ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Extraction Of Antidandruff Compound From *Lawsonia inermis.*

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ABSTRACT

The present research focused to evaluate the *utilization of henna leaves extract* as sources of *natural anti-dandruff compounds*. *Malassezia furfur (M. furfur)* isolated from scalp of subjects and medium standardization was done. The growth curve of the fungus was observed. Henna extracts were prepared using different solvents. Antifungal activity of various crude extracts were detected by well and disc diffusion methods. Comparison on various concentration of henna extracts and known ketoconazole compound stabilized by BHT (an antioxidant) were studied. Partial fractionation of the crude extract was done by Thin layer chromatography (TLC) followed by characterizing the maximal antifungal activity compound by ^{1}HNMR and ^{13}CNMR. Microscopic examination revealed dermatophytes and presence of small, smooth, pale white colonies on selective media. Growth curve were observed approximately 65 hrs of inoculation. The zone of inhibition was observed by 60% hexane extracts of Henna leaves by Disc diffusion method. The Ketoconazole was also stabilized by BHA and respective activity was found to be increased. TLC analysis showed the presence of similar compound in NMR revealed that the *imidazole* was found to resemble *Ketoconazole.* The air-dried, powdered leaves of *Lawsonia inermis (L.inermis)* were used with the different solvents to observe the antifungal activity against the dandruff-causing dermatophyte, *M. furfur.* The results were then compared with the standard anti-dandruff compound ketoconazole with and without antioxidant stabilization. The extracted anti-dandruff compound is then characterized with the help of NMR Spectroscopy.

Keywords: *Malassezia furfur, Lawsonia inermis*, Ketoconazole, BHT.

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ISSN: 0975-8585

INTRODUCTION

Dandruff, a mild form of *seborrhoea dermatitis*, are common chronic relapsing scalp skin disorders that share some clinical features with psoriasis and atopic dermatitis, as it can affect sebum rich areas other than the scalp (Kathy Kerr James et al. 2011). Therefore scientists have suggested that dandruff may be a hypersensitive reaction to the proliferation of *Pityrosporum ovale*, yeast that occurs naturally on the scalp. However many people with this disorder, been characterized by itching and flaking of the scalp (Chen et al. 2002). The common symptoms of dandruff include itching and white, oily flakes. There are several conditions, including psoriasis, dry skin, seborrheic dermatitis, or contact dermatitis etc (Reich and Szepietowski 2007; Ashida and Denda, 2003).

The fungus, *M. furfur* a lipophilic, dimorphic and yeast-like fungus, is the cause of dandruff metabolizes triglycerides present in sebum by the expression of lipase, resulting in a lipid by-product oleic acid (OA). Penetration by OA of the top layer of the epidermis, results in an inflammatory response in susceptible persons which disturbs homeostasis, therefore results in erratic cleavage of stratum corneum cells (Warner et al. 2001).

The treatments for dandruff include Coal tar, Zinc pyridinethione (ZPT) or Miconazole medicated shampoos. The most common antifungal agents used are Zinc pyrithione, Selenium sulfide and Ketoconazole (Peter and Richarz Barthauer, 1999). Other products used include Tea tree oil (Hammer et al. 2000) and Piroctone olamine (Octopirox). Anti-fungal/anti-dandruff shampoos containing ketoconazole have been shown to be more effective than ZPT. Although one study has reported selenium sulfide as being the most effective of the tested shampoos at treating dandruff, a later comparative study concluded that ketoconazole was the most effective antifungal agent.

L. inermis commonly known as Henna, a popular skin and hair colouring agent in many parts of the world as it is traditionally used as a medicinal plant (Surveswaran et al. 2007) by diverse group of tribal/ethnic people (Lev and Amar 2008). It is used as an anti-inflammatory, anti-bacterial, antifungal, anti-rheumatic and anti-neuralgic agent (Marc et al. 2008) and also has potential anti-diabetic drug (), there are even few evidence supporting that the plant having wound healing properties (Nayak et al. 2007). Betulin, xanthones, coumarins (plant); beta-sitosterol, esculetin, gallic acid, glucosides, lawsone, luteolin, laxanthones I and II, scopoletin (leaf); linolenic acid (seed) were the active compounds found in *L. innermis* (Sakakibara et al. 2003). Humans have used henna extracts containing lawsone as hair and skin pigments for more than 5000 years, as it reacts with the protein keratin in skin and hair resulting in a strong permanent stain that lasts until the skin or hair is shed (Ali Hussain and Nawaz 2009).

The present work aimed to prove the antifungal activity of *L. inermis*, as the *M. furfur* were isolated from scalp of subjects with dandruff and followed by medium standardization. Different crude henna extracts were prepared to detect the antifungal activity by well diffusion and disc diffusion method and thereby comparing the results with the pure ketoconazole compound. BHT (antioxidant) were used to stabilize ketoconozole and their respective antifungal activity was determined as well followed by partial fractionation of extraction through TLC and characterizing it by ^{1}H NMR and ^{13}C NMR.

MATERIALS AND METHODS

SAMPLE COLLECTION

L. inermis (Henna) plant was collected from D.G Vaishnav college campus, Arumbakkam, Chennai-106, TN, India.

Preparation of Sample

The leaves of *L. inermis* were washed and air dried followed by oven drying. Then the leaves were crushed and converted into powdered form and stored for further analysis with reference cited by Gunjan Guha (2009).

Fungi

It was collected from the scalps of subject with dandruff. The standard culture from Microbial Type Culture Collection, Chandigarh (MTCC 1765 Strain – *M. furfur*).

ISOLATION OF *M. furfur* FROM SCALPS OF SUBJECTS WITH DANDRUFF

Dandruff samples were collected from the scalps of 3 subjects with the help of sterile swabs or cotton buds with reference to Kathy Kerr et al. 2011. Dandruff is caused by lipid-dependent yeasts (lipophilic yeasts) called *M. furfur* that requires long chain fatty acids for in-vitro growth. Thus the collected sample was cultured on following mediums with lipids base like milk or oils.

- Emmon's Modified Medium by Saravana Kumar and Vijayalakshmi (2006).
- Leeming & Notmann Agar by Leeming and Notman (1987).
- IMU-Mf Medium (International Medical University-*M. furfur* Medium standardised by Chua (2005).

GROWTH CURVE OF *M. furfur*

UV-Visible spectrophotometer was first scanned for appropriate wavelength for maximum absorbance (peak). The scanned wavelength was found to be 340nm. 2ml of broth without inoculum was taken as control. Every 8th hour reading was taken till the end of 96 hrs. Readings were noted down and were plotted on a graph to obtain the growth curve by Mayser et al. 1988.

PREPARATION OF CRUDE EXTARCT FROM PLANTS

Extraction from Lawsonia

Fresh leaves of *L. inermis* were collected and air-dried in a shaded place for around 7-8 days. Dried leaves were ground mechanically, powdered and sieved to get fine powder. 5g of dried leaves powder was then mixed with 50ml of sterile distilled water, Ethyl acetate, 60% Hexane and 70% Methanol in separate beakers and stirred well. The beakers are covered and kept undisturbed for 24 hrs followed by filtering the extract through filter paper. The 1st filtrate was taken in separate porcelain dishes and kept for solvent evaporation. The residue was mixed with 25ml of all these solvents (Ethyl acetate, 60% Hexane and 70% Methanol) and was stirred well, left for 2nd extraction, then the beakers were covered and kept undisturbed for 24 hrs followed by filtering it again with filter paper. The 2nd filtrate was then taken in separate porcelain dishes and kept for solvent evaporation and the dried extracts were collected in separate glass vials and labelled properly. The same procedure was followed with commercially available henna powder as well by Gunjan Guha et al. 2009.

DETERMINATION OF ANTI-FUNGAL ACTIVITY OF THE CRUDE EXTRACT

The dried extracts were dissolved with appropriate solvents like ethyl acetate, acetone and milliq water. Sterile discs were kept in the various herbal extract solutions for 1hr and various concentrations of ketoconazole were prepared with the following stock – 80µg/ml, 100µg/ml, 200µg/ml and 400µg/ml. 400 µl of various herbal extracts and different concentrations of ketoconazole were mixed with 100 µl of BHT Stock (0.1%). The standardized specific growth media was autoclaved and poured into sterile plates. Wells were punctured into the solidified media followed by 3rd day broth culture and was swabbed on to the plates. In well diffusion method, 35µl of herbal extract solutions with various ketoconazole concentrations and the solutions stabilized by BHT were poured into the punctured wells in separate plates to check the respective activity, likewise in disc diffusion method, saturated sterile discs were carefully taken out of the solutions using sterile forceps and kept on the inoculated plate. All the plates were incubated for 4-5days at 30^{o}C in an incubator.

THIN LAYER CHROMATOGRAPHY

Commercially available aluminium plates coated with silica gel (Merck) were used for thin layer chromatography analysis. Solvent systems (Mobile Phase) Chloroform: Ethyl Acetate (7:3) and Ethyl Acetate:

Methanol (6:4) was used.

The plates were cut into required size and were activated in hot air oven at 70 ^{o}C for 30mins. The solvent systems were prepared and poured into TLC developing tanks followed by saturating the tank. The 3 crude extract (dissolved in ethyl acetate, acetone and milliQ water). The samples were spotted on the line at equal distances using capillary tube and the spots were air-dried followed by keeping it in the tank such that the line doesn't touch the solvent. The tank was kept covered to prevent evaporation and the plate was kept undisturbed for 15 mins till the bands get separated. The plate was visualized under UV Trans-illuminator & was photographed referred by Mayser et al. 1996.

NUCLEAR MAGNETIC RESONANCE (NMR) ANALYSIS

The samples were given to IIT, Chennai, TN, India, for the NMR Analzysis. $CDCl_3$ solvent system was used for the ethyl acetate dried Henna extract sample. The NMR instrument used for the analysis is of 500MHz methodology referred by Wei Zheng and Shiow Y. Wang (2001).

RESULTS

Growth of *M . furfur*

Fig1. *L. inermis* Fig 2. Dandruff collected from scalp of different subjects, *M. furfur* was isolated on the Leeming-Notman agar, IMU-Mf agar and the selective growth media specified by MTCC. Fig 3 & 4 shows smooth, pale yellowish, round colonies on the specific growth media specified by MTCC on 3rd and 7th day of culture.

Fig 5. Shows growth of *M. furfur* as small, smooth, pale white colonies on selective media called IMU-Mf on the 4th day.. Fig .6 shows Lacto-Phenol Cotton Blue Staining of *M .furfur* and the 40X microscopic view of the spores of the dermatophyte. Fig. 7 shows the growth curve of *M. furfur*, as maximum cell growth and cell division was attained after approximately 65hrs of inoculation of the yeast. The active metabolites present in *L. inermis* were extracted using aqueous and various solvent systems like polar solvents (ethanol & 70% methanol), mid-polar solvent (60% Hexane) and non-polar solvent (Ethyl Acetate). These dried extracts were then used to check their antifungal and anti dandruff activity.

Crude extract yield

Fig. 8 shows the yield of the dried henna extract with different solvents. Fig. 9 showed the comparison of the Anti-Dandruff activity of various solvent Extracts graphically, 1) Ethyl acetate extract (dried leaves powder), 2) Ethyl acetate extract (commercially available henna powder), 3) 60% Hexane extract, 4) 70% Methanol extract, 5) Ethanol extract, 6) Aqueous extract.

Ketoconazole is a highly lipophilic imidazole antifungal agent. In the present study, it was used as standard anti-dandruff compound and its activity was measured as minimum inhibitory concentration against the dandruff causing organism *M. furfur.* The ketoconazole was also stabilized by BHA and the activity was found to increase. Fig. 10 shows the activity of ketoconazole on *M. furfur* by Disc Diffusion method. 80µg/ml concentration was found to be MIC. Fig. 11 shows the activity of ketoconazole stabilized by BHT on *M. furfur* by disc diffusion method. The activity of ketoconazole was found to increase in the presence of BHT. Fig. 12 shows the comparative study of activity of standard anti-dandruff compound ketoconazole) with and without BHT on *M. furfur.*

Thin Layer Chromatography and NMR Analysis

Partial fractionation of the crude extract was done through Thin Layer Chromatography (TLC) Analysis. Various extracts were run along with the standard in different solvent systems. The result showed the presence of various compound in the ethyl acetate extract of henna leaves in comparison with the standard. Fig 13 & 14 shows TLC Plate with solvent system A & B respectively viewed under normal light and UV light. Solvent system A → Chloroform: Ethyl acetate (7:3), Solvent system B → Ethyl acetate : Methanol (6:4).

ISSN: 0975-8585

Fig. 15 and Fig. 16 shows the chemical shift as interpreted by NMR. Fig. 17 revealed that the extracted compound was found to be imidazole. The expected structure of the compound showed the resemblance with the ketoconazole with mol weight of 531.44D and molecular Formula as $C_{26}H_{28}Cl_2N_4O_4$. Thus it is interpreted that anti-dandruff compound (ketoconazole) was present in the ethyl acetate extract of henna leaves. From the proton integration values, it is seen that 2-3 % of the expected product has been found in the crude ethyl acetate extract of dried Henna leaves.

Fig. 1

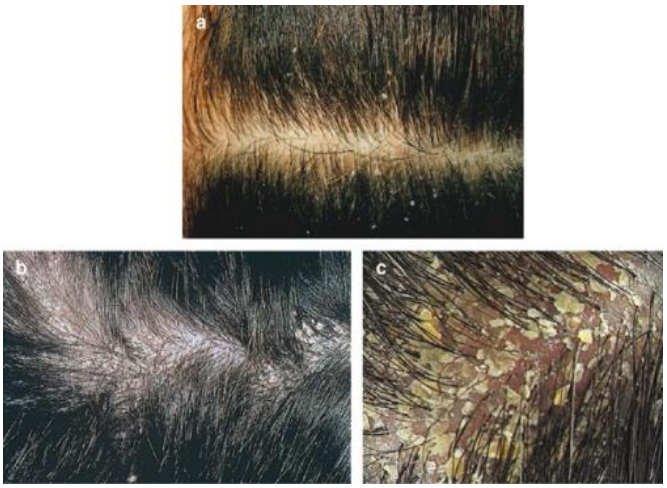

Fig. 2

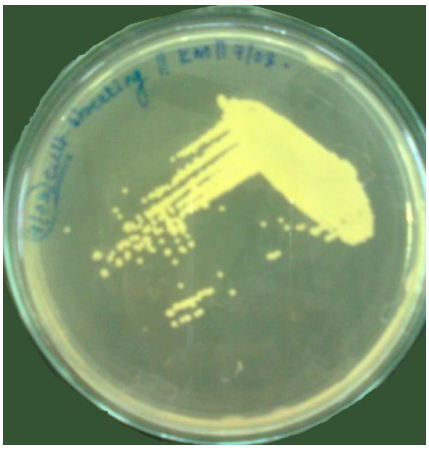

Fig. 3

Fig. 4

Fig. 5

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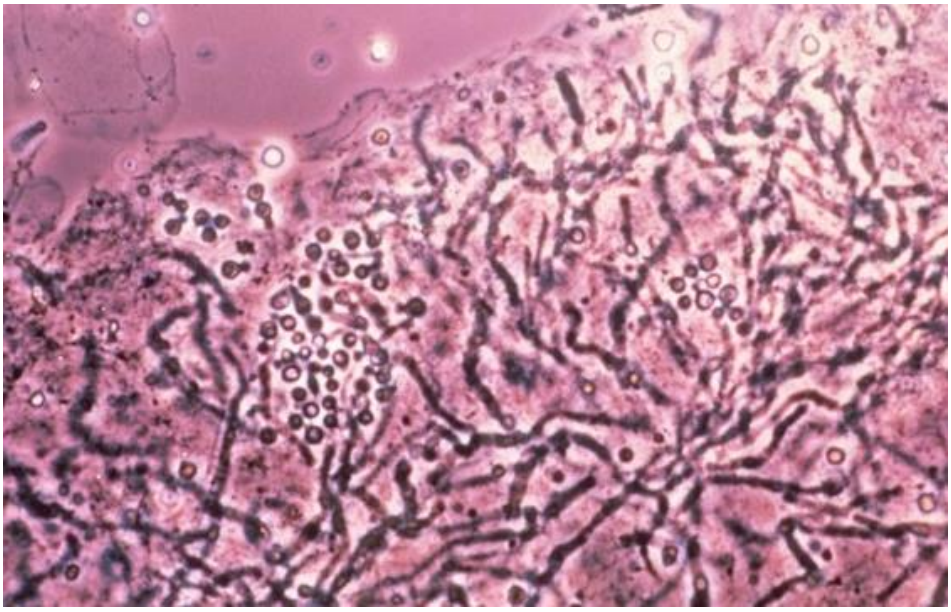

Fig. 6

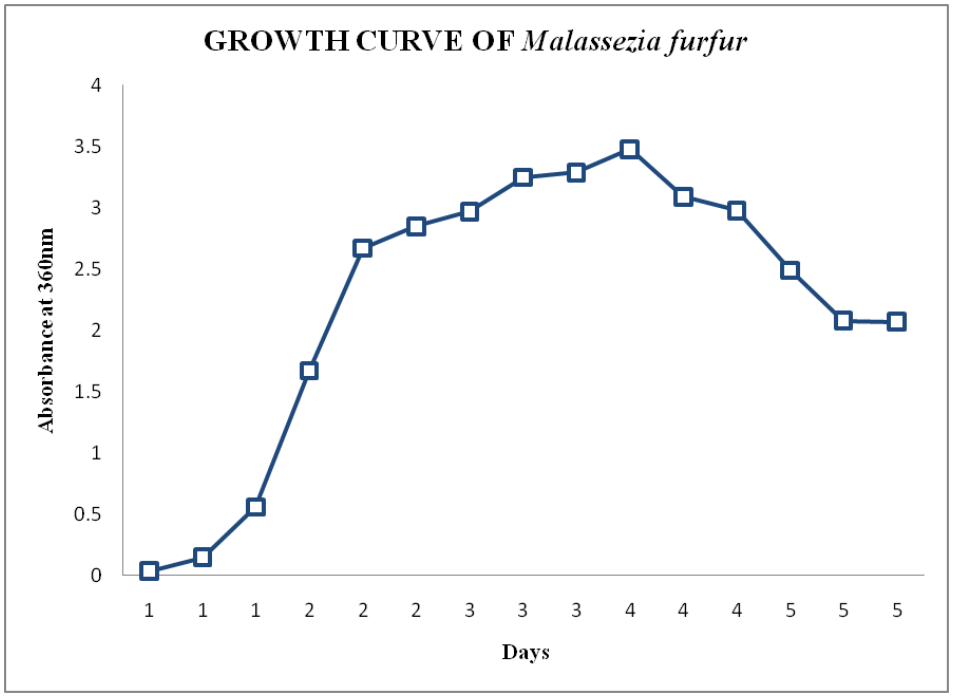

Fig. 7

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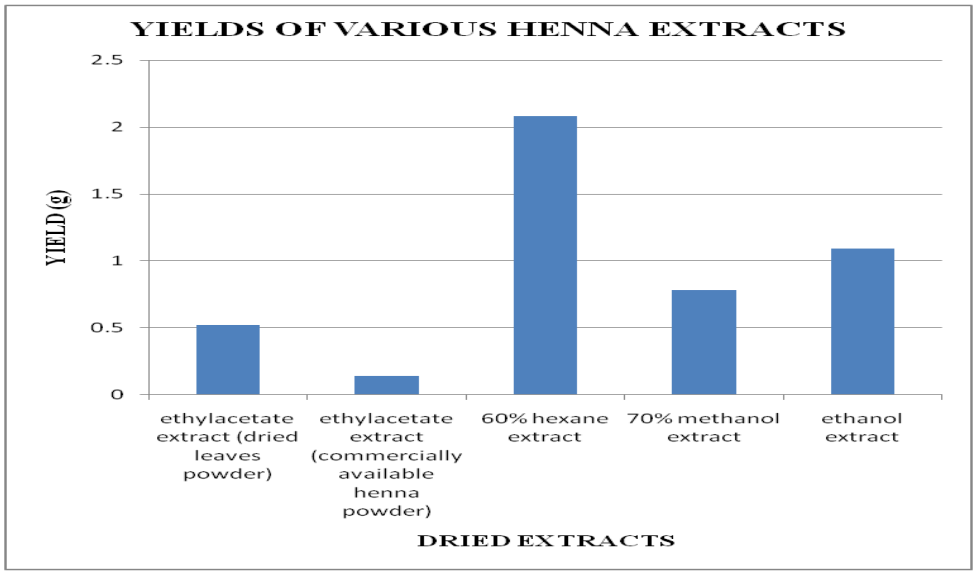

Fig. 8

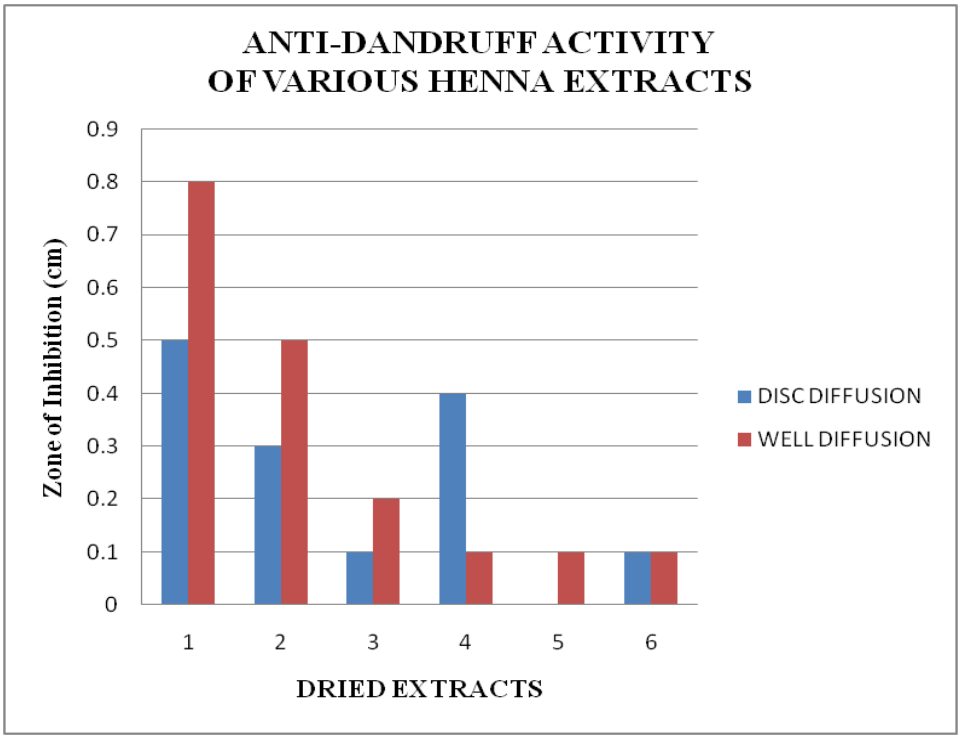

Fig. 9

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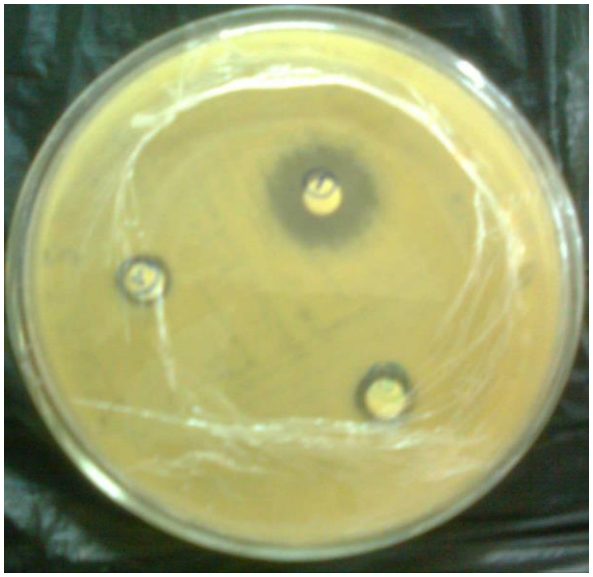

Fig. 10

Fig. 11

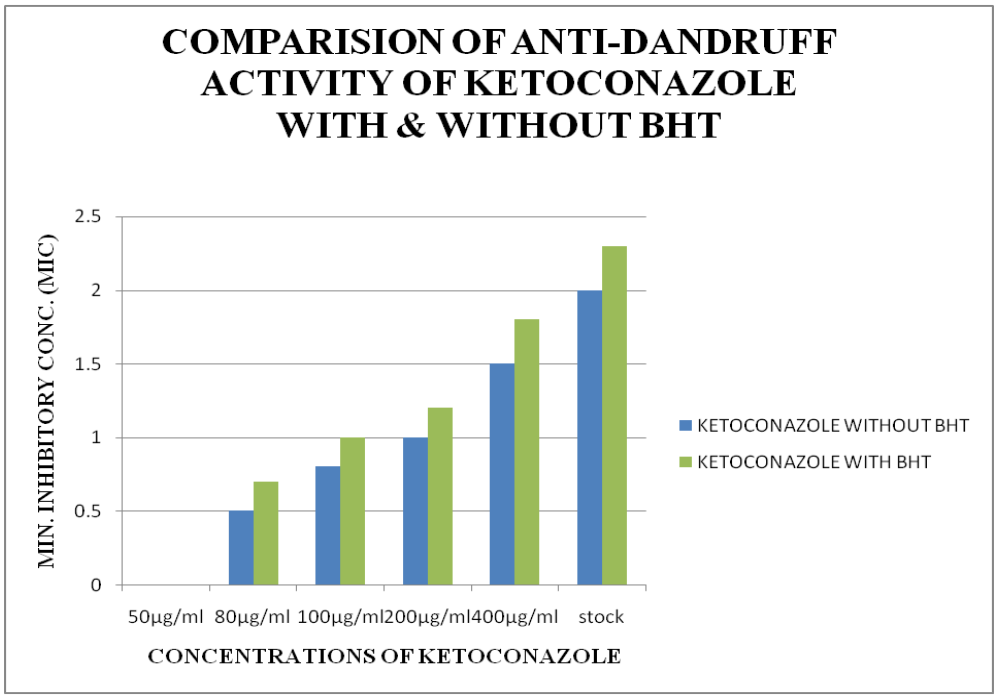

Fig. 12

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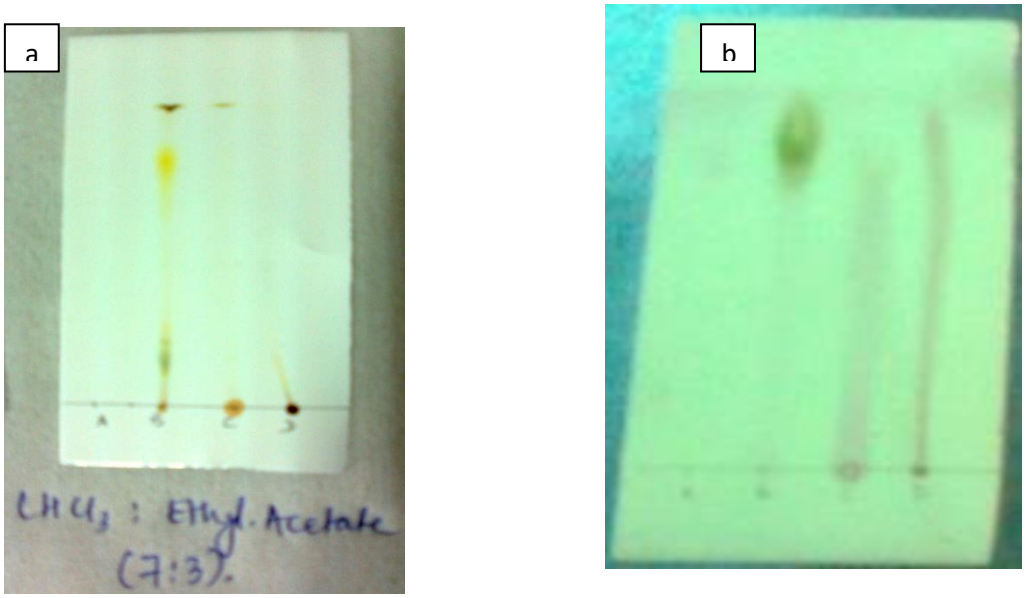

Fig. 13

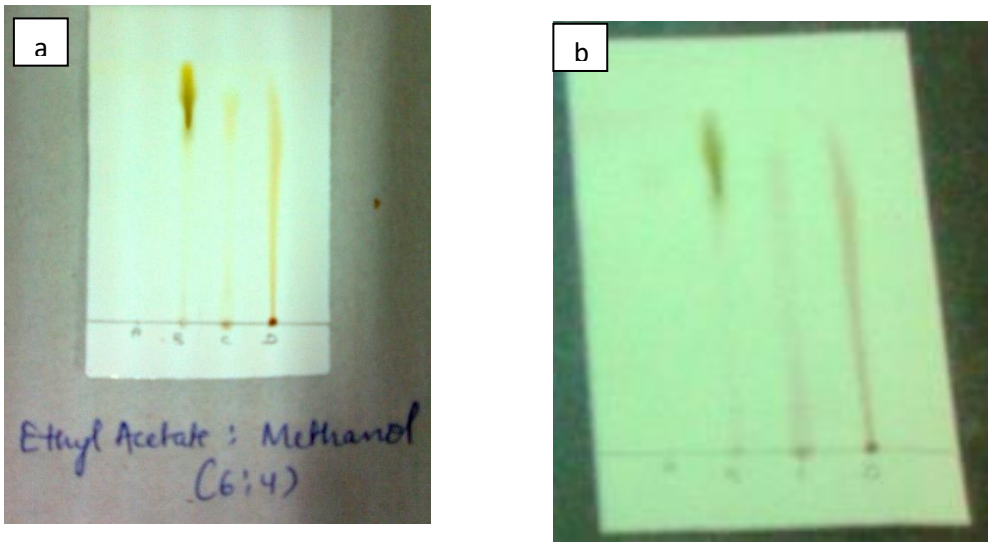

Fig. 14

ISSN: 0975-8585

Chemical shift	Significance
2.09	Methyl group attached to carbonyl carbon
3.4,3.6,3.7,3.8,3.9	Methylene groups attached to oxygen/nitrogen
4.1-4.3	Methylenes attached to oxygen
6.1-6.4	Aromatic hydrogens
7.2,7.7	Aromatic hydrogen, ortho to halogen(chlorine)

Fig .15

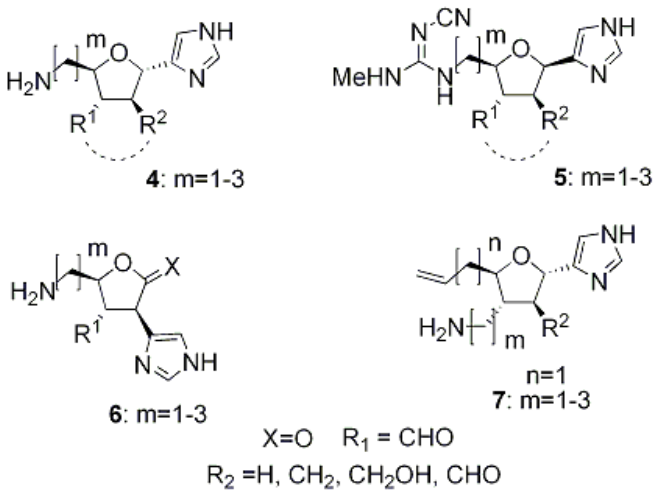

Fig. 16

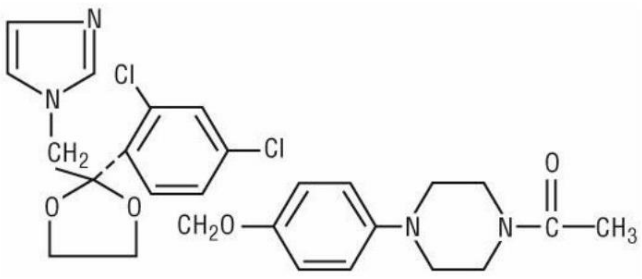

Fig. 17

DISCUSSIONS

The focus of this study was to find out whether the anti-dandruff compounds were present in the *L. innermis* (Henna plant) or not and if present then to identify, extract and characterize these anti-dandruff compounds from the same. Anti-dandruff compounds are active anti-fungal chemical compounds, which

inhibit the growth of dandruff causing organism *M. furfur.*

It is known that *M. furfur,* a lipophilic, dimorphic and yeast-like fungus, occurring on human skin as an opportunistic pathogen, causes diseases such as dandruff, pityriasis, versicolar, seborrheic dermatitis etc as discussed by Saravana Kumar and Vijayalakshmi (2005). Mycological medium was developed for primary isolation and culture of such lipophilic yeasts and suitable medium were standardized for culturing the organism by Chua et al. 2005. Growth of the fungus was also determined in the presence of different carbon sources under the influence of different temperature, pH and salinity by Saravana Kumar and Vijayalakshmi (2005). In the current study *M. furfur* was isolated from the collected dandruff sample from the scalps of the subjects and as MTCC 1765 Strain. The samples were cultured on various media and thus the media was standardized to get the proper isolates of the yeast.

Ketoconazole is an imidazole antifungal agent, imidazoles have five-membered ring structures containing two nitrogen atoms and ketoconazole is a highly lipophilic compound. Christensen's Urea and CLSI broth method were used for testing susceptibilities of six *Malassezia* Species to Voriconazole, Itraconazole, and Ketoconazole. The MIC range (µg/ml) of Voriconazole, Itraconazole and Ketoconazole against *M. furfur* CBS 7019b were found to be 0.125–0.5, 0.125–0.25 and 0.125–0.5. The in vitro activities of ketoconazole, econazole, miconazole, and tea tree oil against 54 *Malassezia* isolates were determined by agar and broth dilution methods contraindicated by Wei Zheng and Wang (2001). Ketoconazole was found to be more active than both econazole and miconazole stated by Hammer et al. 2000. In the current study ketoconazole was used as standard anti-dandruff compound to check its efficacy against *M. furfur* and the MIC of ketoconazole was found to be <80µg/ml

The anti-microbial activity of *L. inermis* was done against most gram positive and gram negative bacteria by Mickelsen et al. 1998. This anti-microbial substance is highly soluble in water, partially-soluble in 70% ethyl alcohol and heat-stable. In the current study the anti-fungal activity of henna plant was shown and this anti-fungal agent was insoluble in water, partially soluble in mid-polar solvent (60% Hexane) and fully soluble in non-polar solvent like ethyl acetate. Clear zone of inhibition was observed in case of ethyl acetate extract of dried henna leaves with disc diffusion method.

The partial fractionation of extracted compounds was done through Thin Layer Chromatography (TLC) Analysis. Various extracts were run along with the standard in different solvent systems. The result showed the presence of standard like compound in the ethyl acetate extract of henna leaves when viewed under UV Trans-illuminator. Thus, the crude ethyl acetate extract of henna leaves showed the presence of imidazole like antidandruff compounds by Mayser et al. 1996.

The ^{1}H-NMR spectroscopy of the crude extract indicates that in one measurement broad information of the metabolic profile in the lipophilic fraction of a plant tissue were resolved reported by Tajima (2005). Both ^{1}H-NMR spectroscopy and ^{13}C- NMR spectroscopy was performed with the ethyl acetate extracted sample so as to characterize the antidandruff compound.

From the NMR Analysis, extracted compound was found to be imidazole. The expected structure of the compound showed the resemblance with the ketoconazole. Thus it is interpreted that ketoconazole-like anti-dandruff compound was present in the ethyl acetate extract of henna leaves. From the proton integration values, it is seen that 2-3 % of the expected product has been found in the crude ethyl acetate extract of dried Henna leaves.

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