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Studies on the combinations of some herbals with various chemical entities as a potent antifungal agents

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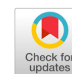

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ABSTRACT

A widespread increase in the prevalence of fungal infections has been documented in recent decades. Candida albicans infections, which are frequently refractory and linked with high morbidity and mortality, place a significant burden on public health, despite the fact that existing antifungal medicines are restricted and associated with toxicity. Fungi are one of the most underappreciated killers, as evidenced by the fact that Amphotericin B and other commercially available antifungal therapies are still recognized as gold standards. The majority of commonly used antifungal medications have toxicity, effectiveness, and cost disadvantages. As a result of these limitations, there is a growing demand for the development of a novel antifungal medication treatment that acts selectively on new targets while having the fewest adverse effects. Natural goods, whether as pure phytocompounds or regulated plant extracts, give prospects for the development of lead compounds that may subsequently be turned into diverse synthetic medications with the appropriate alterations. These herbs can also be used as a component of a herbal synthetic combination, lowering the minimum required dose of the synthetic medicine (when taken singly) and reducing the risk of adverse effects. The goal of this research is to reduce the minimum required concentrations of today's antifungal medications by mixing them with a few less well-known herbal extracts while maintaining their efficacy.

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1. Introduction

A fungus causes a fungal infection, also referred as mycosis, which is a skin infection. Fungi are found in millions of different species. They can be found in the earth, on plants, on surfaces around the house, and on your skin. They often can cause skin issues like rashes or bumps. Fungi are eukaryotic, meaning they have nuclei, mitochondria, and cell membranes, much like human cells. Their membranes, however, take two main sterols, ergosterol and lanosterol. Antibacterial therapy's effectiveness has produced an environment conducive to the spread of opportunistic fungal infections. Furthermore, powerful immunosuppressive and cytotoxic medications lead patients to have severely weakened immune systems, allowing fungi that are non-pathogenic in healthy people to become infectious and cause illness in them.[1] This project aims at lowering the concentration of the antifungal drug required for the inhibitory action of the fungi by virtue of some herbal plants. Fungi usually make their homes in moist areas of the body where skin surfaces meet: between the toes, in the genital area, and under the breasts. Common fungal skin infections are caused by yeasts (such as Candida or dermatophytes), such as Epidermophyton, Microsporum, and Trichophyton.

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Many such fungi live only in the topmost layer of the epidermis (stratum corneum) and do not penetrate deeper. Fungal infections are typically treated with antifungal drugs, usually with antifungal drugs that are applied directly to the affected area (called topical drugs). Topical drugs may include creams, gels, lotions, solutions, or shampoos. Antifungal drugs may also be taken by mouth. Antifungal creams, liquids or sprays (also called topical antifungals) these are used to treat fungal infections of the skin, scalp and nails, includes clotrimazole, econazole, ketoconazole, miconazole, tioconazole, terbinafine and amorolfine. But these antifungal agents may cause many serious side effects such as a bit of itch, burning or redness where the antifungal preparation has been applied, may cause blisters and some allergic reactions in few people. Some antifungal preparations cause liver problems or more serious side-effects in a small number of people when orally administered.[2]

Candida albicans and Candida glabrata account for the majority of the fungus infected population, with C. albicans accounting for 49% and C. glabrata for 22%, respectively. As a result, the remainder of this article's research will focus solely on Candida albicans. Candida is a yeast species (fungus) generally lives in small amounts on your skin. They can multiply and cause infection under certain circumstances. Candida infections most commonly occur in the vaginal (vaginal thrush), oral (oral thrush), and skin. Terbinafine, Itraconazole, Clotrimazole, Econazole, and Amphotericin B are some of the medications that are accepted and used to treat candidiasis.[3] But considering the treatment which includes Terbinafine, it has many serious side effects and may cause stomach aches, loss of appetite, feeling sick (nausea), tummy upsets, diarrhea, headache, rash, taste disturbance and muscle or joint pains.[4] Renal insufficiency, hypokalemia, hypomagnesemia, metabolic academia, and polyuria related to nephrogenic diabetes insipidus are all symptoms of Amphotericin B nephrotoxicity.[5] As a result, Terbinafine and Amphotericin B are considered to be the most important drugs in this initiative. Terbinafine has a MIC of 12 g/ml, according to multiple publications, and the concentrations in marketed formulations are approximately identical to the MIC value.[6] The MIC of Amphotericin B has been reported to be 1.09g/ml in multiple journals, and the concentrations in commercially available preparations are virtually identical to the MIC.[7] But it is still unknown and undiscovered as to how the side effects associated with the Terbinafine and amphotericin B can be reduced without co-administration of other pharmaceuticals which thereby increase the chances of drug interactions. This project directs towards decreasing the concentration of the Terbinafine and Amphotericin B while associating it with the use of herbal plant extract. In a generalized manner the project is intended to focus on researching over use of combination of drug and herbals as antifungal treatment. The majorly caused and commonly found fungal infection includes Candidiasis. This project points at reducing the toxic effects of Terbinafine and Amphotericin B by decreasing its MIC and making combinations with three different herbs so as to get the additive effect or at least the equivalent effect. This guarantees that, even if the concentration of a drug that produces significant toxicity is reduced, an equivalent or additive effect can be reached using a few herbals with high antifungal activities. Terbinafine and amphotericin B's adverse effects are proportional to their concentration, therefore they can be reduced to some extent.

2. Experimental

The experiment is set up in such a way that finding the most reliable, intermediate combination concentration among all potential combinations and concentrations is possible. Assume a general experiment response when Terbinafine is used in conjunction with other medications. Each series is built by combining Terbinafine doses in ascending order with supporting Extract concentrations in descending order, with the goal of identifying the combination concentration that provides the best overall response. This design effectively emphasises solutions to issues such as,

If the plant extract has any action against the fungus individually — As the highest dose of plant extract (F) is paired with blank dose of Terbinafine (A), the response shown by this combination will only be due to the action of plant extract.

Because the highest dose of Terbinafine will be combined with Blank concentration, the response in this plate will be comparable to the medium combination responses if any additive activity against fungus occurs as a result of the combination. So, if the previous plates show action near or equivalent to the highest dose of terbinafine, it's safe to believe that Terbinafine and extract have synergistic effects.

Selection of the best combination that can effectively replace Terbinafine as a single medicine - The combination pair that demonstrates action in the desired range with a lower dose of Terbinafine may be clearly recognized and chosen.

The experiment can also be categorized under

1. Correlation — The experiment intends to identify and produce a combination product, that is nearly as equal or better than marketed fungal creams.
2. Experimental — The experiment intends to find out whether the herbal products work against fungus.

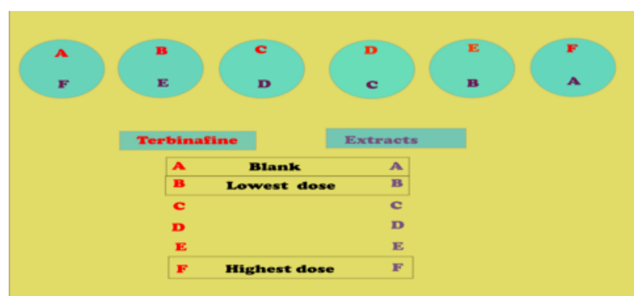

Fig. 1: Pictorial representation of research design.

Fig. 2: Powdered stems and leaves of heliotropium indicum linn

3. Preparation of Plant Extract

3.1. *Heliotropium* ***i****ndicum linn*

The plant for this reports the plant was collected from the garden areas of Koperkhairane, Navi Mumbai. For the preparation of plant extract the leaves and the stem were separated. The collected plant parts were separated from undesirable materials or plants or plant parts. They were sun-dried for one week after cutting into small pieces. Once dried the leaves and the stems were grinded to form a moderately coarse powder with the help of a suitable mechanical grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. The weight of the powder obtained was obtained as 19.79gm and 20.36gm of stems and leaves respectively.

For the extraction of plant material, the powder of dried leaves and stems were processed with the help of Soxhlet apparatus. Extract was prepared using 300ml of ethanol. Extract was filtered using Whatman No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent

The extract obtained is then carried out in a suitable evaporator which will concentrate bulky solution down to small volumes. The further studies were done using this concentrated extract.[8]

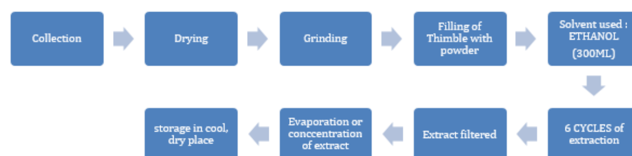

Fig. 3: Flow chart depicting extraction sequence of *Heliotropium Indicum Linn.*

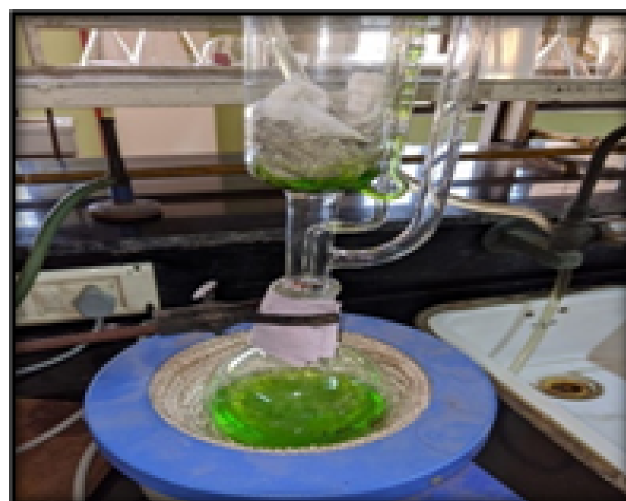

Fig. 4: Soxhlet extraction Process of *HELIOTROPIM INDICUM* Leaves and Stems

3.2. *Thunbergia grandiflora* ***r****oxb*

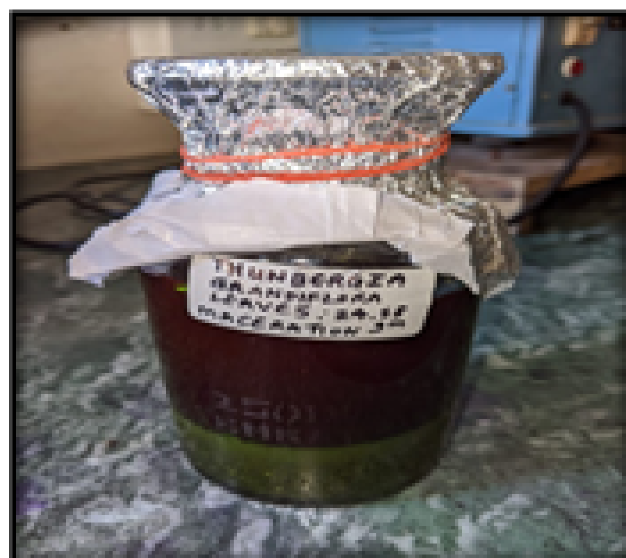

Fig. 5:

Fig. 6: Powdered leaves of thunbergia grandiflora Roxb & maceration of thunbergia grandiflora roxb

4. Extraction: Cold Maceration

Leaves of T. grandiflora were collected, washed and rinsed properly. They were dried in shade and powdered mechanically. About 24.16gm of the leaves powder was macerated with pet. ether, ethyl acetate and ethanol and stored for 72 hours in ice cold condition for the extraction of phytochemicals. At the end of the third day extract was filtered using Whatman No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using electric water bath and stored in an air tight container free from any contamination until it was used.

5. Lepidagathis Trinervis Nees

5.1. *Extraction: Cold maceration of stem*

Dried stems of plant were grinded and powder was macerated using 250ml of Ethanol (95%) and kept for 72 hours in 2-8 degree Celsius. The extract was filtered using Whatman filter paper number 1 and concentrated using suitable evaporator.

5.2. *Ash procedure*

Dried flowers were grinded and converted to ash by heating for 1hr in a muffle furnace. Per batch has an average of 5.4grams of plant material converted to 1.12 gram of ash. Total weight of Ash obtained is 11.6 gram

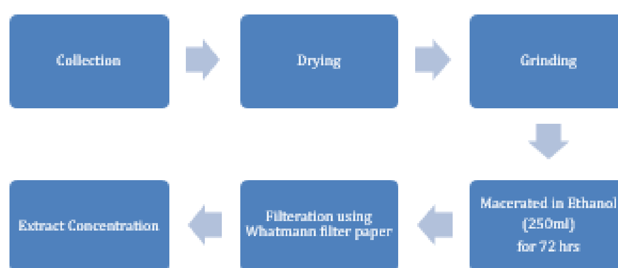

Fig. 7: Flow chart depicting extraction sequence of *Lepidagathis trinervis*

6. Concentrating Extracted Samples

6.1. *For liquid extracts*

1. Electric Water bath is switched on and set-to desired temperature (40-50 °C
2. The extracts containers were removed from freezer and kept in room temperature for few seconds. Meanwhile, Label and weigh empty Porcelain dishes.
3. Approximately 100ml of each extract was placed in labeled dishes and put on electric water bath.
4. Each extract being different in composition, alcohol evaporation rate and time required for slurry formation may differ, so careful monitoring is essential.
5. As soon as any extract has converted to slurry with a approx. 4-5ml alcohol content left, stop heating and let it to cool down in room temperature.
6. After extracts cool down, Weigh Each slurry (Approx 1-2ml liquid with residue
7. Each Weighed slurry is dissolved in 3-4ml of distilled water, and concentration is noted –
8. Concentration of Extract (w/v — (Weight of slurry / Volume of solvent
9. The Mixture container is transferred covered with foil

6.2. *For ash sample*

1. The Prepared Ash was weighed and delivered to the zone of inhibition, initial weight being .02gm, with an increment of .01gm in successive combinations.
2. Quantities of 0.02gm, 0.03gm, 0.04gm, 0.05gm and 0.06gm will be measured on a sensitive weighing machine and pack separately on small butter papers.
3. While administering in combination, pour the desired amount of ash in the hole before adding liquid samples.

6.3. *Procedure for stock solution (100ug/ml) Preparation for Terbinafine*

1. Weigh 1 gm Terbinafine API powder
2. In a 100ml flash dissolve the measured powder in 50ml of water and 50ml of methanol, and shake for 5 minutes. (Concentration achieved — 10mg/ml).

Fig. 8: The ash procedure for the plant *Lepidagathis trinervis nees*

3. Filter this solution through a Whatman paper
4. Pipette out 1ml of this solution and dissolve in 100ml of Methanol and water (1:1) in a 100ml Flask
5. (Concentration achieved — 100ug/ml).
6. Use the stock solution for making sample solutions

6.4. *Procedure for sample solution preparation*

1. Take five washed 10ml flask, and label them as 5ml, 10ml, 15ml, 20ml ,25ml
2. In each of the flask add amount of solvent (1:1 Methanol: water) and stock solution as mentioned in table.
3. Use the prepared samples for combination zone of inhibition study
4. Also use these samples for analytical measurements (At Lambda Max- 283nm) and report in observation section below.

Table 1: Details of component quantity for each concentration

Concentration (ug/ml)	Volume of stock solution added (ml)	Volume of Solvent added (ml)
5	0.5	9.5
10	1	9
15	1.5	8.5
20	2	8
25	2.5	7.5

6.5. *Procedure for stock solution (100ug/ml) Preparation for Amphotericin B*

1. Weigh 1 gm Amphotericin B API powder
2. In a 100ml flask dissolve the measured powder in 100ml of Water, and shake for 5 minutes. (Concentration achieved — 10mg/ml).
3. Filter this solution through a Whatman paper
4. 4. Pipette out 1ml of this solution and dissolve in 100ml of Water in a 100ml Flash
5. (Concentration achieved — 100ug/ml).
6. Use the stock solution for making sample solutions

6.6. *Procedure for sample solution preparation*

1. Take five washed 10ml flask, and label them as 5ml, 10ml, 15ml, 20ml ,25ml
2. In each of the flash add amount of solvent (Water) and stock solution as mentioned in table.
3. Use the prepared samples for combination zone of inhibition study
4. Also use these samples for analytical measurements (At Lambda Max- 361nm) and report in observation section below.

Table 2: Details of component quantity for each concentration

Concentration(ug/ml)	Volume of stock added (ml)	Volume of Solvent added (ml)
5	0.5	9.5
10	1	9
15	1.5	8.5
20	2	8
25	2.5	7.5

7. Preparation of Zone of Inhibition Plates and Introducing Combination

7.1. *Preparation of candida albicans plates*

1. Take and wash 24 Petri dishes, Dry and properly label them according to Coding as mentioned in earlier Observation table.
2. Pour the SDA (Sabouraud dextrose agar medium

3. After the poured medium dries out use a platinum loop to spread out Fungal sample (Candida Albicans) on the plate using streak plate method, keeping in mind the proper spreading of sample.
4. With the tip opening of a 10ml ampoule make 3 holes in each dish with proper distance.
5. Introducing Combination to the Microbial species
6. Weighed ash is to be administered before Terbinafine, in Ash + Terbinafine combination
7. Terbinafine is to be administered in ascending order, and the complimentary sample of the combination is to be administered in descending order.

Note — Streaking of microbes on plate and sampling into the plates is strictly to be done in aseptic conditions with proper protective measures (Gloves, Cap and Mask).

8. Observation Table (Terbinafine + Extracts)

8.1. Coding

T — Terbinafine (Always first in coding, followed by extract)

Combination of each extract with Terbinafine is done on 2 petri dishes with 6 holes total

8.2. Observation table (Amphotericin B + Extracts) Coding

Amphotericin B (Always first in coding, followed by extract)

Combination of each extract with Amphotericin B is done on 2 petri dishes with 6 holes total

8.3. Phytochemical tests

Heliotropium indicum linn:

8.4. Thunbergia grandiflora roxb

Lepitagathis trinervis

8.5. Chemical test for detection of inorganic constituents

8.6. Procedure

Prepare ash of drug material. Add 50% v/v HCl or 50% HNO_3 to ash. Keep for 1 hour or longer. Filter. With filtrate perform the following test.

9. Result and Discussion

Out of the five plant extracts tested against Candida Albicans in combinations with standard antifungal drug Terbinafine, the three combinations showed prominent response as additive and individual agents. Their overall response (in combination with Terbinafine and

Table 3: Observations of the response obtained for each combination (T+E) with vary concentrations.

Zone of Inhibition/Respective Extract = E Terbinafine T	**Well 1 (ZOI in mm) T-25ug/ml + E-0gm/ml**	**Well 2 (ZOI in mm) T-20ug/ml + E-0.3gm/ml**	**Well 3 (ZOI in mm)T-15ug/ml + E-0.6gm/ml**	**Well 4 (ZOI in mm) T-10ug/ml + E-0.9gm/ml**	**Well 5 (ZOI in mm) T-5ug/ml + E-1.2gm/ml**	**Well 6 (ZOI in mm) T-0.0ug/ml + E-1.5gm/ml**
Heliotropium Indicum Leave (E-HL)	31 mm	29mm	30mm	30mm	28mm	25mm
Heliotropium Indicum Stem(E-HS)	30mm	23mm	20mm	15mm	12mm	09mm
Lepidagathis Trinervis Stem (E-LL)	32mm	25mm	19mm	14mm	11mm	04mm
Lepitagathis Trinervis Ash(E-LA)	33mm	30mm	31mm	30mm	31mm	30mm
Thunbergia Grandiflora Leaves(E-TL)	32mm	30mm	30mm	29mm	30mm	29mm

Table 4: Observations of the response obtained for each combination (AB+E) with vary concentrations.

Zone of Inhibition/Respective Extract = E Amphotericin B = AB	Well 1 (ZOI in mm) AB-25ug/ml + E-0gm/ml	Well 2 (ZOI in mm) AB-20ug/ml + E-0.3gm/ml	Well 3 (ZOI in mm) AB-15ug/ml + E-0.6gm/ml	Well 4 (ZOI in mm) AB-10ug/ml + E-0.9gm/ml	Well 5 (ZOI in mm) AB-5ug/ml + E-1.2gm/ml	Well 6 (ZOI in mm) AB-0.0ug/ml + E-1.5gm/ml
Heliotropium Indicum Leave (E-HL)	31 mm	29mm	30mm	30mm	28mm	25mm
Heliotropium Indicum Stem(E-HS)	30mm	23mm	20mm	15mm	12mm	09mm
Lepidagathis Trinervis Stem (E-LL)	32mm	25mm	19mm	14mm	11mm	04mm
Lepitagathis Trinervis Ash(E-LA)	33mm	30mm	31mm	30mm	31mm	30mm
Thunbergia Grandiflora Leaves(E-TL)	32mm	30mm	30mm	29mm	30mm	29mm

Table 5: Chemical analysis of *Heliotropium Indicum Linn.*

Test	Observation	Inference
Alkaloids		
Dragendorff's	Orange brown ppt	Positive
Wagner's	Brown ppt	Positive
Hager's	Yellow ppt	Positive
Mayer's	No ppt	Negative
Flavanoids		
Shinoda's Test	No colour change	Negative
Tannins		
Lead Acetate's Test	No White ppt	Negative
Cardiac Glycosides		
Legal's	No Pink/Red Colour	Negative
Keller-Killiani	Bluish Green	Negative
Anthraquinones		
Borntrager's	No pink or Red colour	Negative
Modified Borntrager's	No pink or red colour	Negative

Table 6: Chemical analysis of Thunbergia grandiflora Roxb

Test	Observation	Inference
Alkaloids		
Dragendorff's	Orange brown ppt	Positive
Wagner's	Brown ppt	Positive
Hager's	Yellow ppt	Positive
Mayer's	No ppt	Negative
Flavanoids		
Shinoda's Test	No colour change	Negative
Tannins		
Lead Acetate's Test	White ppt	Positive
Cardiac Glycosides		
Legal's	No Pink/Red Colour	Negative
Keller-Killiani	Bluish Green	Negative
Anthraquinones		
Borntrager's	No pink or Red colour	Negative
Modified Borntrager's	No pink or red colour	Negative

Table 7: Chemical analysis of *Lepitagathis Trinervis*

Test	Observation	Inference
Alkaloids		
Dragendorff's	Orange brown ppt	Positive
Wagner's	Brown ppt	Positive
Hager's	Yellow ppt	Positive
Mayer's	No ppt	Negative
Flavanoids		
Shinoda's Test	No colour change	Negative
Tannins		
Lead Acetate's Test	White ppt	Positive
Cardiac Glycosides		
Legal's	No Pink/Red Colour	Negative
Keller-Killiani	Bluish Green	Negative
Anthraquinones		
Borntrager's	No pink or Red colour	Negative
Modified Borntrager's	No pink or red colour	Negative

Table 8: Inorganic test for Ash of *Lepidagathis Trinervis*

Test	Observation	Inference
1)Test for potassium a) To 2-3 ml test solution, add few drops sodium cobalt nitrate solution	Yellow ppt. of potassium cobalt nitrate is observed.	Potassium may be present.
2)Test for iron a) To 5 ml test solution add few drops 2% potassium ferrocyanide. Dark blue coloration is observed. b) To 5ml test solution, add few drops 5% ammonium thiocyanate.	a) Dark blue coloration is observed. b) Solution turns blood red.	Iron may be present.
3) Test for chloride a) To 3ml of test solution prepared in HNO_3, add few drops 10% $AgNO_3$ solution.	White ppt. of $AgCl_2$ is observed. Ppt. is soluble in dil. Ammonia solution.	Chloride may be present.
4) Test for carbonate a) With dil. acid liberate carbon dioxide	Effervescence observed.	Carbonate may be present.
5) Test for phosphates To 5ml test solution prepared in HNO_3, add few drops ammonium molybdate solution. Heat for 10 minutes. Cool.	Yellow crystalline ppt. of ammonium phosphomolybdate is observed.	Phosphate may be present.
6) Test for sulphates To 5ml filtrate, add few drops 5% $BaCl_2$ solution.	White crystalline $BaSO_4$ ppt. appears, insoluble in HCl.	Sulphate may be present.
7) Test for nitrates Heat with sulphuric acid and copper.	Red fumes observed.	Nitrates may be present.

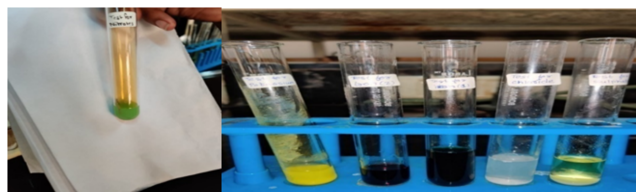

Fig. 9: Observation of the inorganic test

Amphotericin B) was in the order –Lepitagathis trinervis ash > Thunbergia grandiflora leaves > Heliotropium indicum leaves.

Based on the observations above, it is clear that the selected Herbal species when combined with present day antifungal drugs Terbinafine and Amphotericin B, gave good or nearly as good actions as compared to higher concentration of Terbinafine and Amphotericin B given individually. Another Herbal species that showed no additive response but did show slight response as an individual drug is Heliotropium Indicum Linn stems and Lepidagathis Trinervis Stem. This suggests that on increasing the concentration of the extract, Heliotropium Indicum Linn stems and Lepidagathis Trinervis Stems may show better response as Individual or as a combination.

10. Conclusion and Outcomes

This project aimed to find and propose novel alternatives to present conventional antifungal drugs. To be able to compare together all the herbal species response on varying concentration and with different drugs, concentrations for herbal and drugs in each dose was standardized. Though standardization did help in comparing all the responses and also eliminating assumptions, this does not with certainty account if remaining Herbal extracts can show Antifungal

action (Individually or in Combination) at much higher concertation, For example Even after decrease in response concentration in both Amphotericin B and Terbinafine (with rising extract concentration) *Heliotropium Indicum Linn.* stem did show slight rise in response as pure extract which suggests higher concentration of this drug have chance of showing adequate action. With obtaining positive results to many of the combinations, this project can be further be extended to research in multiple direction. As subject of our experiment is an ideal Fungal species (Candida albicans), possessing many similar features with other fungal species of same or other genus, we are very hopeful of future use of our research against other pathological fungus that may cause common diseases or fatal infections. To make the extracts more potent, separation and purification of all potential active substance in extract can be utilized. Purification will make the Herbal Formulation / Combination much more specific in its action and can also be used as parent moiety for molecular modelling which if successful can be developed into series of such new drugs (with necessary modification) with a main chemical base frame. Antifungal combinations used in our experiment can be commercialized into various dosage forms, for both topical (Gels, creams, ointments against topical fungal infections) and systemic use (after invitro test). In the end, we summarize by stating that the experiment proved three of the five extracts obtained from three different Species to be Novel antifungal agents in combinations with present antifungal agents like Terbinafine and Amphotericin B or individually.

11. Source of Funding

None.

12. Conflict of Interest

None.

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