

Evaluation of Hyaluronic Acid Biological Activity against *Malassezia furfur* and *Malassezia pachydermatis*

Project Report

*Submitted in partial fulfillment of the requirements for the award of Bachelor of Sciences
degree in Biotechnology*

By
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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Mohak Udiniya (41770038) who carried out the project entitled "Evaluation of Hyaluronic Acid Biological Activity against *Malassezia furfur* and *Malassezia pachydermatis*" Under our supervision from 12th October 2023 to 28th March 2024.

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TO WHOMSOEVER IT MAY CONCERN

It is my pleasure to write this letter for **Mr. Mohak Udiniya**, pursuing Bachelor of Sciences in Biotechnology at Sathyabama Institute of Science & Technology, Chennai, TN. He did his final year project from 12-10-2023 to 28-03-2024 under my supervision on “**Antimicrobial activity studies of Hyaluronic acid against *Malassezia furfur* and *Malassezia pachydermatis***”. During his internship program, he has gained sufficient expertise in various microbiological techniques, more specifically in handling **BSL-2 pathogen**, antimicrobial susceptibility testing, and sub-culturing.

He has been very systematic in his choice of various biochemical and biophysical characterization techniques which include Spectroscopy, Electrophoresis, Circular Dichroism, and FT-IR. He properly timed himself in performing various laboratory tasks which include inoculation, incubation, sub-culturing, antimicrobial susceptibility testing, and so on. He also gained sufficient knowledge in the fundamentals of Protein X-ray crystallography which includes crystallization, X-ray diffraction, and powder X-ray diffraction. He has also been involved in ongoing laboratory research projects on developing novel silk fibroin-based biomaterial and its applications in tissue engineering. Also, he has a tremendous amount of knowledge in the data analysis program Origin.

Mr. Mohak, a sincere, hardworking, highly motivated, and disciplined student who is always eager to learn new techniques to explore new research areas. He has an excellent analytical mind with a lot of perseverance and has the potential to become a good researcher. He has been proficient in applying his knowledge and skills where required and comes up with productive ideas. He is steadfast and diligent in learning new techniques, showing an increase in diligence with relevant literature reading and consistent practice. He maintains a cordial relationship with colleagues and performs well with good communication and optimistic involvement in promoting a holistic research environment at the lab.

I wish all the very best for his academic degree.

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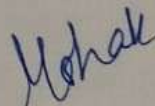
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DECLARATION

I hereby declare that the Project Report entitled **Evaluation of Hyaluronic Acid Biological Activity against *Malassezia furfur* and *Malassezia pachydermatis*** done by me under the guidance of Dr. V Ramesh Kumar (Internal) and Dr. S. M. Jaimohan (External) at CSIR-Central Leather Research Institute, Adyar, Chennai is submitted in partial fulfillment of the requirements for the award of Bachelor of Sciences degree in Biotechnology.

DATE: 25-04-2024

PLACE: Chennai



SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to The Board of Management of Sathyabama Institute of Science and Technology for their encouragement in doing this project and for completing it successfully. I am grateful to them.

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I would like to express my sincere and deep sense of gratitude to my Project Guides **Dr. V Ramesh Kumar (Internal)** and **Dr. S M Jaimohan (External)** for their valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT:

The aim of this study is to find the effect of hyaluronic acid in different solvent media like water, glycerine and Dimethyl Sulfoxide (DMSO) and, concentrations on *Malassezia furfur* and *Malassezia pachydermatis*. Hyaluronic acid is a known novel material which is a glucosaminoglycan, naturally found in skin's extracellular matrix (ECM). This complex polysaccharide has various molecular weights and for the purpose of this study we have used high molecular weight hyaluronic acid (~1,000,000Da – 2,000,000Da). *Malassezia furfur* and *Malassezia pachydermatis* are species of *Malassezia* genus, which are lipophilic, basidiomycetous yeasts. This genus of yeast is naturally found on skin of many mammals and is commensal in nature. In patients with weakened immune system and with appropriate growing conditions, these yeast can cause skin conditions like otitis and dermatitis. *Malassezia furfur* is a known pathogen of human skin, manifesting in pityriasis versicolor and dandruff; whereas, *Malassezia pachydermatis* is a zoonotic pathogen mainly affecting cats and dogs. This study found that high molecular weight hyaluronic acid is not effective against either of the species under laboratory conditions on Dixon's agar medium. The study uses 0.5%, 1%, 2% & 4% solutions of hyaluronic acid in water, 0.5% & 1% solutions of hyaluronic acid in 10% DMSO and 1% & 2% solution of hyaluronic acid in glycerine. None of the concentrations in any solvent returned a positive result, i.e., no inhibition circles were observed. The positive control established was 30µg/mL and 3mg/mL concentration solutions of imidazole antibiotic, Itraconazole.

Keywords: *Malassezia furfur*, *Malassezia pachydermatis*, *Hyaluronic acid*, *Dermatitis*, *Yeast*.

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
DMSO	Dimethyl Sulfoxide
ECM	Extra-Cellular Matrix
LNA	Leeming Notman Agar
SCID	Severe Combined Immunodeficiency
AIDS	Acquired Immunodeficiency Syndrome
HIV	Human Immunodeficiency Virus
PSI	Pound - force per Square Inch (Pressure)
TLR	Toll Like Receptors
Da	Daltons (molecular weight)
w/v (%)	Weight by Volume (percentage)
v/v (%)	Volume by Volume (percentage)

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CHAPTER 1

INTRODUCTION

Malassezia is a genus of lipophilic commensal yeast that is found on human skin. It is part of normal microbiota on healthy human skin. Members of this genus are opportunistic and can cause infections in patients with weak immune system. It is a genus of basidiomycetous yeast with 18 members (Rhimi *et al.*, 2020). Different species of this genus can be identified in other domestic organisms like dog, cat, horse and cattle. This study will be focussing on two species, i.e., *Malassezia furfur* and *Malassezia pachydermatis*.

Malassezia furfur is an opportunistic pathogen that causes problems like psoriasis, dermatitis, tinea versicolor and pityrosporum (*Malassezia*) folliculitis. It is also one of the first in the genus to be discovered and isolated among the genus. *Malassezia furfur* can be easily isolated from a patient with pityrosporum folliculitis and tinea versicolor (McGinley *et al.*, 1975; Theelen *et al.*, 2018). Since it is lipophilic in nature, it requires special growth media for culturing under laboratory conditions. Two of the most commonly used media are Leeming Notman agar (LNA) and Dixon's agar. Both agars contain lipid supplements in the form of tween 40, 60 or 80, glycerol stearates and free fatty acids (palmitic acid and oleic acid). Desiccated ox bile is also an important component of the aforementioned agars (Chua *et al.*, 2005). This yeast is also notorious for causing systemic infections in patients with impaired immunity, due to severe combined immunodeficiency (SCID), human immunodeficiency virus (HIV) causing acquired human immunodeficiency syndrome (AIDS), autoimmune disorders and immunosuppressants. Systemic infections of the lung and heart, by the yeast can be fatal if left untreated. But, infections of skin and system by it are easily cured by imidazole antibiotics and polyene (amphotericin B) (Velegarki *et al.*, 2015). Despite being more toxic and consequential, amphotericin B is highly effective in treating systemic infections by fungal pathogens. This drug is administered intravenously or intradermally.

Malassezia pachydermatis is also a member of the same genus, but it is lipid independent and does not require lipid supplementation. It was first isolated from skin of an Indian rhinoceros with severe exfoliative dermatitis. This species

has been found on many carnivores including, bears cats and dogs. It is a medically important species especially for dogs, since it causes severe cases of otitis externa and *Malassezia dermatitis*(Guillot & Bond, 1999). This species is seldom found on human skin, but when found its source can be attributed to pets. This species can be grown easily on Sabouraud's dextrose agar, Leeming Notman agar and modification of Dixon's agar; with or without lipid supplementation (Chua *et al.*, 2005; Puig *et al.*, 2017).

Malassezia pachydermatis is a zoonotic and commensal in nature. Despite being transmitted to humans, it is transient and is unable to infect, unless the person is immunocompromised. According to reports, infections in human by this species have mostly been seen in infants, old people and immunologically impaired patients (Huang *et al.*, 2020; Rhimi *et al.*, 2020). The point of transmission in health care facilities was found to be intravenous catheters and the infection was majorly systemic in nature. Both pets and human patients respond to anti-fungal therapy and tend to recover, but, rarely are anti-bacterial therapies for such infections are seen working solely. Use of anti-fungal and bacterial therapies in synergy can lead to swift recovery in many cases. Terbinafine is an anti-fungal medication which was found to be extremely helpful for treatment of pets, but not in humans. Simultaneously ketoconazole, itraconazole and miconazole-chlorhexidine treatments are also very effective. Selenium sulphide and zinc pyrithione shampoo were also prescribed routinely in both human and animal infections (Cafarchia *et al.*, 2012; Velegraki *et al.*, 2015).

This study will focus on innate ability of high molecular weight hyaluronic acid to show antimicrobial activity. Hyaluronic acid is a very versatile complex polysaccharide with anti-inflammatory, anti-angiogenic, antimicrobial and wound healing properties. Moreover it is a constituent of ECM and is non-toxic (Frenkel, 2014; Kawano *et al.*, 2021). Hyauronic acid has also been used in a significant number of studies against *Malassezia spp.*, caused infection like seborreheic dermatitis. In such studies it has shown a significant activity, as topical formulation, dermal filler and various biomaterial films (Campione *et al.*; Garg *et al.*, 2020; Zamboni *et al.*, 2021; Zhang *et al.*, 2022; Zhang *et al.*, 2020). Many of such studies have used low molecular weight hyaluronic acid with a mixture of other antimicrobial and anti-inflammatory compounds in the form of gels and creams

(Gariboldi *et al.*, 2008; Schlesinger & Rowland Powell, 2014). Since, high molecular weight hyaluronic acid also has its benefits and is relatively unused in non-cross linked form against *Malassezia*; this study will use it in form of solutions of varying concentration in water, 10%DMSO and glycerine against *Malassezia furfur* and *Malassezia pachydermatis*, to check for antimicrobial activity in-vitro.

CHAPTER 2

AIMS AND SCOPE:

The aim of this study is to find out the biological activity of non cross linked, high molecular weight hyaluronic acid against two species of *Malassezia* genus, i.e., *Malassezia furfur* (MTCC 1765) and *Malassezia pachydermatis* (MTCC 1369). Hyaluronic acid is known for its antimicrobial activity against many bacteria and is also a natural constituent of the skin's extracellular matrix. Hence, the scope of this study will be to find a safer and as effective substitute, to antibiotics, against *Malassezia spp.*, in form of hyaluronic acid solutions of different concentrations in Water, 10% DMSO and glycerine and to check their activity in in-vitro conditions on agar plate.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS USED:

For the purpose of this study high molecular weight hyaluronic acid (~ 1,000,000Da – 2,000,000Da) was used. 1 gram of hyaluronic acid and glycerine for solutions, was procured from a local vendor with 98% purity. Dixon's agar medium for cultivation of *Malassezia furfur* and *pachydermatis* was procured from *HiMedia Laboratories pvt. Ltd.*; which was divided in two parts: part 'A' served as the agar base with required nutrients and Part 'B' served as the lipid supplement. Ampicillin for adding to broth was by Sigma-Aldrich. Membrane filter used for filter sterilizing solutions was *Whatman Uniflo* 0.2µm polyethersulfone filter. Autoclaved Millipore water was used for preparation of solutions. DMSO solution of 10% in water was made from *Emparta* grade reagent. *Malassezia furfur* MTCC 1765 and *Malassezia pachydermatis* MTCC 1369 were provided by CSIR- Institute of Microbial Technology [Microbial Type Culture Collection and Gene Bank]. Itraconazole for positive control was made from *Itrosted®* Leeford Healthcare Ltd. 100mg capsule.

3.2 METHODOLOGY:

3.2.1 Solutions:

Four corresponding concentrations; 0.5%, 1%, 2% & 4% (w/v) of hyaluronic acid in autoclaved Millipore water were made. Since hyaluronic acid is insoluble in DMSO, 10% (v/v) concentration solution of DMSO in water was used to make hyaluronic acid solution. 0.5% & 1% (w/v) concentration of hyaluronic acid solutions were made in 10% DMSO. For glycerine based solutions 1% and 2% (w/v) hyaluronic acid concentrations were used. All the solutions were filter sterilized using *Whatman Uniflo* 0.2µm membrane filter and sterile syringe, and refrigerated for storage. A 50µg.mL⁻¹ solution of ampicillin to avoid bacterial contamination was also made. For positive control 100mg itraconazole tablet was diluted to lower concentrations of 30µg.mL⁻¹ and 3mg.mL⁻¹ for testing.

3.2.2 Cultivation and Plating:

For preparation of Dixon's agar plates, part A and part B were mixed according to the measurements provided and sterilized at 121°C (15 psi) in an autoclave. Before pouring plates 50µg.mL⁻¹ of ampicillin was added to the agar to protect it from bacterial contamination. For test tubes, Dixon's agar preparation was diluted with autoclaved Millipore water.

Malassezia furfur MTCC 1765 and *Malassezia pachydermatis* MTCC 1369 were obtained as lyophilized spores in vacuum sealed glass ampoules. Sterile 0.9% saline was used for reviving the spores in test tubes. A minimum time of 20 minutes was given for activation of spores at room temperature. Two separate test tubes of Dixon's agar in broth form were prepared and labelled appropriately with numbers 1765 & 1369, before transferring the activated spores of both species to the broth.

The species were incubated in test tubes at 37°C for 1 week, until turbidity was seen. Later, these test tubes were used for streaking petri plates with Dixon's agar. 200µL of solution from the either of the test tubes was poured onto the plates before streaking the solution evenly using L-rod. These plates were incubated at 37°C for 5-6 days and were checked regularly for growth. After 6 days the plates with *Malassezia furfur* showed numerous colonies whereas, plates with *Malassezia pachydermatis* showed no sign of growth. Plates with *Malassezia pachydermatis* were re-incubated for further 2 days to check for growth. After two days the plates were checked for growth but, no growth was obtained. Upon further trials using nutrient agar, Czapek Dox agar and non-lipid supplemented Dixon's agar, the best growth for *Malassezia pachydermatis* was obtained for non-lipid supplemented Dixon's agar. This stands clear with the fact that *Malassezia pachydermatis* can grow without lipid supplementation.

Since, the test tubes yielded saturated number of colonies on agar plates they were used as mother source for plating. Before further plating the mother source was diluted for plating. For *Malassezia furfur* 10⁻³ dilution in 0.9% saline was used for subculture and for *Malassezia pachydermatis* 10⁻² dilution was used. These dilutions values were confirmed as feasible through repeated plating and

will be followed as uniform values throughout the study. For plating 200µL of dilution was pipette on the plates and uniformly streaked using glass L-rod.

3.2.3 Testing Susceptibility to Hyaluronic Acid:

For testing susceptibility, Whatman filter paper discs were used. Using a hole punching machine, filter paper was carved for discs. These discs were then sterilized in an autoclave and later stored in hot air oven at 70°C to maintain sterility.

3.2.3.1 For *Malassezia furfur*:

Petri plates at 10^{-3} dilution were streaked and incubated at 37°C for 3-4 days until small log phase colonies were observed. Upon confirmation that the colonies are present, all the solutions were loaded onto filter discs, put on the agar plates and labelled water, 10% DMSO and glycerine corresponding to the plates with respective solutions. Concentrations were clearly marked on the plates to avoid confusion. Each plate was sealed with parafilm and all the steps were carried out in a laminar air flow to avoid contamination.

3.2.3.2 For *Malassezia pachydermatis*:

Petri plates at 10^{-2} dilution were streaked and incubated at 37°C for 3-4 days until small, transparent, log phase colonies were observed. Upon confirmation that the colonies are present, each solution was loaded on the sterile filter paper discs. Later, the plates were labelled as water, 10% DMSO and glycerine corresponding to the solution used on the plate and concentrations were marked clearly for better observation. For avoiding contamination and to maintain sterile conditions all the steps were carried out in a laminar air flow and the plates were sealed using parafilm.

3.2.4 Control plates:

Three plates were used as control; an empty plate, a plate with native solvents loaded on filter discs, i.e., water, 10% DMSO and glycerine loaded filter discs and a positive control of itraconazole antibiotic. As already discussed, itraconazole is a commonly prescribed imidazole antibiotic for *Malassezia* infections. Two concentrations of itraconazole were used; 30µg.mL⁻¹ and 3mg.mL⁻¹.

All the plates were checked for results on the 6th or 7th day from the initial streaking. This is because, the two *Malassezia* species used in the study take 6-7 days to reach stationary phase and then the culture declines, reaching death phase by 8th or 9th day. Hence, 3rd or 4th day from streaking gave the right opportunity for putting filter discs to observe inhibition zones.

CHAPTER 4

RESULTS AND OBSERVATIONS

4.1 RESULTS:

4.1.1 For *Malassezia furfur*:

From *figure 4.1.1*, it can be clearly elucidated that no inhibition zones were observed for any of the solvents nor were there any differences in growth due to variation in concentrations. Growth of the yeast was very evident on the filter discs as well, suggesting that hyaluronic acid does not have any biological activity against *Malassezia furfur*.

Simultaneously, it was observed that none of the solvents had any effect for the formation of inhibition zones on the plate [figure 4.1.2 (A)]. On the other hand, inhibition zones can easily be established and marked around the filter discs containing itraconazole [figure 4.1.2 (B)].

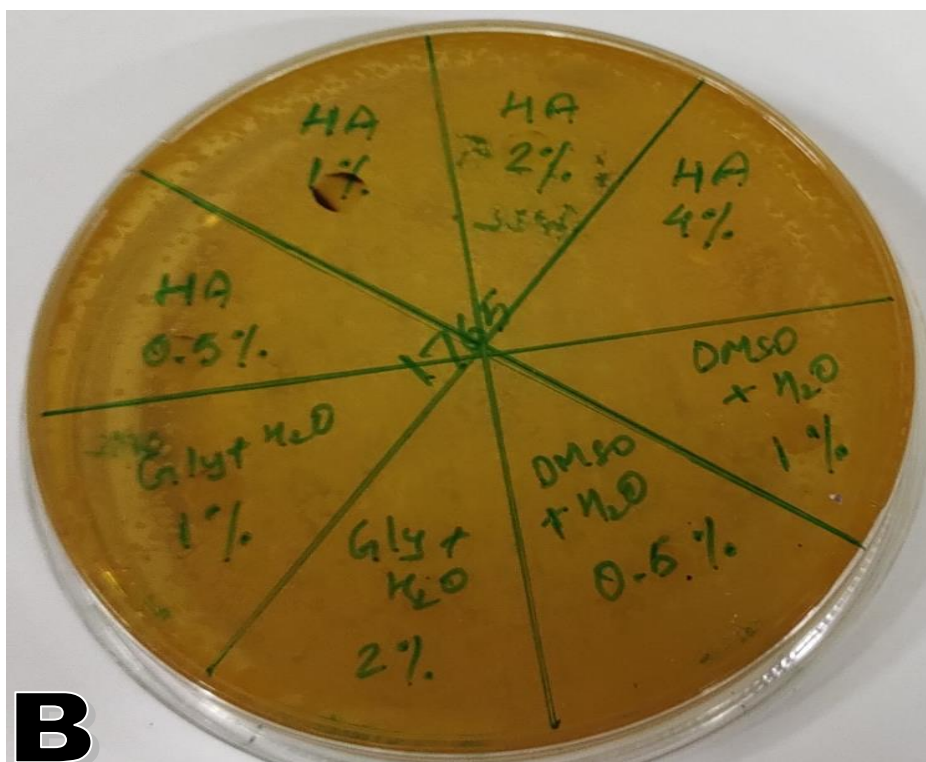
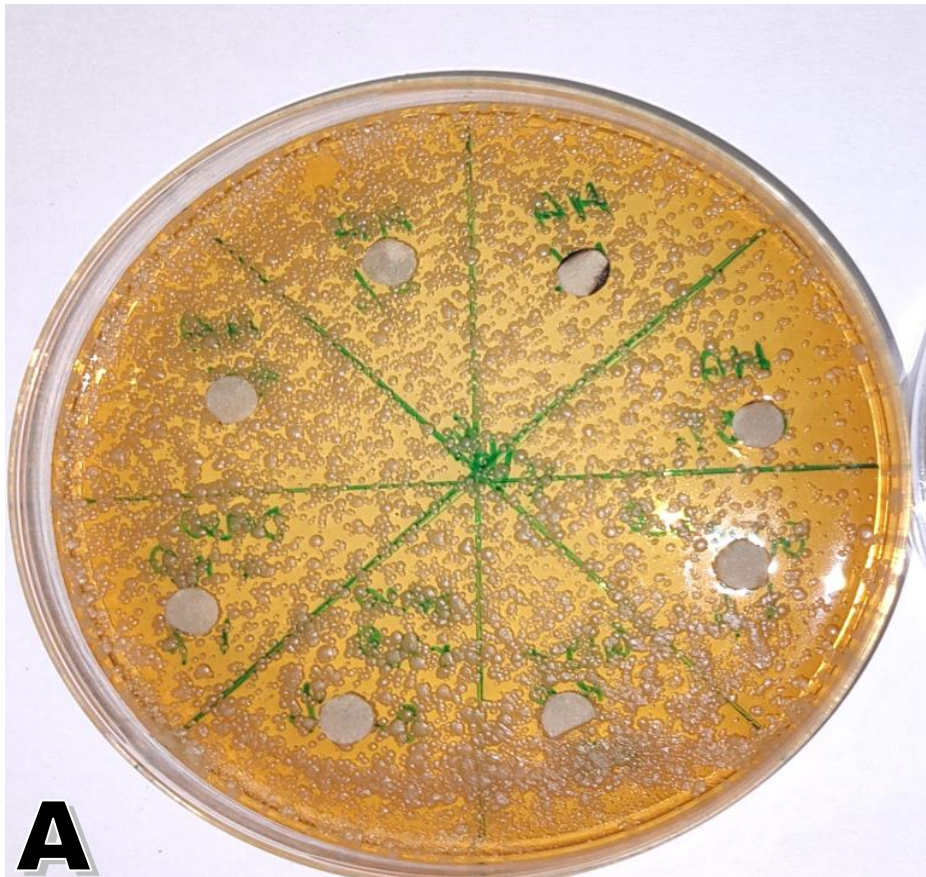


Figure 4.1.1: A) an image of agar plate with *Malassezia furfur* and filter discs loaded with different solutions. B) This image shows all the labels corresponding to the solutions

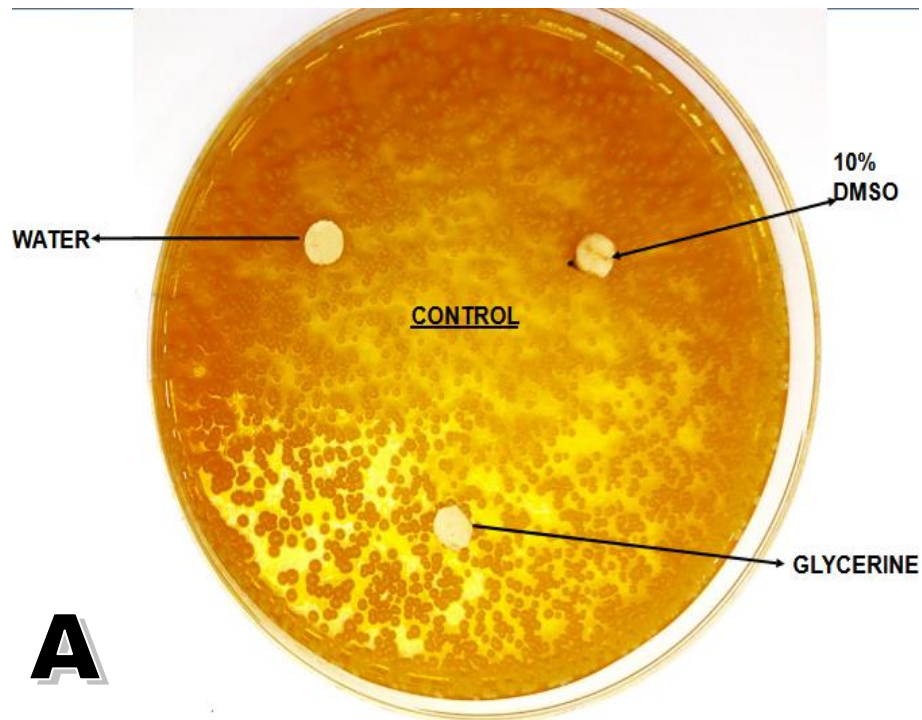


Figure 4.1.2: *Malassezia furfur*: A) this is an image of control plate with native solvents of water, 10% DMSO and glycerine. B) An image of positive control itraconazole with given concentrations and corresponding inhibition zones.

4.1.2 For *Malassezia pachydermatis*:

From figure 4.2.1, it is visible that there is no inhibition circle formation. No effect was seen for any of the concentrations of hyaluronic acid in water, 10% DMSO and glycerine. Moreover, the control established with native solvents of

water, 10% DMSO and glycerine without hyaluronic acid didn't show pattern or activity either [figure 4.2.2 (A)]. The positive control established as itraconazole antibiotic of $30\mu\text{g}.\text{mL}^{-1}$ and $3\text{mg}.\text{mL}^{-1}$ concentration showed clear zone of inhibition [figure 4.2.2 (B)].

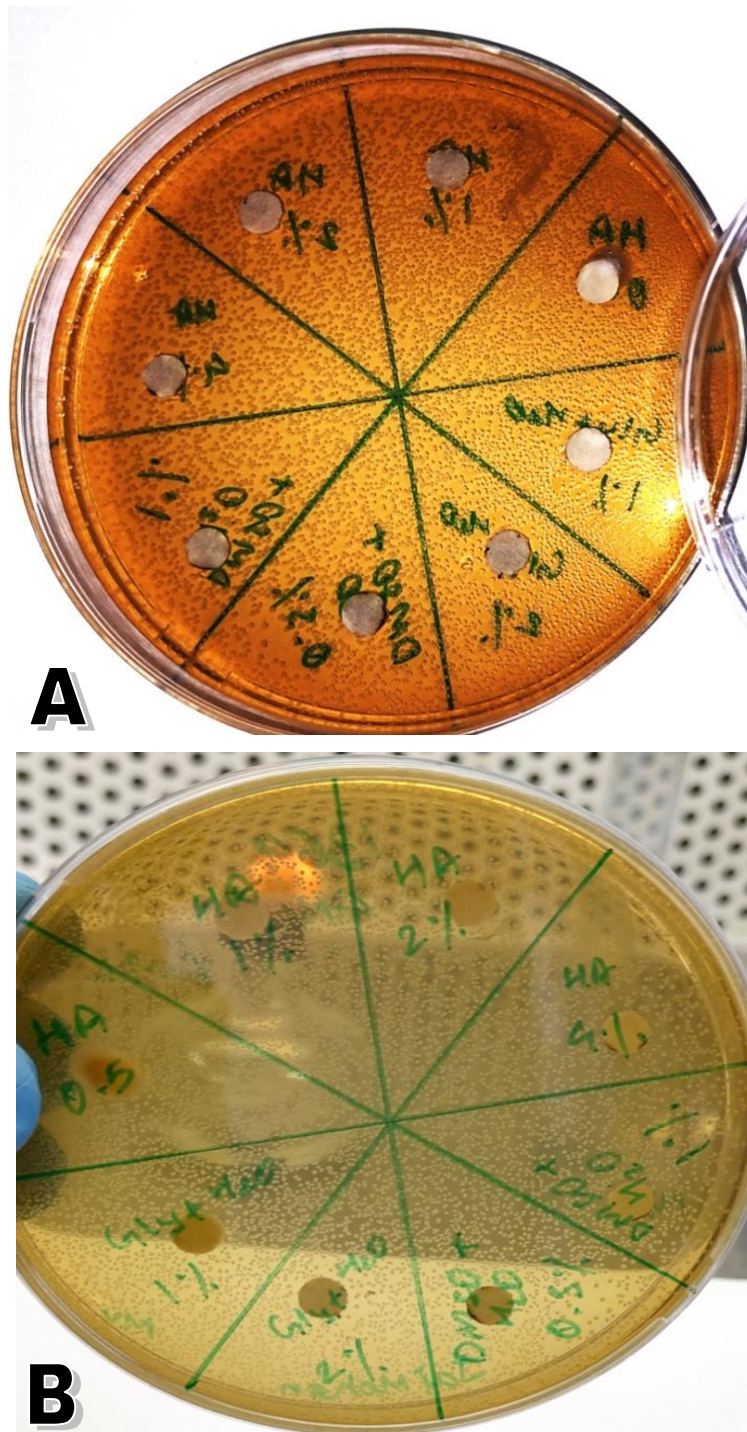




Figure 4.2.2: *Malassezia pachydermatis*: A) this is an image of control plate with native solvents of water, 10% DMSO and glycerine. B) An image of positive control itraconazole with given concentrations and corresponding inhibition zones

4.2 OBSERVATIONS:

All the plates obtained after 7-8 days of incubation for both *Malassezia furfur* and *Malassezia pachydermatis* gave negative results. Hyaluronic acid in water at 0.5%, 1%, 2% and 4% concentration did not show any inhibition zones. Hyaluronic acid with 10% DMSO as solvent did not show any activity at both 0.5% and 1% concentrations. Both the species did not show any susceptibility against hyaluronic acid dispersed in glycerine at 1% and 2% concentration as well. Moreover, upon microscopic examination of plates for all the solutions, colony formation was clearly observed on the filter discs as well.

Positive control established at $30\mu\text{g.mL}^{-1}$ and 3mg.mL^{-1} concentration of itraconazole had very clear inhibition zones and the other control with native solvents showed similarity to the plate with respective solvents of water, 10%DMSO and glycerine.

In general, the colony morphology of *Malassezia furfur* was white, circular, shiny and smooth; whereas, Colony morphology for *Malassezia pachydermatis* was transparent and wrinkled on Dixon's agar. On microscopic examination powdery, rough and round colonies were seen for *Malassezia furfur* beside mycelia like structures [figure 4.3] and Snowflake like colonies were seen for *Malassezia pachydermatis* [figure 4.4].

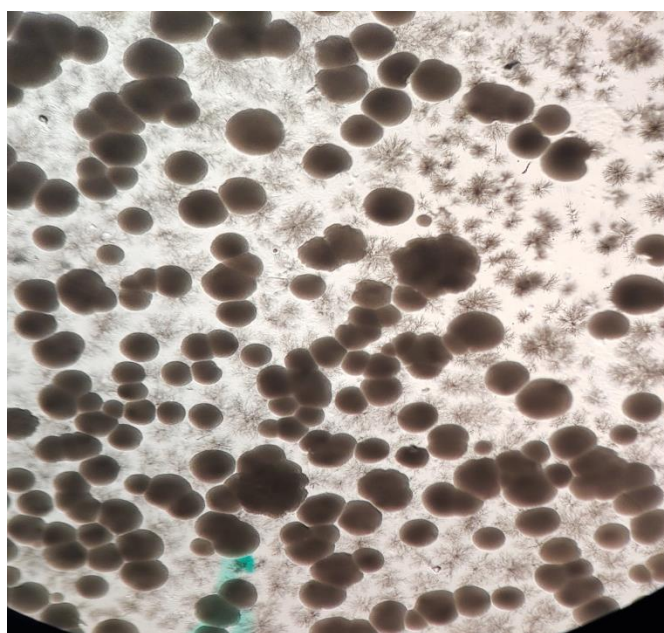


Figure 4.3: Microscopic image showing powdery, rough and round colonies of *Malassezia furfur*.



Figure 4.4: Microscopic image showing snowflake like colonies of Malassezia pachydermatis.

CHAPTER 5

CONCLUSION

According to a previous study, that uses non-cross linked hyaluronic acid dermal filler on reconstructed human epidermis against *Malassezia furfur* and other inflammation causing organism of the human scalp; antimicrobial, anti-inflammatory and antibiofilm activities were seen (Sommatis *et al.*). Another study that uses low molecular weight hyaluronic acid on human subjects returned similar results (Schlesinger & Rowland Powell, 2014). The above studies use conditions similar to human skin or a live subject for their study along with lower concentration and molecular weight hyaluronic acid. This is in the light of a study that demonstrated that, low molecular weight hyaluronic acid can activate many toll like receptors (TLRs) which in turn secrete β -defensins (Gariboldi *et al.*, 2008). β -defensin is a group of antimicrobial peptides showing high activity against many scalp bacteria and fungi like *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Candida albicans* and *Candida parasilopsis* (Pazgier *et al.*, 2006). The above mentioned bacteria and fungi along with *Malassezia spp.*, are known for regulating skin microenvironment. A significant change in populations of bacteria can change *Malassezia* populations on scalp and hence, a decrease in population of above mentioned bacteria due to β -defensins can lead to a decrease in *Malassezia furfur*'s population (Clavaud *et al.*, 2013). Thus, positive results can be seen in studies which use human scalp like conditions. Meanwhile, strains of *Malassezia pachydermatis* isolated from dogs are known for producing enzymes that can digest components of ECM., including hyaluronidase which can digest hyaluronic acid. Since, the strain used in this study was isolated from ATCC 14522, which was previously isolated from a dog with otitis externa; it is very viable that despite after long time of sub-culturing, it can still secrete the enzymes which resulted in negative result (Coutinho & Paula, 2000).

It is to be considered, this study uses high molecular weight hyaluronic acid on an agar medium, and is an in-vitro study. In case of *Malassezia pachydermatis* it can produce hyaluronidase and would have broken down high molecular weight

hyaluronic acid into short fragments of low molecular weight (Maharjan *et al.*, 2011). These smaller fragments on a skin or skin-like model could have activated TLRs, which in turn can start a cascade that can lead to a decrease in populations due to β -defensin production and eventually alleviating of symptoms from infection. In the previous studies conducted against *Malassezia furfur*, the hyaluronic acid used is mixed with other materials to increase the efficacy of the biopolymer, and also the preparations were in gel or cream form and not pure solution (Puviani *et al.*, 2019; Schlesinger & Rowland Powell, 2014; Sommatitis *et al.*). The aforementioned studies not only used live organisms or skin models, but, also took longer periods of time to show any palliative changes.

All the solvents used in the study have very low toxicity to humans and were merely used for their inherent properties, to increase activity or efficacy. But, with this study, it can be concluded that high molecular weight hyaluronic acid in water at 0.5%, 1%, 2% & 4%; in 10% DMSO at 0.5% & 1% and in glycerine at 1% and 2% concentrations does not have any antifungal activity against *Malassezia furfur* and *Malassezia pachydermatis*, in-vitro on Dixon's agar. These same solutions can be used on human skin-like models or patients in future studies to conclusively prove presence of any activity or not. Moreover, use of high molecular hyaluronic acid can be prescribed rather than steroids for decreasing inflammation of skin in *Malassezia* infections (Kastarinen *et al.*, 2014; Litwiniuk *et al.*, 2016).

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