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Study on The Effect of Temperature, pH And Antifungal Activity of Fruit Peel Extracts on The Dandruff Causing Fungi

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Abstract

Dandruff is caused when scalp shed epidermal cells rapidly in the form of large clumps. Dandruff is a sebaceous gland or skin scaling disorder caused by *Malassezia*, a dimorphic fungus. The present study is to analyze the morphology and colony characteristics of dandruff causing fungi from different individuals and to monitor the growth of dandruff causing fungi by altering physical parameters such as pH and temperature. Plant extracts contain broad spectrum of bioactive compounds which are often referred to as vitamins, phytochemicals or phytoconstituents which have the ability to overcome the resistance in causative organism and are of great therapeutic use. Some phytochemicals such as phenolic acids help in the reduction of the adherence of organisms. Citrus fruit are rich in vitamin C which maintains the pH level of scalp and prevents itching and shedding of flakes. The anti-dandruff and anti-fungal property of citrus fruit peel extract against dandruff causing fungi was examined. Among all the fruit peel extracts used, lemon peel extract was able to inhibit the fungi in all the concentrations. The anti-fungal compound was characterized.

Keywords

Anti-dandruff, Anti-fungal, Dandruff, Phytochemicals, Nuclear Magnetic Resonance

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INTRODUCTION

Dandruff is a common scalp disorder which is affecting half of the population, but it occurs more in males in comparison to females because male have a weaker scalp barrier and has more sebum production (50%) than women that makes them more prone to dandruff. Dandruff is caused when scalp shed epidermal cells rapidly in the form of large clumps. The skin of scalp regenerates itself once in a month. Mostly scalp sheds dead cells in an invisible way, but sometimes cell turnover is surprisingly faster and dead cells are shed as visible flakes called dandruff [1]. *Malassezia*are budding yeasts found on the skin of patients with dandruff[2].Seborrheic Dermatitis (SD) and dandruff

share many common features but differ only in their locality and severity that affect the seborrheic areas of the body.In clinical manifestation it is found that *Malasseziafurfur* plays an important role in dandruff and SD as an intensifying factor. It is said that these two types are identical whereas dandruff is mild non-inflammatory in comparison to SD [3].In comparison to SD, dandruff is more common and affects worldwide. Incidence approximately up to 50% of the general adult population. The incidence is seen at pubertal age and reaches peaks up to 20 years and becomes less prevalent at the age group of 50 years [4].

The cluster of dandruff scale is called as corneocytes which showed high degree of cohesion and dissociate from surface of substratum corneum. The size and number of scales are variable from one region to another [5].

Dandruff is a common skin disease with high level of sebum secretion. The role of sebum is closely associated with sebaceous gland activity. The causes of dandruff are also reliant upon three factors that are sebaceous gland secretions, microfloral metabolism and individual susceptibility[6]. In dandruff, *Malassezia* is common scalp yeast. By effective therapies used in pretreatment level reduces the load of *Malassezia* and re-colonization of *Malasezzia* whereas in post treatment it results in the reoccurrence of the condition. When *Malassezia* is secreted in conditions of dandruff, free fatty acids are released from sebaceous triglycerides by lipase activity; these fatty acids especially the unsaturated fatty acids induce inflammation and hyper proliferation. The rate of hair fall is high and common in case of dandruff. When the properties of hair was compared from the normal population it was found that the hair was more narrow, with more brittle surface and less shine in case of dandruff which may be due to the presence of *Malassezia*. Anti-dandruff shampoos containing anti-fungal activities reduce hair loss in androgenic alopecia populations, further supporting a potential involvement of *Malassezia*. Therapeutic considerations involve the use of active materials such as zinc pyrithione, ketoconazole and other azoles, selenium sulfide and piroctoneolamine which help to reduce hair fall and the occurrence of dandruff [7].

The aim of taking treatment is to prevent itching and burning sensation and also the recurrence of disease. There are wide options available for the treatment of dandruff. The primary treatment involves the use of antifungal and topical corticosteroids. Other therapeutic modalities which can be topically applied to soften and remove the thick hardened crusts are salicylic acid, zinc pyrithione, and coal tar. In severe conditions or when the patients don't respond to traditional topical therapies they take up oral medications like antifungal and retinoid [8].In day to day life cosmetic products are used to avoid the dandruff. The Food and Drug Administration (FDA) recommend agents that help to miniaturized *Malassezia* level (e.g. anti-fungal) or help in eradication of flakes (e.g. kerotolytic agents or combinations of anti-inflammatory and anti-proliferative agents with antimicrobials). Similar actions or methods are employed throughout the world to control dandruff associated flaking and pruritus.

There are so many natural effective remedies available in market to control dandruff. Instead of using antidandruff shampoos or commercial products, we can use variety of plants and fruits because they have ability to synthesize some substances which are useful to control the growth of causative organisms used, they can be possible source of antimicrobial agents [9]. Phytochemicals possess many ecological and physiological roles and they are widely distributed as plant constituents. Phytochemical constitutes alkaloids, cyanogenic glycosides, flavonoids, terpinoids and phenolic compounds these are not directly involved in the process of growth but act as detergents to insects and microbial attack; hence these compounds are called as secondary metabolites which plant produce as chemicals. Citrus plant synthesizes and accumulate in their cell as a large number of phytochemicals including low molecular phenolic such as hydroxyl benzoic and hydroxycinnamic acids, acetophenons, terpenoids, flavonoids, stilbenes and condensed tannins [10]. Flavonoids have the ability to modify the body's reaction to allergens, viruses and carcinogens. They also exhibit anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity. Flavonoids are known for their ability to enhance the effects of ascorbic acid. The anti-microbial activities of citrus fruits have been investigated and the extracts are useful in treating viral, bacterial and fungal diseases [11].

The present study is to concentrate more on plant extracts because they contain broad spectrum of bioactive compounds which are often referred to as vitamins, phytochemicals or phytoconstituents which have the ability to overcome the resistance in causative organism. The classes of phytochemicals are alkaloids, flavonoids, phenolics, saponins, tannins, terpenes, essential oil and steroids [12]. The use of plant extracts that contain phytochemicals are of great therapeutic use. Some phytochemicals such as phenolic acids help in the reduction of the adherence of organisms. Citrus fruit are rich in vitamin C which maintains the pH level of scalp and prevents itching and shedding of flakes. It also helps to reduce the dullness of hair and makes it look thicker and shinier [13].

Flakes or scales were collected from six individuals scalp and were streaked on different fungal media by altering the physical parameters such as pHand temperature to analyze the growth of dandruff causing fungus. Methanolic fruit peel extract of orange, lime, banana, chikoo was used to analyze the antifungal property against the dandruff causing fungus. The present investigation shows the antifungal property, zone of inhibition, of the methanolic fruit peel extract of lemon, orange, banana and chikoo against the dandruff causing fungus. The study was performed to check the growth of fungus at varied pH and temperatures. Among all the fruit peel extracts used, lemon peel extract was able to inhibit in all the concentrations. Dandruff was able to grow faster in incubator within 24 hours where as in room temperature the growth was observed after 2 days. It was observed that growth of dandruff causing fungus was less at pH below 4.5 and at pH above 7.0 the growth of dandruff causing fungus was found to be more prominent.

MATERIALS AND METHOD

Isolation of Dandruff Causing Fungus

Sample Collection

Flakes or scales were collected from six individual's scalps. Scrapping approximately one-inch area using a sterile cotton swab. The samples were streaked on Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), Czapekdox agar (CDA) and Dixon's agar (DA) in sterile Petri plates. The petriplates were then incubated at 35°C and 25°C for 24 hours.

Culture Medium and Inoculums

Inoculum was prepared by suspending loopfull of cultures in 15ml of broth and was incubated at 35°C and 25°C for 24 hours [14].

Catalase Test

Catalase test was performed to confirm the presence of *Malassezia* species. Except for *Malassezia restricta* which is catalase negative. A loopfull of culture was transferred on a clean glass slide using a sterile loop. A drop of 3% $H_2O_2$was placed on to the slide and mixed with a sterile loop. Active effervescence confirms positive result [1].

Physical parameters (Temperature, pH)

pH

The media was altered by changing the normal pH range as shown in **Table 1.**and then the media was inoculated and kept for incubation to observe the growth of dandruff causing fungus in different fungal media.

Table 1: Different pH range maintained

Sl.No	Media	pH range				
		Normal	Acidic		Basic	
1	DA	6.0±0.2	4.5±0.2	4.0±0.2	7.5± 2	8.0±0.2
2	SDA	5.6±0.2	4.4±0.2	3.6±0.2	7.1±0.2	7.6±0.2
3	PDA	5.6±0.2	4.4±0.2	3.6±0.2	7.1±0.2	7.6±0.2

Temperature

The inoculated media was kept at two different temperatures (35 °C as well as in normal room temperature 25 °C) for 24 hours to check the growth of dandruff causing fungus.

Preparation of Extract

Different fruit peels **(orange, lime, bananaand chikoo**) were collected, washed, cut into small pieces and dried in hot air oven at 50°C temperature for 2-3 days, which were observed regularly and was ground to a powder form by using an electric mixer grinder. About 20g of each powder was added in 40ml of methanol solvent

and kept for 48 hours at room temperature in a dark condition. The extracts were filtered through Whatman filter paper and stored in refrigerator at 4 °C [15].

Minimum Inhibitory Concentration-Anti-fungal Activity

Minimum Inhibitory Concentration was done on Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA) and Dixon's agar (DA) plates. 24 hours active culture was spread using a sterile glass L spreader rod over the surface of agar media plates. Each plate contained a well of 0.6 cm in diameter in which different concentrations of fruit peel extracts were added using a micro-pipette. Direct concentrations of extracts were used to check MIC using fruit peel extracts against dandruff causing fungus. The concentrations were 20µl, 40µl, 60µl, 80µl and 100µl respectively. Experiments were performed in triplicates.

Zone of Inhibition

Zone of Inhibition (ZOI) was done on Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA) and Dixon's agar (DA) plates. 24 hours active culture was spread using a sterile spreader over the surface of agar media plates. Each plate was punched with a well of 0.6 cm in diameter in which different concentrations of fruit peel extracts were added using a micropipette. Direct concentrations of extracts were used to check the ZOI using the fruit peel extracts against dandruff causing fungus given. Experiments were performed with suitable controls [15].

Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance is the analytical technique used to identify the monomolecular organic compound present in the sample. Similarly, biochemists use NMR to identify proteins and other complex molecules. Besides identification, NMR spectroscopy provides detailed information about the structure, dynamics and reaction state, chemical environment of molecules [16].

In this technique, the sample is placed in a magnetic field and the NMR signal is produced by excitation of the nuclei sample with radio waves into nuclear magnetic resonance, which is detected with sensitive radio receivers. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its individual functional groups.

RESULTS AND DISCUSSION

Characterization of Dandruff causing fungus

The samplescollected from different individuals were streaked over the surface of different fungal media and the colonies morphology was observed.In Dixon agar, the colonies were observed as creamish, round, mucoid, gummy and elevated from the surface with glassy translucent appearance **(Fig.1).**In Sabouraud dextrose agar, the colonies were observed as pale white, yellow and orange in colour, round shape, mucoid, gummy and elevated from the surface with glassy translucent appearance **(Fig. 1).**In Potato dextrose agar, the colonies were observed as pale white, round, mucoid and elevated from the surface. In all the fungal media plates, the growth of dandruff causing fungus is almost similar in morphological characteristics which are gummy, mucilaginous and elevated from the surface with glassy translucent appearance **(Fig 1).**

Catalase test

In catalase test, active effervescence was observed (**Fig 2)** when the isolated colony of fungi was smeared on the slide and to which a drop of 3% H_2O_2, hence the result was positive in Sabouraud dextrose agar and Dixon agar plates except Potato dextrose agar **(Table. 2).**

Table 2: Catalase test

Sl.No.	Fungal Media	Catalase Test
1.	DA	+
2.	SDA	+
3.	PDA	_

(+ Positive, **-** Negative)

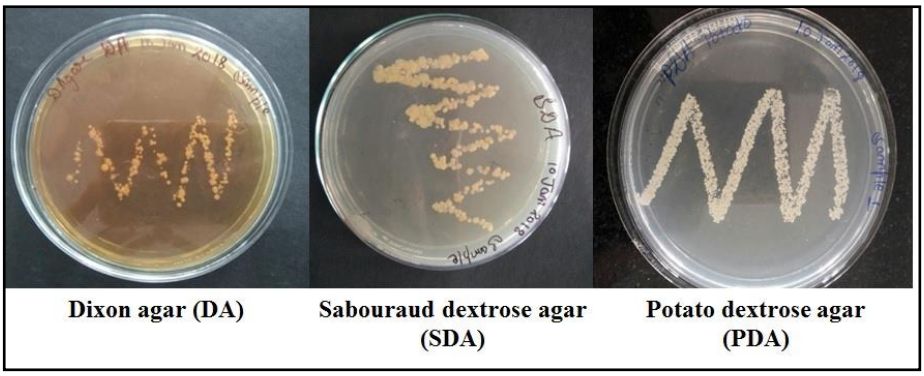

Fig. 1- Characterization of Dandruff causing fungus

Fig. 2 Catalase test positive for SDA and DA

Physical Parameters

pH

Different fungal media were altered by changing its normal pH range. After three days of incubation, the three media namely Dixon agar (DA), Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) showed different growth conditions.

a) The colonies in SDA plates were observed as pale white and creamish due to the change in pH.
b) In Dixon Media, the colony morphology was observed same after the formulation of pH.
c) As well in PDA plates, the colony morphology remains same even after change in the pH range.

In Dixon agar,the changes of pH from normal (6.0±0.2) to acidic pH (4.0±0.2) causes low solidification in the media as shown in **Fig. 3 (a),** Whereas at pH (4.5±0.2) the media solidified but no growth was observed due to the increase in acidity as shown in **Fig. 3 (b).**The media was found to be solidified, when the pH was increased to (7.5±0.2 and 8.0±0.2). Colony characteristics of the fungus were found to be pale white, mucoid with the increase in pH conditions which is similar to the colonies found in normal pH as shown in **Fig. 3 (c)**and **Fig. 3 (d).**

In Sabouraud dextrose agar and Potato dextrose agar media, the changes of pH from normal (5.6±0.2) to the acidic pH (3.6±0.2) showed no solidification in both the media at pH 3.6±0.2 due to increase in acidity as shown in **Fig.4(a) and Fig.5(a)** respectively**.** At acidic pH 4.4±0.2 growths occurs but the colony characteristics are different as compared to the colonies found in Sabouraud dextrose agar and Potato dextrose agar media with normal pH as shown in **Fig. 4(b) and Fig.5(b)** respectively**.** The increase in pH form normal to basic (7.1±0.2 and 7.6±0.2) in Sabouraud dextrose agar showed differences in the colonies characteristics and growth as shown in **Fig.4(c) and Fig.4(d).**

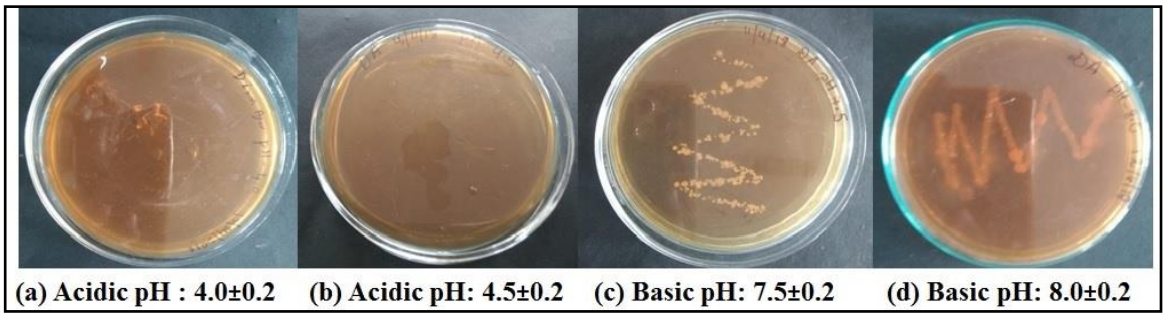

Fig 3. pH range and colony characteristics in Dixon Agar

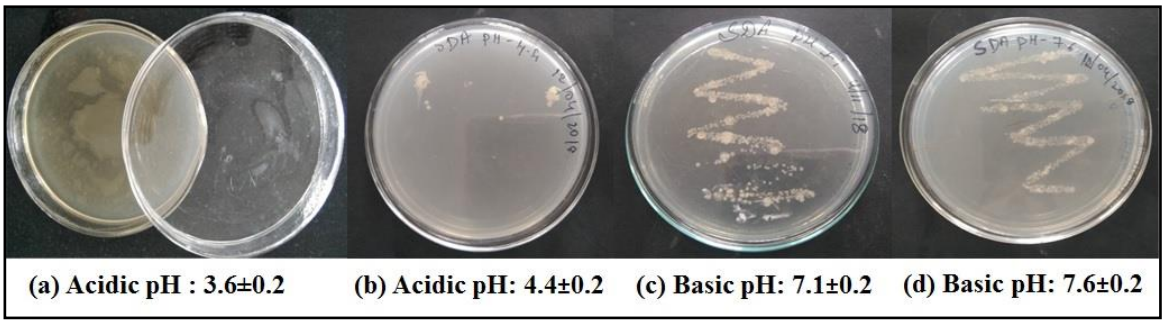

Fig 4. pH range and colony characteristics in Sabouraud dextrose agar

The increase in pH form normal to basic (7.1±0.2 and 7.6±0.2) in Potato dextrose agar media showed growth same as in the plate of normal pH as shownin**Fig.5(c) and Fig.5(d).** The results indicate that the acidic nature of the media does not support any growth of dandruff causing fungus. However, growth appears in the basic pH.

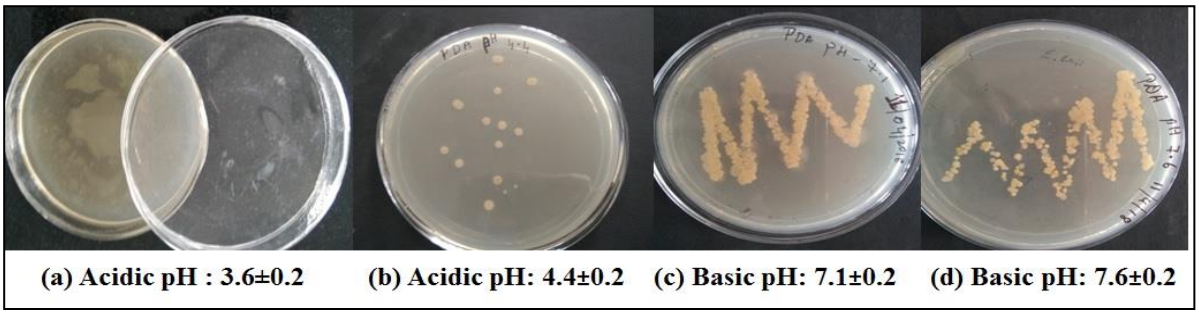

Fig 5. pH range and colony characteristics in Potato dextrose agar

3.2 Temperature

Fungal media plates were kept at two different temperatures (incubation at 35°C and room temperature 25°C) to observe the growth and study on their colony characteristics. The colonies were observed within 24 hours of incubation at 35°Cas shown in **Fig. 6(a), 7(a) and 8(a)**whereas in the plates incubated at room temperature (25° C) the colonies were observed only after 48 hours, **Fig. 6(b), 7(b) and 8(b).**

In Dixon agar (DA) and Sabouraud dextrose agar (SDA) the growth of colony characteristics were observed same as incubation temperature after 48 hours but the growth is less at room temperature as shown in **Fig 6** and **Fig 7**. In Potato dextrose agar, the growth of colony characteristics is same as incubation temperature after 24 hours as shown in **Fig 8**.

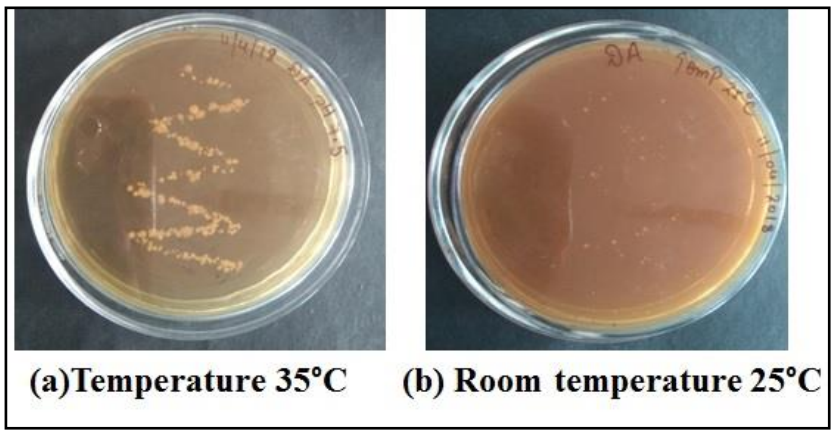

Fig 6. Temperature range and colony characteristics in DA

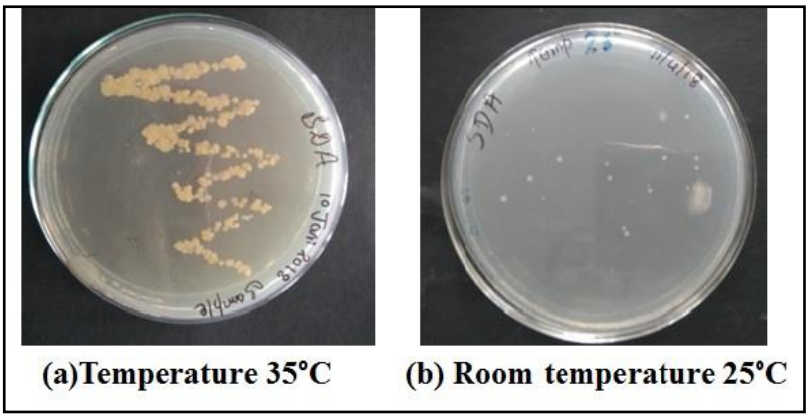

Fig 7. Temperature range and colony characteristics in SDA

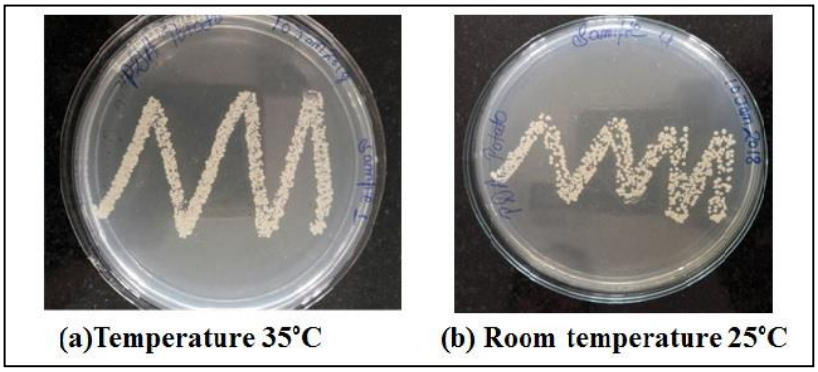

Fig 8. Temperature range and colony characteristics in PDA

Anti-fungal Activity

Minimal Inhibitory concentration

The Minimum Inhibitory Concentration was performed using different direct concentrations of fruit peel extract to check the inhibition against dandruff causing fungus. Prominent zones were observed with extracts of lemon peel. The results have been tabulated below **(Table 3).**

TABLE 3: Effect of different concentrations of different fruit peel extracts on Dandruff causing fungus and the measurement of their zone size in diameter (cm) for Dixon Agar

Fruit peel extracts	Zone of Inhibition in diameter (in cm)				
Amount (µl)	20µl	40µl	60µl	80µl	100µl
Orange	-	-	-	-	-
Lemon	1.3	1.5	1.6	1.8	2.0
Banana	-	-	-	-	-
Chikoo	-	-	-	-	-

Zone of Inhibition for Dixon agar (DA)

Different concentrations (20µl, 40µl, 60µl, 80µl and 100µl) of fruit peel extracts (orange, lemon, banana and chikoo) were used to check the efficiency of fruit peel extracts in terms of inhibition for Dixon's agar. The maximum inhibition was observed in Dixon agar at 100µl using lemon peel extract as shown in **Fig 9 (c).**

Lemon has antioxidant, anti-carcinogenic, antibacterial and antifungal property. Lemon peel methanol extract contain high amount of phenolic compounds which have ability to inhibit the growth of dandruff causing fungus. Hence zone of inhibition was observed for lime at concentrations ranging from 20µl-100µl, whereas zone of inhibition was not observed for the other fruit peel extracts (orange, banana, chikoo) against the dandruff causing fungus. The results have been tabulated below in **Table 3**. and **fig 10.**

Zone of Inhibition for Sabouraud dextrose agar (SDA)

Different concentrations (20µl, 40µ, 60µl, 80µ and 100µl) of fruit peel extracts were prepared to check efficiency of fruit peel extracts in terms of inhibition for Sabouraud dextrose agar (SDA). The maximum inhibition was observed in Sabouraud dextrose agar

(SDA) at 100µl for lemon peel extract as shown in **Fig 11 (c).**

Zone of inhibition was observed for lime at concentrations ranging from 20µl-100µl, whereas zone of inhibition was not observed for the other fruit peel extracts (orange, banana, chikoo) against the dandruff causing fungus. The activity of lemon peel methanol extract was found to be quite effective. The results have been tabulated below **(Table 4).**and in **Fig 12.**

Table 4: Effect of different concentrations of different fruit peel extracts on Dandruff causing fungus and the measurement of their zone size in diameter (cm) for Sabouraud dextrose agar (SDA)

Fruit peel extracts	Zone of Inhibition in diameter (in cm)				
Amount (µl)	20µl	40µl	60µl	80µl	100µl
Orange	-	-	-	-	-
Lemon	-	2.0	2.4	2.6	3.1
Banana	-	-	-	-	-
Chickoo	-	-	-	-	-

Different concentrations (20µl, 40µl, 60µl, 80µl and 100µl) of orange peel extract were prepared to check the efficiency of orange peel extract in terms of inhibition for Sabouraud dextrose agar (SDA). No zone of inhibition was observed in Sabouraud dextrose agar (SDA) for orange peel extract as shown in **Fig 13.**

Different concentrations (20µl, 40µl, 60µl, 80µl and 100µl) of chikoo and banana peel extract were prepared to check the efficiency of chikoo and banana peel extract in terms of inhibition for Sabouraud dextrose agar (SDA). No zone of inhibition was observed in Sabouraud dextrose agar (SDA) for chikoo and banana peel extract as shown in **Fig 14.**

Zone of Inhibition for Potato dextrose agar (PDA)

Different concentrations (20µl, 40µ, 60µl, 80µ and 100µl) of fruit peel extracts were prepared to check efficiency of fruit peel extracts in terms of inhibition for Potato dextrose agar (PDA). No zone of inhibition was observed in Potato dextrose agar for fruit peel extracts as shown in **Fig 15.**

Nuclear Magnetic Resonance (NMR)

The highest peak value of lemon peel extract is shown in **Fig 16** which is for carbon atom. This value corresponds to the standard ascorbic acid [17]. The highest peak value of lemon peel extract is 3.5 ppm which is for hydrogen atom. This value corresponds to the standard ascorbic acid as shown in **Fig 17**[17].

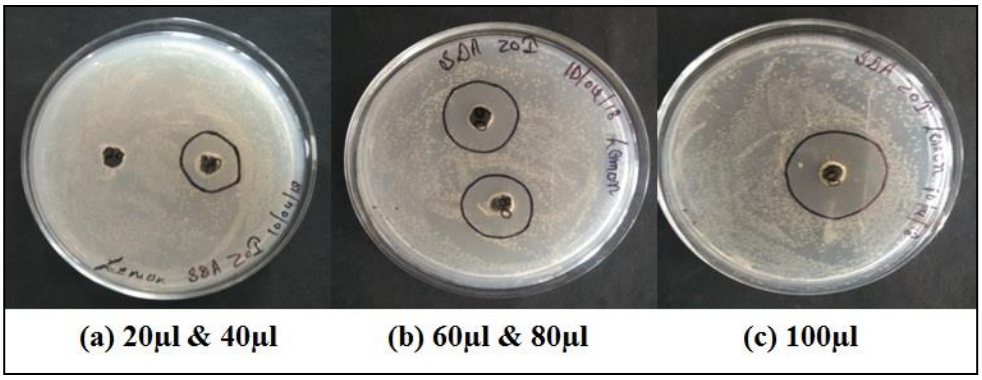

Fig 11. ZOI in SDA- concentration of lime peel extract

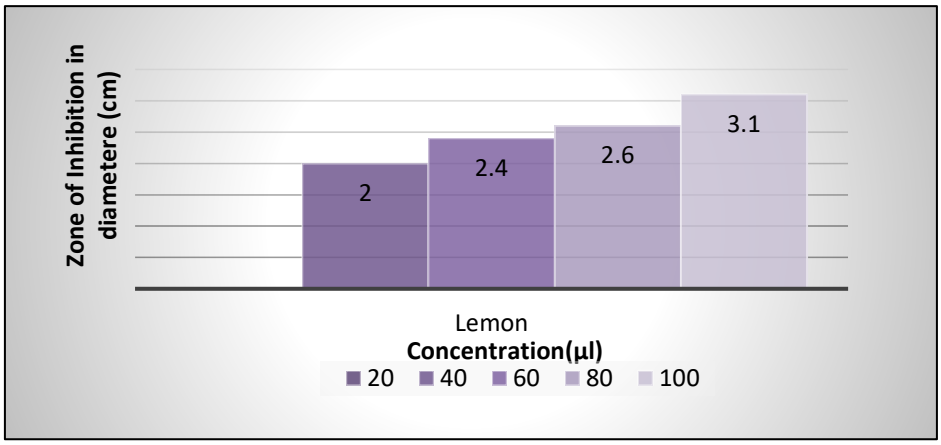

Fig 12. Effect of lemon peel extract against dandruff causing fungus in SDA

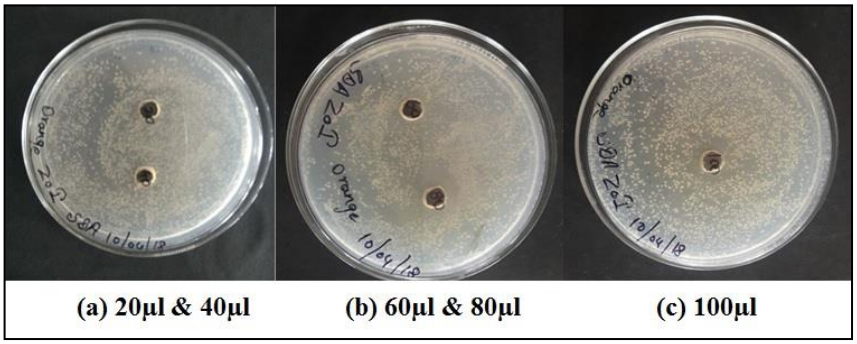

Fig 13. ZOI in SDA- concentration of orange peel extract

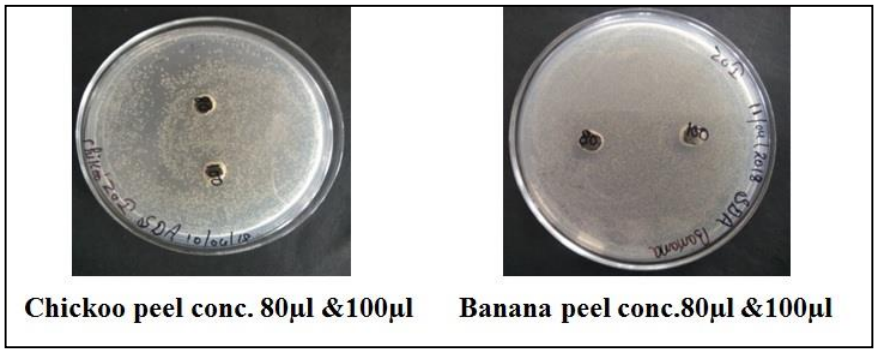

Fig 14. ZOI in SDA- concentration of chickoo and banana peel extracts

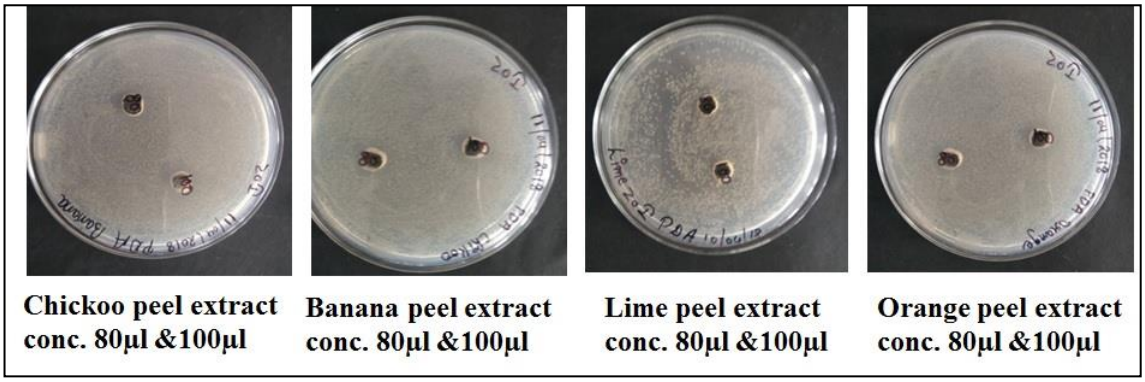

Fig 15. ZOI in PDA- concentration of chickoo, banana, lime and orange peel extracts

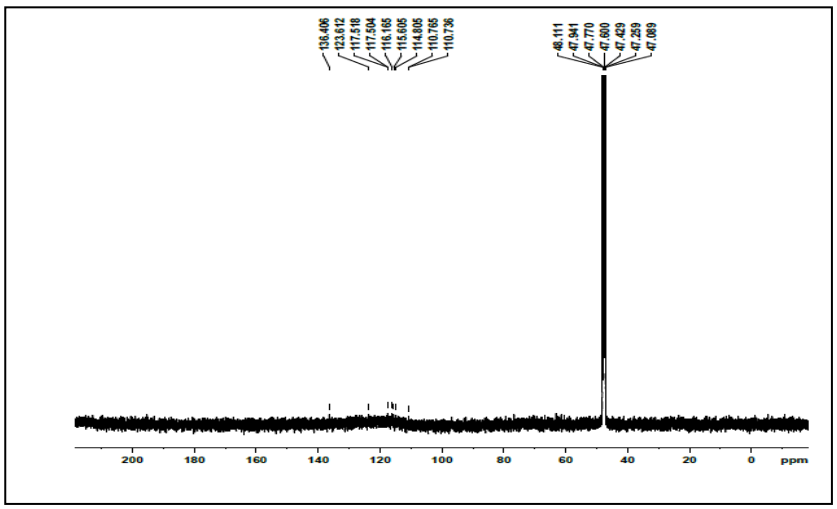

Fig 16. NMR spectroscopy for lemon Methanol extracts showing peak value of carbon atom

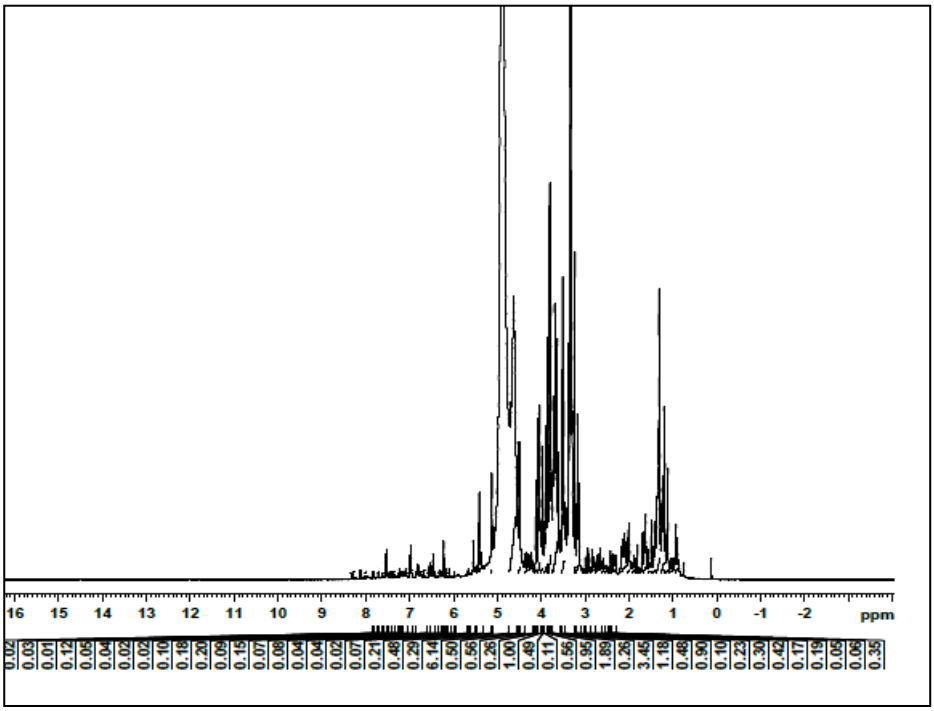

Fig 17. NMR spectroscopy for lemon methanol extracts showing peak value for hydrogen atom

CONCLUSION

Dandruff is a chronic scalp condition characterized by scaling, itching and redness of the scalp. It occurs when dead skin cells are shed in large oily clumps, which appear as white or grayish patches on the scalp, skin and clothes called dandruff. *Malassezia* is a monophylic and unipolar lipophilic yeast. It is a part of natural body flora and associated with a variety of conditions including dandruff, atopic dermatitis, pityriasisversi color, sebhoric dermatitis and folliculitis [18].

The dandruff causing fungi was cultured in different fungal media such as PDA, SDA, and DA, the colonies in Dixon agar were observed as creamish, round, mucoid and smooth regular concave surface. In Potato Dextrose agar, the colonies were observed as pale white, round, mucoid and smooth regular concave surface. In Sabouraud Dextrose agar the colonies were

observed as white, yellow and orange in colour, round shape, mucoid and smooth regular concave surface.

In our study we analyzed the growth of fungus in different fungal media and also by modulating the normal pH range followed by incubating the fungi in two different temperature conditions as discussed earlier. These studies were done to check the growth of fungus at varied pH and temperatures. It was found that by incubating at different environmental conditions, dandruff causing fungi were able to grow faster in incubator within 24 hours where as in room temperature the growth was observed after 2 days. It was observed that at pH below 4.5 the growth of dandruff causing fungus was less and at pH above 7.0 the growth of dandruff causing fungus was more. In Dixon agar by altering the normal pH range the colony morphology did not change while in PDA and SDA the colony morphology were different. The aim is to study the effectiveness of fruit peel extracts because they contain broad spectrum of phenolic compounds and vitamins which have the ability to overcome the resistance in causative organism. Citrus fruits are rich in vitamin C which maintains the pH level of scalp and prevents itching and shedding of flakes. Many fruit peel extract have been used and tested to check their therapeutic activity.

From the study the antifungal activity of the fruit peel extract was judged by performing zone of inhibition method. The samples were collected from the scalp of different individual to conduct the study. The fruit extract was obtained by soaking the powdered fruit peel with methanol. Orange, sweet lime, lemon, grape were evaluated for the physicochemical characteristics. The results were significant at different interval. Among all the citrus fruit lemon showed high content of citric acid but grapes and orange were rich in total soluble solids [19].

Among all the fruit peel extracts used, lemon peel extract was able to inhibit in all the concentrations (20µl -100µl), followed by orange which was able to inhibit at 40µl, 80µl and 100µl concentration in SDA only. NMR analysis was carried out which revealed that Vitamin C is the compound present in lemon fruit peel extract which was found to have anti dandruff activity. Therefore, natural products have relevant advantages over synthetic compounds they are easily available, comparatively cheaper, and can reduce side effects and irritation potential of chemicals to a large extent. Plants are rich in wide variety of secondary metabolites such as tannins, alkaloids and flavonoids found to have antimicrobial properties [20]. Our study helps in choosing the advisable fruit peel extract with anti-dandruff activity which is easily available and helps in assessing the grade of new products containing lemon as an active compound.

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