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Research Article

ISOLATION AND MOLECULAR CHARACTERIZATION OF THE DANDRUFF SAMPLE AND ITS INHIBITION BY MEDICINAL PLANTS

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ABSTRACT

With dandruff being a common everyday problem and the market loaded with antidandruff shampoos and such skin care products; it is obvious to assume resourceful research into this area. Medicinal plants have some natural Antimicrobial property and therefore such combination could be a potential antidandruff activity. To check its antidandruff activity, experiments have been conducted on *Malassezia furfur* the causal organism for seborrheaic dermatitis or dandruff, which has been cultured for such study in our lab and its molecular characterization, is done by Agarose gel electrophoresis method. *Malassezia* spp. is lipophilic unipolar yeasts recognized as commensals of skin that may be pathogenic under certain conditions. The medicinal plants are natural and it has no or fewer side effects. Through synthetic and semi synthetic drugs are available in today's market; there is need for new ones from natural origin like phytoconstituents. Spices are the natural constituent who has contained more amounts of chemical constituents is helpful for antimicrobial or antifungal activity.

Keywords: Antidandruff activity, *Malassezia*, Agarose gel electrophoresis, Phytoconsituents

INTRODUCTION

Dandruff is a common scalp disorder affecting almost half of the population at the pre- pubertal age and of any gender and ethnicity. Dead skin cells are shed in large oily clumps, which appear as white or greyish patches on the scalp, skin and clothes is called dandruff.[1-6]There are some factors for dandruff formation: Oily skin due to sebum or sebaceous secretion.[7-8] There are different types of *Malassezia* yeast example-*M. dermatis,M. furfur,M. globosa,M. japonica,M. nana,M. obtusa,M. pachydermatis,M. restricta,M. slooffiae,M. sympodialis. Malassezia* is a genus of fungi. *Malassezia* colonies grow rapidly and mature in 5 days at 30 to 37^{0}c.*M.furfur* colonies are creamy yellow brown and red.[9-12]The lipase gene which have an important role in dandruff formation. Which is an extracellular enzyme degraded the long fatty acid chains and cause dandruff and other skin diseases. It is also a facultative pathogen, associated with a wide range of skin diseases. It is also a facultative pathogen, associated with a wide range of skin diseases. It has been associated with various diseases ranging from the pigmentation disorder pityriasis vesicular to atopic dermatitis .[13-19]The outermost layer of the complex cell wall consists mainly of lipids which are thought to be involved in the pathogenesis of this fungus. The lipase enzyme necessary for these activities can be considered as virulence factors. Lipases catalyse the hydrolysis of the ester bonds of triacylglycerol, thereby releasing free fatty acids.[20-24]

Many plants synthesize substances that are useful to control the growth of microorganisms and plants are the possible source of antimicrobiaagents. Through synthetic and semi synthetic drugs are available in today's market, there is need for new ones from natural origin like phytoconstituents. Spices are the natural constituent who has contained more amounts of chemical constituents is helpful for antimicrobial or antifungal activity. The major chemical compounds which are present in the spices are flavonoids, tanins, saponins, terpinoids and alkaloids. These are for helpful for antifungal and antibacterial activity.[25-29]The DNA of the Malassezia sample which can identify its molecular characterization. The advance technology for this phylogenetic analysis is 16s rRNA study through PCR method.

MATERIALS AND METHODS

The following samples are collected in the present study; the details of the sample collection are presented below:

Dixon's agar

Firstly a solution of following ingredients with 1000ml of H_2O is prepared. The ingredients are (a) Malt extract 30.0g (b) Peptone 6.0g (c) Oxible/beef extract 20g (d)Glycerol 2.0g (e) Tween-80 10.0ml and (f) Agar 20.0g. Samples were collected by scraping the lesions of patients and stored in sterile containers in refrigerator until used. The plates were incubated at 37^{0}C for 3-5 days.

Identification and characterization of culture

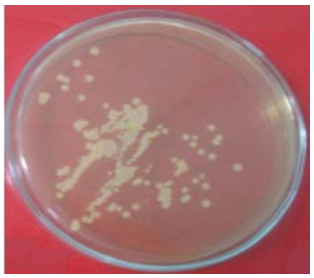

Fig. 1: Culture of dandruff sample after incubation

Culture broth of the organism

Here we are using the Dixon's broth. There are several common formulations of Dixon's broth. Although they are different and the ingredients are used to promote growth, here the solution with 1000ml H_2O including the following i.e. Malt extract 30.0g, Peptone 6.0g, Oxible/beef extract 20g,Glycerol 2.0g and Tween 80- 10.0ml is prepared and is being Sterilize by autoclaving at 121^{0}C.

Extraction of Fungal DNA

Lysis buffer This is prepared using 2% Triton-x-100, 1% SDS, 100mM NaCl, 100mMTris-HCl (p^H-8), 1mM EDTA and Distilled water. The stepwise methodology for extraction of DNA is presented below,

Transfer 15ml liquid culture of yeast grown for 20-24h at 37^{0}c in Dixon broth into a centrifuge tube. Pellet cells by centrifuge at 12,000 rpm for 10-15 min. Add 2ml of lysis buffer to that pellet. Immerse tubes in ice bath for 10min.Transfer to the tube in 95^{0}c water bath for 5min.Repeat the last two steps. Vertex 2mints to that sample. Add 2ml of chloroform and vertex for 5min.Centrifuge 15min at room temp. For 12,000rpm.Transfer the upper aqueous phase to a centrifuge tube containing 5ml ice cold 100% ethanol. Mix by inversion or gentle vertexing Incubate at RT, 10 min. Centrifuge at 12,000 for 15 min. Remove the supernatant with pasture pipette. Wash the pellet with 5ml in 70% ethanol. Centrifuge 15' at RT for 12,000 rpm. Remove supernatant from the centrifuge tube. Air dries the pellet at RT or 5' in 60^{0}c in a vacuum dryer. Suspended in 200µl TE (p^H-8) or water.

Agarose Gel Electrophoresis

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products. To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product this method is useful.[30]

The following materials are considered for the experiment i.e. TAE buffer :- (p^H-8.0) which contains 40mM Tris-48.5gm,1mM EDTA-6.37gm,Acetic acid-11.42gm and distilled water-1000ml. For the Gel loading buffer, we have used Bromophenolblue (BPB)-0.25%, Xylenecynol-0.25%, Glycerol/glycerin-50%, Distilled water-100ml. Similarly the TE buffer 10mM Tris HCl (p^H-8),1mm EDTA (p^H-8) is used for 1000ml solution where as for 500ml, we have used 1M Tris-HCl-5ml,0.5M EDTA -1ml added with d/w up to 500ml.

In the next phase the electrophoresis protocol of Agarose gel is prepared and the stepwise methods are presented below,

Weigh 0.6gm agarose powder and add it to a 250ml conical flask. Add 60ml TAE buffer to the flask with that 0.6gm agar. Melt the agarose in a microwave or hot water bath until the solution become clear. Let the solution allow cooling about 50 to 55^{0}C under running top water. Then add two drops of Ethidium bromide by the capillary tube. Mix well the gel. Pour the gel inside the casting tray. Place the comb in the gel casting tray. Allow it for solidify. Carefully pull out the combs and remove the tape. Place the gel in the electrophoresis chamber. Add enough TAE buffer so that there is about 2 to 3mm of buffer over the gel.

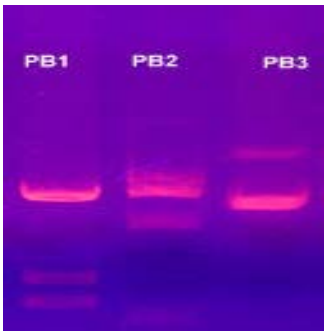

Fig. 2: Identification of DNA bands through UV transilluminator

Next the gel is loaded by adding5µl of 3xdye with 2µl of extracted DNA and by carefully pipette 7µl of each sample. Then the gel is allowing it for running at 100v for 45' to 1 hour. Finally when we view under UV transilluminator, the sample looks like orange color and the sample is presented in figure 3 below. After this stage generally the sample is used for PCR analysis for 16s rRNA study which will can be done for further analysis.

Phytochemical Analysis

In the final stage the phytochemical analysis has been carried out and the step wise descriptions are explained below:

Material collection and extraction

Different spices were collected, ground into powder and passed through 60 meshes. About 5g of each powder added in 10ml of methanol solvent and kept it for 48 hours at room temperature. The extracts were filtered through Whatman No.1 filter paper. This extracts were tested for the presence of active chemical compounds by the following the methods described by **Trease and Evans (1989)**. The different tests i.e. from test are carried out and the details about the test are presented.[31]

Tests for Tannins

One millilitre of the extract was added with 5ml of distilled water and kept for boiling in hot water bath. After boiling, sample was cooled down and to these two to four drops of 0.1% ferric solution was added. Appearance of brownish green or blue black coloration confirms the presence of tannins.

Test for Phlobatanins

One percentage of HCl was added to the extract (1ml) and boiled in hot water bath. Formation of red precipitation indicates indicates the presence of phlobatanins.

Test for Saponins

One millilitre of the extract was taken in a test tube and distilled water (2ml) was added to it. The test tube was then kept in boiling water bath for boiling and was shaken vigorously. Existence of froth formation persisted for next one hour confirms the presence of saponins.

Test for Flavonoids (Ammonia test)

One millilitre of the extract was taken in the test tube and ammonia solution was added (1:5)followed by the addition of conc. sulphuric acid. Appearnce of yellow colour and its disappearance on standing indicates the positive test for flavonoids. Here the ammonia is kept into the refrigerator for 30mints before use.

Test for Terpinoids

Five millilitre of extract was taken in a test tube and 2ml of chloroform was added to it followed by the addition of 3ml of conc. Sulphuric acid formation of reddish brown layer at the junction of two solutions confirms the presence of terpinoids.

Test for Glycosides

Five millilitre of each extract was added with 2ml of glacial acetic acid which was followed by the addition of 2ml glacial acetic acid, 1drop of ferric chloride solution and 1ml of conc. sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Test for alkaloids (Hager's test)

The0.5ml of the extract. Few drops of 0.1% picric acid were added. Formation of the yellow colour which indicates the presence of the alkaloids.

Test for phenols (Ferric chloride test)

The0.5ml of the extract was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green colour indicates the presence of the phenolic compounds.

Test for steroids

Two millilitre of acetic acid was added to 0.5ml of the extract and then added 2ml of H_2SO_4 change of colour form violet to blue or green indicate the presence of steroids.

In the present analysis mainly we considered 7 spices (medicinal) plants and they are Pepper, Cardamom, Clove, Cinnamon, Fennel, Star Anise, and stone flower. Table 7 presents the presence (+) or absence (-) of different compounds using the spices in different tastings and it is found that in Star Anise the availability of the chemical compound is more followed by stone flower as they have more + in the (table 1).

RESULTS AND DISCUSSION

After the isolation of DNA from dandruff sample and running through Agarose gel electrophoresis, it was confirmed by the orange colour florescence formation under the UV transilluminator. Then by using molecular marker we can further study about the phylogenetic analysis of dandruff sample i.e. *(Malassezia furfur)* by PCR method.

Antidandruff assay

For the study of antifungal activity, it was find out that the more zone of inhibition was shown by stone flower (i.e.34mm, 36mm, 30mm) against different sample followed by clove, cinnamon and fennel. Before this we did phytochemical study to know about which compound present in the different samples. It is concluded that we can widely use the stone flower for the antidandruff activity against *Malassezia* species and it is cheap and less side effect due to its herbal property.

Table 1: Phytochemical analysis of different spices samples

Phytochemical	SAMPLE (Spices)						
	Pepper	Cardamom	Clove	Cinnamon	Fennel	Star Anise	Stone flower
ALKALOIDS	+	−	+	−	−	+	+
FLAVONOIDS	−	−	−	−	−	−	+
GLYCOSIDES	−	−	−	−	−	−	−
PHENOLS	−	−	−	−	−	+	−
PHILOBATANINS	−	−	−	+	−	+	−
SAPONINS	−	−	−	−	−	−	+
TANINS	−	+	+	+	−	+	−
STEROIDS	−	−	−	−	−	−	−
TERPINOIDS	+	+	+	+	+	+	+

+ = presence; - = absence

Table 2: Comparison of zone of inhibition for 3 samples (PB1, PB2 and PB3) with respect to 7 types of different spices

Spices	Zone of inhibition (mm) for sample		
	PB1	PB2	PB3
*Syzygium aromaticum(*Clove)	20±1	21±1	23±1
Cinnamomum verum (Cinnamon)	16±1	20±1	22±1
Foeniculum vulgare (Fennel)	16±1	10±1	14±1
Elletaria cardamomum (Cardamom)	4±1	8±1	10±1
Didymocarpus pedicellatus (Stone flower)	34±1	36±1	30±1
Pimpinella anisum (Star anise)	2±1	2±1	6±1
Piper nigrum (Pepper)	2±1	4±1	7±1

Generally Medicinal plants have the Antimicrobial property and their combination is found to be a potential parameter to control the antidandruff activity in human. In the present study these conclusions are inferred using the various samples and rigorous laboratory experiments. The important point is that as the medicinal plants are natural and having fewer side effects compared to the chemical compounds so the usage of these plants for the removal or eradication of the dandruff in human is considered.

Lipids are essential for growth of most species of the genus *Malassezia*, it must be concluded that these fungi are able to hydrolyse lipids extracellular. It should also be noted that the lipase activity is at 37 ^{o}C.[13-19]Alkaloids, flavonoids, saponins and tannins were present as the major phytoconstituents in different spices. It is also observed that there was an increasing zone of inhibition with increasing concentration of extracts. The maximum inhibitory activity was shown by stone flower (i.e.34mm, 36mm and 30mm for PB1, PB2 and PB3 respectively).

It is concluded by these three types of experiment, the lipase gene which is mainly causing for dandruff activity can be inhibited by the drugs which is extracted by the spices i.e. *Didymocarpus pedicellatus* for the persons belonging from same region or different regions. In the several sample and spices combination the zone of inhibition is presented and still for some sample the zone is not so strong and significant, it is very challenging to optimise the spices for the maximum zone of inhibition by analysing more samples of different clusters comprising of different geographical area (like coastal or dessert), sex (male or female), age group, body parts etc.

After getting a huge database the same process can be modelled and the statistical significance tests and other data mining and gene modification approach needs to be incorporated for the better usage of the present study in future.

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