the 50 % ethanolic extract of *Eclipta alba* have significant anti-fungal activity against *Malassezia* spp.i.e. *M. globsoa*, *M. restricta*, *M. furfur* and *M. sympodalis* with MIC (MIC range 0.625 to 1.25 mg/ml) .Presence of significant antioxidant activity in the 50 % ethanolic extract of *Eclipta alba* is value addition capable of being therapeutic success in near future against human disease associated with *Malassezia* and other dermatophytes.The present study exhibits that *Eclipta alba* is a potential source of secondary metabolites which can be used in developing herbal formulation as a result of multicentral double blind topical testing after successful investigation on animal model.

Author Contributions:
AKT and AD conceived and designed the study. AKT performed the experiments and analyzed the data. AKT, AD and PKD wrote the paper, discussed the results and revised the manuscript.

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