An in Vitro Anti-Dandruff Potential of Moringa Leaves Extract and Its Synergistic Effects with Ketoconazole

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Abstract:
Dandruff, a dermatological scalp ailment, characterized by rapid scalp flaking and itching due to invasion of a lipophilic yeast, Malassezia furfur. Herbal extracts enriched with bioactive phytoconstituents serve as safer alternative over synthetic antidandruff agents. Although the role of Moringa leaves as anti-fungal agent has been established, its efficacy against Malassezia furfur remained elusive. Hence, the present investigation was undertaken to unveil in-vitro anti-dandruff potential of moringa leaves. In the present study, dried moringa leaves powder was extracted in the ethanolic solution using maceration method. A preliminary qualitative phytochemical screening of the ethanolic extract of Moringa leaves was conducted. Moreover, in-vitro anti-fungal activity of Moringa leaves extract against Malassezia furfur was evaluated using broth dilution and agar cup plate method. The synergistic activity of moringa leaves extract in combination with ketoconazole was investigated using agar cup plate method. The phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, steroids, tannins, proteins, carbohydrates, and glycosides. The ethanolic extract of moringa leaves exhibited antifungal activity at the concentration of 10 mg/ml, 12 mg/ml, 14 mg/ml with zone of inhibition of 8±0.45 mm, 12±0.57 mm and 14±0.25 mm respectively in agar cup plate assay and minimum inhibitory concentration (MIC) was obtained at 12 mg/ml. In combination study, moringa leaves extract with ketoconazole (12 mg/ml+1mg/ml) revealed symbiotic effect with zone of inhibition 20±0.62 mm as compared to ketoconazole control (15±0.34 mm). Findings suggest that moringa leaves extract could be novel herbal anti-fungal substitute over synthetic anti-dandruff agents.

Keywords: Moringa leaves extract, anti-dandruff, anti-fungal, Minimum inhibitory concentration, Zone of inhibition, Phytochemical screening.

INTRODUCTION

Dandruff is a scalp disorder, characterized by itching, irritancy, and rapid turnover of epidermal cells of the scalp [1]. The dead skin is exfoliated usually as patches and flakes of greyish white colour [2]. Yeast like lipophilic basidiomycetous fungus, Malassezia furfur (Pytirosporum ovale) colonization is the key

causative agent for dandruff development [3]. Dandruff was found to be more common among males than females and most predominant in pre-pubertal age. South Asia has reported a greater dandruff prevalence (60.1%) compared to other regions of the world (50%) [4].

Cutaneous Malassezia furfur needs external source of lipids for their survival and hence stimulates the production of lipase enzyme which in turn, converts the sebum lipid into fatty acids and triglycerides, that culminates into hyperproliferation of keratinocytes [5,6]. The cosmeceuticals for dandruff management chiefly contains active ingredients such as zinc pyrithione, salicylic acid, imidazole derivatives, ketoconazole, coal tar, zinc pyrithione, piroctone olamine, triclosan, selenium sulfide and lipase inhibitors and are currently available in the forms of hair serum, lotions, shampoos etc. [7]. These synthetic treatment options have certain limitations, which might be due to poor efficacies, side effects (erythema, dry skin, pruritus, edema, headache) and progression of fungal resistance upon chronic usage [8,9,10]. The preeminent approach to treat dandruff is to use plants or herbal based formulations which possess antidandruff/antifungal properties [7]. This initiative seeks to cultivate remedies characterized by safety, potency, sustainability, and reduced side effects compared to synthetic derivatives. Herbal extracts are enriched with several secondary metabolites like, flavonoids, alkaloids, sterols, carbohydrates, and tannins that contributes to its antimicrobial/anti-fungal properties [11,12].

The drumstick tree leaves are scientifically known as Moringa oleifera, which are native to Indian subcontinent. The Moringa oleifera, is a fast-growing plant belongs to the family Moringaceae [13]. The moringa oleifera is distributed over the Central America and the Caribbean, northern countries of South America, Africa, south and Southeast Asia [14]. The young pods and leaves are used in traditional medicines. The medicinal properties of Moringa oleifera includes, anti-tumor, anti-pyretic, anti-inflammatory, anti-ulcer, antidiabetic, anti-bacterial and anti-fungal activities [15,16,17]. Moringa leaves have shown to contain many active phytoconstituents including, alkaloids (N-benzyl carbamic acid, Aurontiamide acetate, Nicotine), flavonoids (Isoflavone, Neoflavanoids), phenolic compounds (Salicylic acid, Gallic acid, Ellagic acid and Quercetin), saponin, coumarin, glycoside, protein, steroids, and carbohydrates. These bioactive exhibits antimicrobial properties that helps to combat the proliferation of dandruff causing fungi and bacteria on the scalp [18,19].

A plethora of scientific literature demonstrated antifungal potential of Moringa oleifera leaves extract against Fusarium oxysporum, Botrytis cinerea, Aspergillus flavus and Rhizopus stolonifera [20,21,22]. Recently, the antifungal activity of moringa seed oil from East Nusa Tenggara, Indonesia, against dandruff causing M. furfur has been speculated [23]. However, putative role of Moringa oleifera leaves against dandruff causing yeast, Malassezia furfur has remained yet unexplored. Hence, the present investigation was undertaken to unveil in-vitro antifungal potential of ethanolic extract of Moringa Oleifera leaves against Malassezia furfur for dandruff management employing broth dilution and agar cup plate assays. Moreover, the synergistic effect of ethanolic extract of Moringa Oleifera leaves with ketoconazole was also investigated using agar cup plate method.

MATERIALS AND METHODS

Materials

The active Moringa leaves (Figure 1.) were procured from local suppliers and authenticated at Department of Botany, R.T.M., Nagpur University.

Figure 1. The authenticated Moringa oleifera leaves

Preparation of plant extract

The samples of moringa leaves were sorted, washed and shade dried at room temperature for 15 days. The plant materials were crushed well into fine powder in an electronic grinder and kept into airtight polythene bags for further use and stored at room temperature. Approximately 40 gm of moringa leaves powder were soaked and macerated in 400 ml of 70% ethanol for 3 days with occasional stirring (Figure 2.). After extraction, the extract was decanted and filtered through Whatman filter paper no.1. The ethanolic crude extract was obtained by evaporating the solvent using rotary evaporator and water bath at 60°C. The ethanolic extract was weighed (3 gm) and then stored in the refrigerator at 4°C until use [24].

Figure 2. Moringa leaves extraction using maceration method

Phytochemical screening

Moringa oleifera leaves ethanolic extract was subjected to phytochemical screening for presence of phytoconstituents such as alkaloids, flavonoids, saponins, steroids, carbohydrates, tannins, phenolic compounds, and proteins employing the following standard procedures [25,26,27].

1) Detection of flavonoids

A. Alkaline reagent test: To one ml solution of the extract, 1 N NaOH solution was added to give yellow colour. This colour vanishes after addition of few drops of dilute acid indicating the presence of flavonoids.

B. Shinoda test: To the test solution, few magnesium turnings and concentrated HCl was added drop

wise. If the pink, scarlet, crimson red or occasionally green to blue colour obtained after few minutes, shows the presence of flavonoids.

2) Detection of alkaloids

The extract (50 mg) was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents as follows.

A. Mayer's Test: To a few ml of filtrate, a drop or two of Mayer's reagent were added by the side of the test tube. A white or creamy precipitate indicated the positive test.

B. Dragendorff's test: To a few ml of filtrate, 1-2 ml of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive.

3) Detection of carbohydrates

The extract (100 mg) was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

A. Molisch's test

To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

B. Benedict's test

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic-colored precipitate indicated the presence of
sugar.

4) Detection of glycosides

The 1ml of extract was added with 2ml of acetic acid and ferric chloride then add 1ml of conc. sulphuric acid violet colour ring indicates the presence of glycosides.

5) Detection of saponins by foam test

The 2 ml of moringa extract was diluted with 2 ml of distilled water. The suspension was shaken continuously in a graduated cylinder for 15 min. A stable two cm layer of foam indicated the presence of saponins.

6) Test for steroids 0.5 of ethanolic moringa leaf extract was dissolved in 2ml of sulphuric acid and the color change from violet to blue or green, confirmed the presence of steroids.

7) Detection of Proteins and Amino acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatman No.1 filter paper and the filtrate were subjected to tests for proteins and amino acids.

A. **Biuret test:** An aliquot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets, pink colour in the ethanolic layer indicated the presence of proteins.

8) Detection of tannins

A. Ferric chloride test: An extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. Appearance of brownish green or blue black colour

indicated the presence of tannins.

9) Test for phenols
The extract (1ml) was dissolved in distilled water then 3 ml of lead acetate was added, occurrence of bulky white colour, suggested the indication of phenol.

10) Test for Anthraquinone
1ml extract was shaken well in 2ml of chloroform, equal volume of 100% ammonia solution was added. It was mixed well the development of pink, violet or red colour in the ammoniacal layer indicate the presence of anthraquinone.

11) Test for Terpenoids
A. Salkowski's Test: 5ml of the extract were mixed with 2 ml of chloroform and then added carefully the 3 ml of conc. H_2SO_4 to form a layer. An appearance of reddish brown colour in the inner face indicates the presence of terpenoids.

Test microorganism
In the present study, the standard fungal culture of Malassezia furfur (M. furfur) (MTCC No.1374) was procured from Institute of Microbial Type Culture Collection and Gene Bank, Chandigarh (MTCC), India.

Preparation of the media
The culture of Malassezia furfur was prepared by using Sabouraud dextrose agar (SDA). The Loop-full of fungal culture was inoculated in the SDA medium and incubated for 72 hours at room temperature [28].

In vitro antifungal assays of an extract

Preparation of stock solution of moringa leaves extract
The stock solution was prepared by weighing 2 g of ethanolic moringa leaves extract and dissolved in 100 ml of water to obtain 20 mg/ml concentration.

Broth dilution method
The antifungal activity of ethanolic extract of Moringa oleifera leaves were evaluated using broth dilution method according to the procedure described by Shaikh et al. (2010) [29] with some modifications. The broth/tube dilution test is the standard method for determining levels of microbial resistance to an antimicrobial agent. Serial dilutions of the test agent were made in a liquid microbial growth medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. At the end of the incubation period (generally 24-48 hours), the tubes are visually examined for the presence or absence of turbidity.

Determination of minimum inhibitory concentration (MIC)
Minimum inhibitory concentration (MIC) is the lowest concentration of extract that inhibits the growth of test pathogens by inhibiting the visual appearance of turbidity. In the present study, the microbial work was carried out in an aseptic area and the MIC was evaluated by preparing the inoculum of microorganisms from nutrient broth cultures. Initially, the nutrient broth was prepared and sterilized by autoclave using 15 lb pressure at 121 °C for 15 min. The medium was poured into the test tubes. The constant volume of 0.1 ml of standard M. furfur inoculum was added in the test tubes. The extracts were serially diluted, and the exact amount of extract was added using sterile pipettes as indicated in the Table 2. to obtain a final volume of 10 ml. Ketoconazole (1 mg/ml) was used as a reference standard. The tubes were incubated at

temperature 37 °C for 48 hours. The test tubes were evaluated for growth of fungus by observing the presence or absence of turbidity. The test procedure was repeated in triplicates to measure reproducibility of the results. The lowest concentration which showed the absence of turbidity was recorded as the MIC value [30].

Agar cup plate method

The antifungal activity against M. furfur was investigated by agar cup plate method according to the procedure described by Shaikh et al. (2010) and Ruth and Miller, 2015. [29, 31] with slight modifications. Sabouraud dextrose agar medium was poured into the Petri plate. After the medium got solidified, 0.5 ml of diluted fungal suspension was swabbed on respective nutrient agar plates. Then, a hole with a 1 cm diameter was punched aseptically with a sterile cork borer. The 100 µL (0.1 ml) of ethanolic moringa leaves extracts containing 10 mg/ml, 12 mg /ml and 14 mg/ml concentrations were added into each well respectively. Ketoconazole (1 mg/ml) solution was used as a reference standard control. The plates were incubated at 37 °C for 48 hrs. To elucidate synergistic antifungal activity of moringa leaves extract with ketoconazole (12 mg/ml+1 mg/ml), the combination study was performed. The antifungal activity was determined by measuring the diameter of zone of inhibition (mm) around the well by vernier caliper. The data were recorded in terms of mean standard deviation.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The pharmacological characteristics of natural herbs are endorsed by the presence of primary or secondary metabolites (phytoconstituents) which are synthesized naturally by the plants. The qualitative phytochemical analysis of Moringa Oleifera leaves extract was done to test for presence of various phytochemicals. The results of the phytochemical analysis of Moringa Oleifera leaves extract using ethanolic solution has been shown in Table 1. The phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, steroids, carbohydrates, tannins, phenolic compounds, glycosides, and proteins. Our results corroborate previous findings, which suggest that prevalence of several bioactive secondary metabolites in the herbs and Moringa oleifera leaves (flavonoids, phenols, alkaloids and saponins) might be responsible for their antimicrobial traits [21, 32, 33].

In vitro Antifungal/Antidandruff activity of Moringa oleifera leaves extract

In the present research, the antifungal activity of the ethanolic leaves extract of Moringa oleifera against Malassezia furfur was evaluated by broth dilution and agar cup plate method. The broth dilution assay revealed that ethanolic extract of Moringa oleifera leaves dose-dependently inhibited the growth of M. furfur with the increasing concentrations. Table 2. and Figure 3. demonstrates the antidandruff activity of ethanolic extract of Moringa oleifera leaves as revealed by absence of turbidity at 12 mg/ml, 14 mg/ml, and 16 mg/ml relative to control group. These results indicated that the minimum concentration of the extract (MIC) that inhibited the growth of M. furfur was found to be at 12 mg/ml, compared to the control group as evident in the Figure 2. The results are in accordance with previous findings [21]. Demonstrated that the MIC of Moringa oleifera leaf ethanolic extracts against B. cinerea isolates (BCH02 and BCH07) were observed at 10 mg/ml and 15 mg/ml respectively. Moringa seed extracts as well as oil exhibited antifungal effects with MIC at 5% concentration. The discrepancies might be due to different parts of the organized moringa plant and phytochemicals present in it. In the present study, Moringa leaves extract was investigated, wherein, authors used moringa seeds to elucidate its antifungal potential. [34]

In the agar cup plate method, the ethanolic extract of Moringa oleifera leaves exhibited significant

antifungal potential at 10 mg/ml, 12 mg/ml, and 14 mg/ml concentrations with zone of inhibitions of 8±0.45 mm (weak), 12±0.57 mm and 14±0.25 mm (strong; Figure 4.) respectively as compared with ketoconazole control (15±0.34 mm). Table 3. depicts the dose-dependent zone of inhibitions of ethanolic Moringa oleifera leaves extract at different concentrations against M. furfur. The present findings confirmed the antidandruff potential of ethanolic extract of Moringa oleifera leaves as revealed by inhibitory effect against M. furfur. The results of present study are in accordance with the previous findings. Recently, moringa seed oil at 12.5% effective concentration exhibited very strong antifungal activity using in-vitro agar diffusion method [35]. Ethanolic extract of Moringa oleifera leaves demonstrated significant antifungal activity against Fusarium oxysporum at 1 mg/ml concentration with zone of inhibition of 3.50±0.10 [25]. Additionally, Moringa oleifera leaves extract revealed antifungal activity against several fungal species including, Fusarium oxysporum, Botrytis cinerea, Aspergillus flavus and Rhizopus stolonifera [14, 15, 16, 17]. It has been claimed that moringa leaves contain several phytoconstituents (alkaloids, flavonoids, phenolic compounds, tannins, triterpenoids, saponins) which attributes to its antimicrobial and antifungal effects [12,32,33]. In the combination study, ethanolic extract of moringa leaves with ketoconazole at 12 mg/ml+1 mg/ml concentrations exhibited synergistic effect with the zone of inhibitions of 20±0.62 mm (Very strong) as compared with Moringa oleifera leaves extract alone (12±0.57 mm) and ketoconazole control (15±0.34 mm; Figure 5 and Table 4). The current results are in accordance with our previous findings, which speculates that combination therapy of carica papaya leaves extract with ketoconazole displayed synergistic antidandruff effects relative to monotherapy [36]. Altogether, we suggest that moringa leaves extract possess effective anti-dandruff potential against dandruff causing M. furfur. Moreover, combination therapy of moringa leaves extract with synthetic antifungal agents could pose a promising strategy in combating the side effects and minimize or delay the emergence of fungal resistance [37,38].

CONCLUSION

The current study unveils the novel herbal antidandruff potential of moringa leaves extract. Additionally, moringa leaves extract in conjunction with ketoconazole significantly revealed synergistic activity over alone bioactive extract. The current findings validate the relevance of combination therapy of bioactive compound (moringa leaves extract) with synthetic drugs that might improve the therapeutic efficacy and lower the dose and toxicity associated with synthetic antidandruff agents.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgment

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Table 1: Evaluation of phytochemical screening of ethanolic extract of Moringa Oleifera leaves

SECONDARY METABOLITES	CHEMICAL TESTINGS	INFERENCE
Alkaloids	**Mayer's test & Dragendorff's Test**	+
Flavonoids	**Alkaline reagent & shinoda Test**	+
Carbohydrates	**Molisch's & Benedict's Test**	+

Glycosides	**Keller killiani Test**	+
Saponin	**Foam Test**	+
Steroid	**Sulphuric Acid Test**	+
Protein	**Biuret Test**	+
Tannins	**Ferric chloride Test**	+
Phenolic compounds	**Lead Acetate Test**	+
Anthraquinone	**Borntrager's Test**	+
Terpenoids	**Salkowski Test**	+

+ **indicates the presence of phytochemical compounds.**

Figure 3: The minimum inhibitory concentration (MIC) of ethanolic extract of Moringa oleifera leaves using broth dilution method.

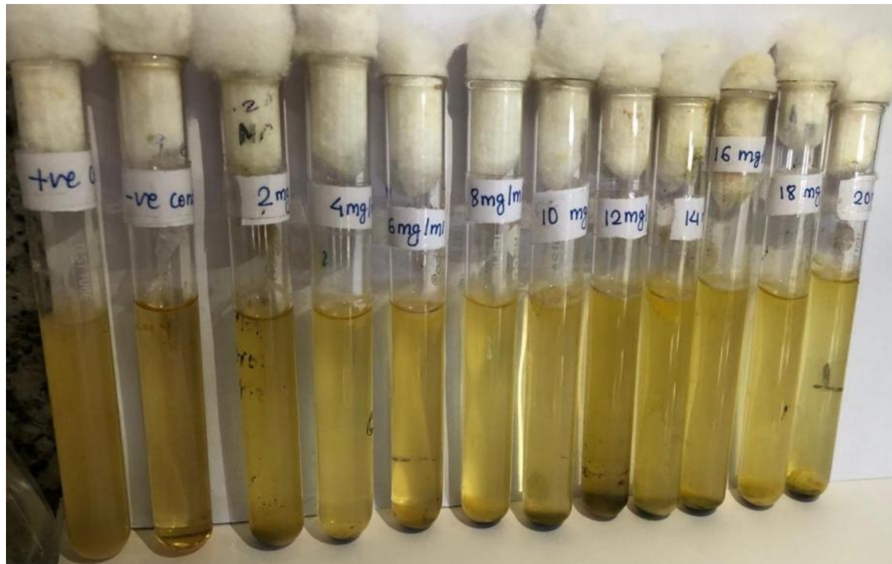

Table 2: Evaluation of minimum inhibitory concentration (MIC) against Malassezia

Sr.no	**Amount of Extract (ml) (Stock:20 mg/ml)**	**Concentration of extract (mg/ml)**	**Amount of medium Broth + inoculum**	**Total volume of solution (ml)**	**Turbidity**
1)	0.1	2 mg/ml	9.9 ml	10 ml	++
2)	0.2	4 mg/ml	9.8 ml	10 ml	++
3)	0.3	6 mg/ml	9.7 ml	10 ml	++
4)	0.4	8 mg/ml	9.6 ml	10 ml	++
5)	0.5	10 mg/ml	9.5 ml	10 ml	++
6)	0.6	12 mg/ml	9.4 ml	10 ml	-
7)	0.7	14 mg/ml	9.3 ml	10 ml	-
8)	0.8	16 mg/ml	9.2 ml	10 ml	-
9)	0.9	18 mg/ml	9.1 ml	10 ml	-
10)	1.0	20 mg/ml	9 ml	10 ml	-

11)	-	0	10 ml (broth only)	10 ml	-
12) Ketoconazole (Standard control)	1 ml (Stock: 10 mg/ml)	1 mg/ml	9 ml	10 ml	-

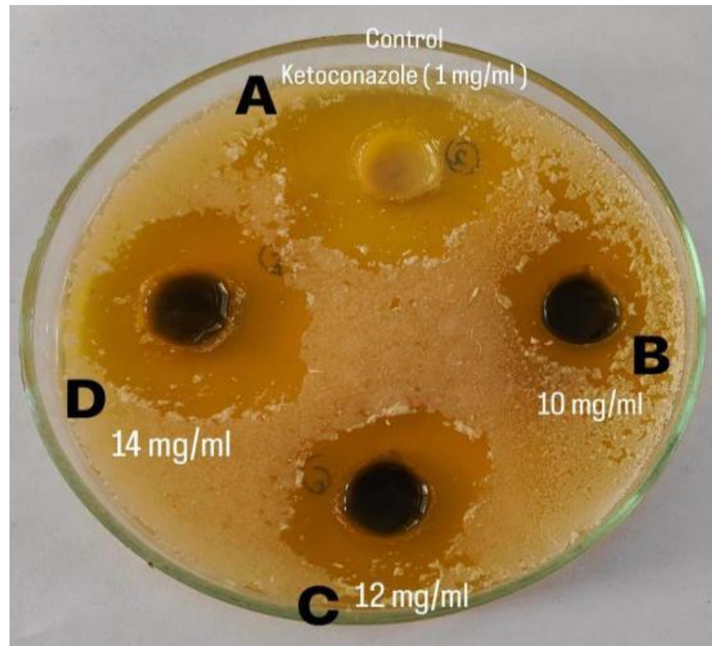

Figure 4: The zone of inhibitions of ethanolic extract of Moringa oleifera leaves against Malassezia furfur at different concentrations using agar cup plate method.

- Standard control Ketoconazole (1 mg/ml)
- Ethanolic Moringa leaves extract (10 mg/ml)
- Ethanolic Moringa leaves extract (12 mg/ml)
- Ethanolic Moringa leaves extract (14 mg/ml)

Table 3: Dose-dependent antifungal activity of Moringa Oleifera leaves extract (Test) against Malassezia furfur using agar cup plate method

Ethanolic moringa leaves extract (Test)	Concentration			Control (ketoconazole)
	10 mg/ml	12 mg/ml	14 mg/ml	
Zone of inhibition (Mean±SD)	8±0.45 mm,	12±0.57 mm	14±0.25 mm	15±0.34 mm

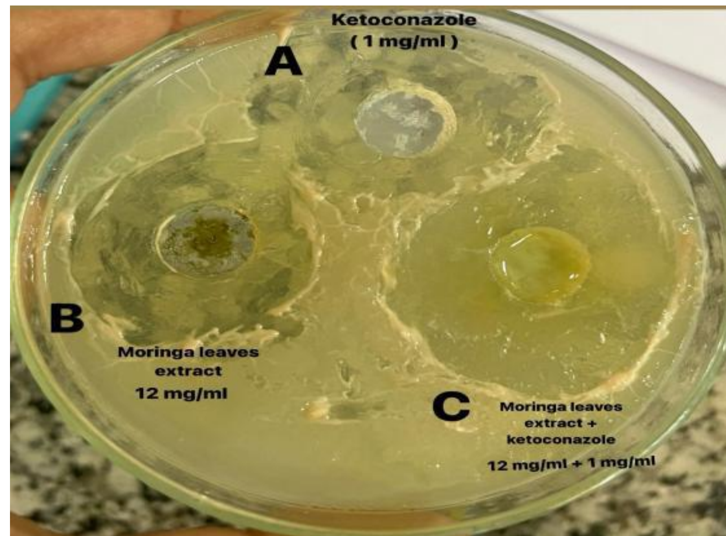

Figure 5: The zone of inhibitions of combination of moringa leaves extract with ketoconazole against Malassezia furfur at different concentrations using agar cup plate method.

- Ketoconazole (1 mg/ml)
- Moringa leaves extract (12 mg/ml)
- Ketoconazole (1 mg/ml) + Moringa leaves extract (12 mg/ml)

Table 4: The zone of inhibitions of ethanolic extract of Moringa oleifera leaves (Test) in combination with control against Malassezia furfur using agar cup plate method.

Concentration	**Ethanolic moringa leaves extract alone (Test)**	**Ethanolic moringa leaves extract (Test) + Control**	**Control (Ketoconazole)**
	12 mg/ml	12 mg/ml+1mg/ml	1 mg/ml
Zone of inhibition (Mean±SD)	12±0.57 mm	20±0.62 mm	15±0.34 mm

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