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Study of Inhibitory Effect of Plant Extracts on Lipolytic Enzymes Secreted By *Malassezia furfur*

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ABSTRACT: *Malassezia fungus yeast has many species as constituent of human scalp dandruff. But Malassezia furfur is predominant in the locally observed infected scalp of select individuals. The species survives effectively due to the organism's ability to secret extracellular lipolytic enzymes degrading oily substrate on the scalp. The present experiment verified this fact in in-vitro condition and attempted to inhibit the enzyme activity by application of select plant extracts (nine) through the assay medium. The collected enzyme lipase of M. furfur, was qualitatively, morphologically and biochemically characterized, purified and confirmed through protein estimation of the crude extract, dialysis of the content, column chromatography fractionation and finally by SDS PAGE in sequence to study the objectives. Among nine medicinal plant extracts tested for inhibitory effect on lipase activity, the percent inhibition was found maximum by the extract of Piper longum (Linn.) as 35.32%, followed by Ficus benghalensis (Linn.) aerial roots extract = 32.18%, and Phyllanthus emblica (Linn.) = 26.62%. These inhibitory effects of plant extracts open up the new possibility to control malassezial effect on human scalp through newer and safe herbal formulations over chemical therapeutics.*

Key words *– Malassezia, Dandruff, Lipase, Medicinal plants, Inhibitory assay*

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I. Introduction

There are more than 300,000 species of fungi found all over the world, of which some 200 species have been made known to cause infections in humans. All pathogenic fungi have one/other feature in common (Kerridge et al., 2012). These human pathogens range from disfiguring dermatophytic fungi to fungi responsible for life threatening systemic mycoses. Such organisms are normally harmless but capable of causing life-threatening diseases in the compromised host i.e. one, where the normal defense mechanism is impaired or the microenvironment in which the fungus can grow is altered (Bossche et al., 1993). The most important fungal pathogens linked to human health are *Aspergillus fumigatus, Aspergillus niger, Candida albicans* and *Malassezia furfur* and have been responsible for the upsurge in research interest. *Malassezia* fungus is an exception because it occurs as opportunistic yeast in humans as well as in other animals. *M. furfur* is of significant importance for its action on human scalp causing dandruff and responsible for hair disorders. Present day youth and old are equally concerned for their self-esteem due to untimely hair loss. It is clinically observed that approximately 75% of women are suffering from hair disorders at some stage in their lifetime (Stöppler, 2017).

Phospolipases are the group of enzymes that hydrolyze one or more ester bonds of phospholipids to liberate fatty acid and lysophospholipids, which serve as precursors for a diversity of bioactive lipid intermediaries (Prohic, 2016). Phospholipids and proteins are the key chemical components of the cellular membranes of the host. The phospholipases and proteases are concerned with the destruction progression of such cellular membranes. But these two enzymes have distinct biological roles (Pini et al., 2011). All lipases and esterase boast the catalytic triad Ser–Asp (Glu)–His, where serine is frequently used as a component of the highly preserved pentapeptide Gly–X–Ser–X–Gly. At some stage of infection, lipases may interfere with phagocytosis, indicating a direct involvement of lipase in pathogenesis (Lowe et al., 1998). *Malassezia* is having species which are lipid dependant and some other species are lipid independent making this organism unique and opportunistic. The ability of this organism to hydrolyze lipid and lipophilic form is often considered as an important virulence attribute, but the evidence is not historic and over highlighting. The morphological and biochemical changes that occur during culturing were not clearly understood, so molecular study of the yeast induced lipase to reduce lipid substrate is a mystification in the literature associated with scalp infection. It is therefore a need to correctly understand the lipolytic enzyme activity of lipid dependant species like *Malassezia furfur.* Furthermore, effort to inhibit lipolytic enzyme action by medicinal plant extracts than chemical treatments is necessary to check the spread of dandruff, the root cause of hair disorders at unnatural age groups

from human health care prospects. The present paper systematically attempts to these objectives and opens the future possibility of controlling malassezial menace through herbal remedy.

II. Materials And Methods

In order to progress in the defined objectives of this piece of research, the following methods and materials were used as per requirements.

Chemicals

Tween 20, 40, 60, 80, Cremophore, Tributyrin (Glycerol tributyrate), Triton X-100 and DEAE-Cellulose were purchased from Himedia Laboratories Pvt. Ltd. and Olive oil from Fisher Inorganic and Aromatics Ltd., Mumbai.

Yeast strains and media

Malassezia furfur strain CRS-39 was used in this study. This strain was grown on yeast extract peptone dextrose (YEPD) agar medium (1% yeast extract, 2 % peptone, 2 % glucose/dextrose, 2.5 % agar) as per Juntachai (2009) supplemented with 0.1% of Tween 20,40,60,80 and cremophore along with 1% olive oil at 32^0 C for 7 days.

Culture conditions

5ml of the yeast was precultured for 7 days in the YEPD media at 32°C and 120 r.p.m. shaker in 100 ml flasks and maintained in the research laboratory, COCSIT Biotechnology research centre, Latur.

Lipolytic enzyme activity of *Malassezia furfur* CRS-39

To observe lipolytic enzyme activity of *Malassezia furfur*, optimized YEPD agar and Tributyrin agar plate supplemented with Tween 20, 40, 60, 80 and cremophor EL were used. The inoculated plates were kept for incubation at 32^0C and results were noted daily from day 3 to 15 days. The formation of precipitation zones around the colony was considered positive for lipolytic activity (Annemarie, 1992).

Lipase enzyme (EC 3.1.1.3) assay

The lipase enzyme assay was performed with titrimetric method as per Sigma Aldrich ACS Specification (1993).

Extraction and Purification of Lipase

The culture supernatant (100 ml) was obtained by centrifugation of the culture broth at 10,000 rpm at 4^0C for 15 min. The supernatant was subjected to ammonium sulfate salt precipitation with constant stirring to bring the saturation to 40 % with overnight incubation at 4^0C and the precipitate was removed by centrifugation at 10,000 rpm and lipase activity of precipitate was determined. The supernatant containing lipase was precipitated again with ammonium sulfate to bring 80 % saturation. The mixture was left overnight by constant stirring with magnetic stirrer and re-centrifuged at 10,000 rpm for 15 minutes at 4°C. The precipitate was collected and dissolved in 0.2 M Tris-HCl buffer and was dialyzed against distilled water with continuous stirring for 24 hours. Further purification of lipase thus collected was done by column chromatography. The dialyzed enzymatic fractions were applied to DEAE-cellulose chromatography (column size 1.5 x 25 cm). The column was previously equilibrated with Tris-HCl buffer pH 7.2. The fractions were eluted at a flow rate of 20 ml/ hour with elution buffer (Tris-HCl buffer pH 7.2). The fraction content subjected to positive lipase activity was again applied to DEAE-Cellulose matrix column (Bangalore Genei). The column was previously equilibrated with 50mM phosphate buffer pH 7.0. The enzyme bound to the column was eluted with a linear gradient of 25 mM phosphate buffer containing 0.1-0.5 M $NaCl_2$ at a flow rate of 10 ml/hour. 2ml of each fraction was collected and checked for enzyme activity (Yasuhide, 2014). The active fraction was collected and characterized for molecular confirmation of lipase enzyme by SDS-PAGE 12% polyacrylamide gel (Gallagher, 2012).

Estimation of protein

Protein content was estimated (as per Lowry et al., 1951) at different stages of purification of the culture supernatant to know the concentration of the enzyme using BSA as the standard.

Effect of pH on lipase

In order to study the effect of pH on lipase activity, various pH ranges i.e. from pH 6.0-12.0 was made by adjusting addition of 0.1 M hydrochloric acid and 0.1 M sodium hydroxide to achieve acidity and alkalinity respectively. Then the effect of pH was measured using the standard assay conditions (Behal et al., 2006 and Yasuhide et al., 2014).

Effect of Temperature on lipase

The stability of lipase was determined at a temperature range from 10-80^0C at pH 8 using 0.2 M Tris-HCl buffer (Ghaima et al., 2014). Enzyme stability was performed by incubating lipase at different temperatures for 30 min. and then the effect was measured using the standard assay conditions (Ota et al., 2014).

Effect of substrate concentration on lipase

The effect of substrate concentration on lipase activity was carried out according to the method of Ghaima et al., (2014). Different substrate concentration range from 0.1-1ml on the activity of lipase was carried out. Further maximum and optimum activities of lipase with stability were also observed.

Effect of incubation time on activity of lipase

The effect of incubation time interval for optimum and maximum activity of the enzyme was conducted according to the method of (Ghaima et al., 2014). The activity was determined between the ranges of 5-60 minutes at pH 8.0. Phenol red was used as indicator (Behal et al., 2006).

Determination of molecular weight of lipase by SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of lipase was performed. The experiment was conducted using a 12% separating gel and 3% stacking gel under non-reducing conditions. Purified protein was concentrated by steam evaporator at temperature 0-4^{0}C. After evaporation of the solvent, dried concentrated protein was obtained. 50µl protein with Tris HCL buffer was used to run the sample. The standard protein molecular weight marker was run along with the enzyme samples. Electrophoresis was stopped when the tracking dye (Bromophenol blue) reached the bottom of the gel. The proteins in the gel were stained with Coomassie brilliant blue R-250 and de-stained with a solution containing 5% methanol and 7% acetic acid (Laemmli, 1970 and Sean, 2012).

Plants and extraction of crude drugs

Nine medicinal plants chosen for the study were based on ethno botanical survey with description of their predictable uses and reported bioactivities (Sahib, 2011) and found mentioned in Sanskrit medical text of *Mādhava cikitsā* (Bhavarlal Dugad, 1976) and in the content statements of marketed hair management products. These were *Piper longum* Linn. *Tribulus Terrestris* Linn., *Withania somnifera* Dunal., *Hibiscus rosa-sinensis* Linn., *Jasminum officinale* Linn., *Phyllanthus emblica* Linn., *Ficus benghalensis* Linn., *Curcuma longa* Linn. and *Terminalia chebula* Gaertn. The crude drug part extract of each plant was made by different solvents (Water, Methanol, Ethanol, Hydroalcohol, Ethyl acetate) in Soxhlet extraction procedure (Survase et al., 2013).

Lipase Inhibitory Assay

The inhibitory lipase assay activity was conducted with modified procedure of Sharma et al. (2010). A control was prepared using the same procedure replacing the plant extract with distilled water. The lipase activity was determined qualitatively by observing the change in color from yellow to pink using phenol red as indicator. The inhibitory activity was further measured at the absorbance 410 nm (Kim et al., 2005) The results were calculated for Lipase inhibitory activity as percent inhibition in the following formula (McCue et al., 2004 and Kazeem et al., 2013).

%Inhibition= [Abscontrol−Absextracts /Abscontrol] ×100

III. Results

The experiments conducted as per the materials and methods with the objectives kept in mind in this piece of research, following results were recorded and expressed in graphs or tables.

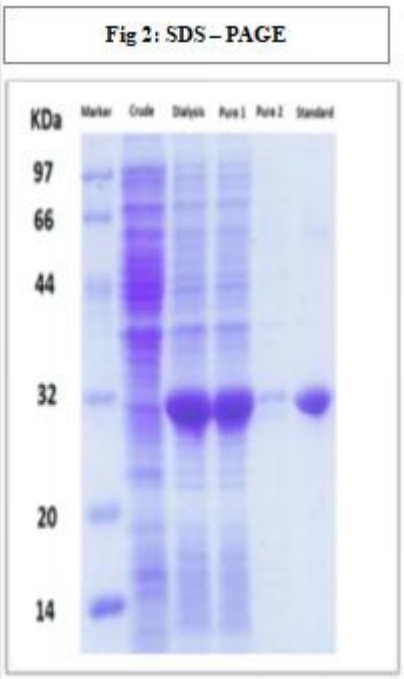
Table 1: Estimation of Lipase protein from *Malassezia furfur* in optimized medium

Protein concentration of Malassezial lipase	Crude	After Dialysis	Column purified
	3.14 µg/ml	1.90 µg/ml	0.685 µg/ml

In order to produce sufficient amount of lipolytic enzyme by *Malassezia furfur*, the optimized media defined in the process was found to be most effective.

The qualitative test conducted using assay method confirmed positive for lipase enzyme produced by the organism. Simultaneously, morphological character like precipitation zones around the colony area was also observed on the culture media (Fig. 1).

Further characterization of lipase conducted through protein estimation of the crude extract, dialysis of the content, column chromatography fractionation (Table 1) and finally by SDS PAGE in sequence confirmed band with control and standard marker shows presence of 32 kDa lipase (Fig. 2).



The study to get best or maximum performance of lipolytic enzyme by *M. furfur* on a range of pH, temperature and incubation period, revealed that the optimum pH was 8; the optimum incubation time was 30 min.; and at temp 40^0C, the enzyme activity was more over other ranges in a optimum substrate concentration at 0.6ml for the assay with 20µl of enzyme in 1920 µl of total volume (Graph A-D).

The inhibitory effect studies on the activity of lipase using extracts of nine mentioned plants individually are presented in Fig 3 and Fig 4. The percent inhibition activity was found (Table 2) maximum by the extract of *Piper longum* (Linn.) as 35.32%, followed by *Ficus benghalensis* (Linn.) aerial roots extract = 32.18%, and *Phyllanthus emblica* (Linn.) 26.62% (with minimum standard deviations).

Graph A-D: Effect of pH, temperature, incubation time and substrate concentration on lipase activities

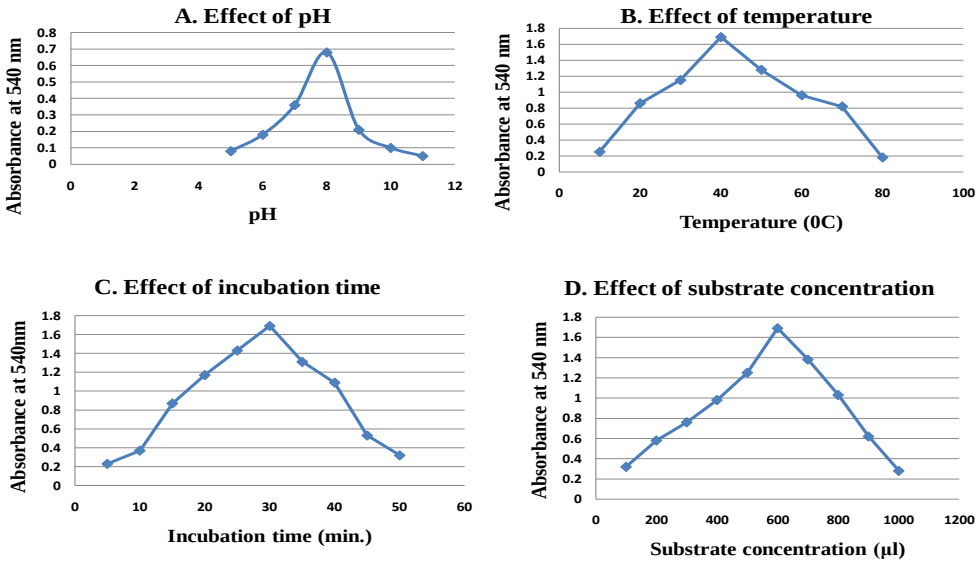


Table 2: Inhibitory effect of different medicinal plants on lipase (Msp) of *Malassezia furfur*

Plants name	Family	Plant part	Type of solvent	Absorbance at 410 nm (Mean±SD)	% Inhibition
Piper longum **(Linn.)**	Piperaceae	Fruits	Methanol	1.093±0.011	35.32%
Tribulus terrestris **(Linn.)**	Zygophyllaceae	Fruits	Water	1.656±0.057	2.36%
Withania somnifera **(Dunal.)**	Solanaceae	Root	Hydroalcoholic	1.513±0.011	10.65%
Hibiscus rosa-sinensis **(Linn.)**	Malvaceae	Flower	Ethanol	1.466±0.041	13.60%
Jasminum officinale **(Linn.)**	Oleaceae	Flower	Ethanol	1.500±0.022	11.24%
Phyllanthus emblica **(Linn.)**	Euphorbiaceae	Fruits	Water	1.246±0.046	26.62%
Ficus benghalensis **(Linn.)**	Moraceae	Aerial root	Ethanol	1.146±0.041	32.18%
Curcuma longa **(Linn.)**	Zingerberaceae	Rhizomes	Ethyl acetate	1.680±0.017	0.59%
Terminalia chebula (Gaertn.)	Combretaceae	Fruits	Water	1.680±0.005	0.59%

Fig 3: Lipase enzyme inhibition assay

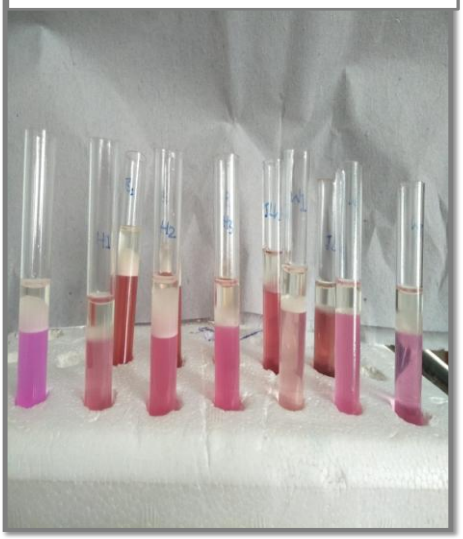
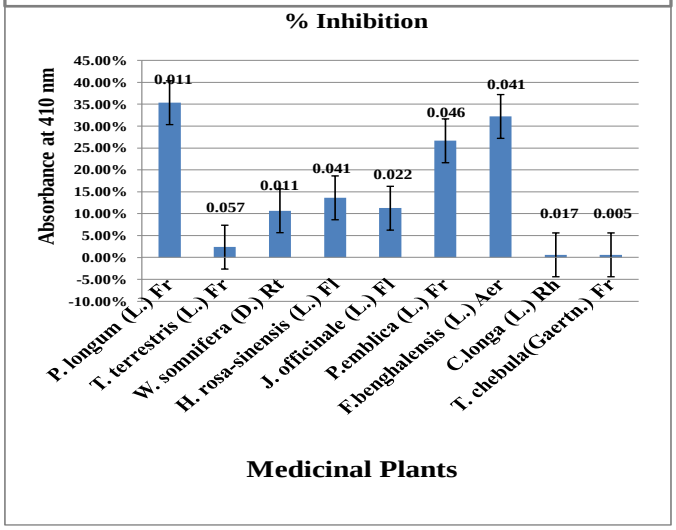


Fig 4: Inhibitory effect of plant extracts on lipase



IV. Discussion:

Now a day, hair loss is a major problem with prime age and genetically predisposed individuals. As per clinical study and survey, 16% men between 18-29 yrs., 50% females below 50 yrs and 53% men between 40-49 yrs are most commonly affected with hair disorder problem. Malassezial activity on human scalp is an identified cause of hair disorder. Its growth has been medically controlled by use of antifungal chemicals prescribed by modern medical practitioners or by herbal products that are explained in the Ayurvedic preparations. According to Tsujita (1996), presence of lipolytic enzymes produced by *M. furfur* was observed by the hydrolysis of tween opaque medium and the liberated fatty acids appear as insoluble precipitation zone around the inoculated colonies. Malassezial lipase was also produced *in-vitro* first time. DeAngelis (2007) had given the expression of lipase gene LIP1.

Barret-Bee (1985) correlated the production of phospholipase from *Candida albicans* with its pathogenic nature, signifying that isolates with a high pathogenic prospective. Gabriella (2011) stated that the

phospholipase activity of *Malassezia* sps. take part in the initiation of skin lesions, particularly in case of pityriasis versicolor (PV), although phospholipases should be measured as only one of the many factors involved in the complex interaction between the yeast and its host leading to the expansion of skin lesions. According to Sparber (2017), *Malassezia* species show lipid-dependency and lipolytic enzymes, such as lipases are required for the organism to obtain fatty acids from the surroundings. *Malassezia* may also have beneficial as well as harmful effects for the host. Recently, Li et al. (2017), reported that *Malassezia globosa* has capability to secrete an aspartyl protease (MgSAP1) that could inhibit *Staphylococcus aureus* biofilm formation. This biofilm formation is a virulence attribute of *S. aureus*. The results of Johansson (2018) present an increased understanding of the nature of MalaEx, which seem to be morphological diverse, enriched with certain proteins, and have the ability to interact with skin cells. These results give us further clues for understanding more about host-microbe interactions in the sensitization.

As per literature, it has been observed that many medicinal plants are having inhibitory effects on lipolytic enzyme activity produced by different fungal species (Muhammad et al., 2013). All the plants studied in the project for inhibition assay of lipase are mainly used in hair and skin disorders as part of oils, shampoos, conditioners, creams and gels, etc. But first time, the studied nine plants are reported for the inhibitory effects on *Malassezia furfur*'s lipolytic enzyme. Lai et al. (2014) screened lipase inhibitory activity of methanolic extracts of different parts of 32 selected medicinal plants in Malaysia against porcine pancreatic lipase and p-nitrophenyl butyrate in an *in-vitro* assay. Among the 32 extracts, 27 crude extracts showed evidence of inhibitory activity against porcine pancreatic lipase. Toma et al. (2014) explored the inhibitory activity of the ethanolic extract of leaf of *Moringa stenopetala* on pancreatic lipase using spectrophotometric assay. The plant extract to some extent inhibited pancreatic lipase with IC_{50} value of more than 5 mg/ml. Ekanem et al. (2007) determined the inhibition of pancreatic lipase of ethanolic extracts of *Aframomum melegueta* (seeds) and *Spilanthes acmella* (flower buds) at different concentrations. Kumar et al. (2013) screened lipase inhibitory activity of different parts of thirty three medicinal plants from India (n-hexane, dichloromethane, methanol, and ethyl acetate extracts) *in vitro*.

During the present study, it was found that the optimum pH for lipase activity was 8 but there was no activity detected when the reactions were performed at pH 5 - 6. At pH 9 -11, the substrate was unstable. The optimum temperature was 40^0C for lipase action but no activity was detected at temperature below 20^0C and above 50^0C. The optimum incubation time was 30 min. and the enzyme activity was more over other ranges in an optimum substrate concentration at 600 µl for the assay with 20ul of enzyme in 1920 µl of total volume (Graph A-D). The percent inhibition activity of select medicinal plants was found (Table 2) maximum by the extract of *Piper longum* (Linn.) in methnol as 35.32%, followed by *Ficus benghalensis* (Linn.) aerial roots extract in ethanol = 32.18%, and *Phyllanthus emblica* (Linn.) extract in water = 26.62%. Plotkin et al. (1996) have reported in the characterization of lipase activity extracted from *Malassezia furfur*, similar observations about the optimum enzyme activity w.r.t. to pH and different cations. They opined that lipase activity of *M. furfur* is dependent on the pH of the environment and it is affected by cations present in the medium. Some studies showed inhibitory effects of different medicinal plant extracts on lipase activity and have remarked that such plants are potential and convenient sources of anti-obesity agents (Muhammad et al., 2013). Our findings of optimum conditions and inhibitory effects of nine medicinal plants on malassezial lipolytic enzymes (Lipase) produced from dandruff isolate could be useful in herbal drug designing and formulation of herbal products related to hair disorder management.

V. Conclusion

The results of this study signify the possibility of different medicinal plants to be considered for the improvement of herbal formulations against scalp infections caused by *M. furfur*. With the increasing interest for gaining acceptance over synthetic drugs as therapeutic agents, the outcome of this research provides scientific justification of traditional uses of these nine plants against lipolytic enzyme of *M. furfur*.

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