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IN-VITRO STUDY ON THE PRESERVATIVE EFFECT OF ANTI-MICROBIAL COMPOUNDS EXTRACTED FROM *ACALYPHA INDICA LINN.* ON COSMETIC PREPARATIONS

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ABSTRACT

Cosmetics are personal care products which containing water and organic / inorganic compounds that are sensitive to microbes, due to the possibility of cross contaminations during manufacturing, filling, and packing process. Hence, it required the right preservation against microbial contamination to ensure the safety and shelf life of the product. The most common strategies are the application of anti-microbial agent, either by using Natural or synthetic compounds including multifunctional ingredients. Therefore, the present study is to evaluate the preservative effect of the methanolic leaf extract of *Acalypha indica L.,* collected from Pallipattu village of Thiruvallur district of Tamil Nadu, India. In Cosmetic preparation by in-vitro study, the crude methanolic extract re-dissolved in propylene glycol was examined for Preservative effect (challenge and re-challenge test) by *In-vitro* method followed the method of Cosmetic, Toiletry, and Fragrance Association 2007. (CTFA) Microbiology Guidelines. The anti-microbial effect of *Acalypha indica L.*, effect of 1.00 to 2.00% of concentrations tested against the gram-negative bacteria *Pseudomonas aeruginosa* and the fungal species *Aspergillus niger* by using Agar plate count method at various intervals up to 28 days. The result of the study indicates that decreasing of microbial cells at increasing intervals such as 24hrs, 48hrs, 5days, 7days, 14days, 21days and 28days. However, after 5 days of incubation, the microbial growth is completely inhibited as nil growth observed even up to 28days period. The present study concludes that the compounds extracted from *Acalypha indica L* has moderate to significant level of antimicrobial effect against the microbes in the cosmetic preparations which could be used as a good natural preservative in various personal care products especially cosmetic preparations.

Key words: Cosmetics preparation, Antimicrobial activity, *Acalypha indica L*, Preservatives

INTRODUCTION

The global cosmetics market was $460 billion in 2014 and is expected to reach $675 billion by 2020 at an estimated growth rate of 6.4% per year **[1]**. This was educating to market which requires continuous multidimensional control, like to monitor toxic ingredients and environmental contamination such as physical, chemical, and biological contamination. Some of the cosmetics found with hazardous chemical compounds which is risk to consumer due to the presence of prohibited or restricted substances in accordance with cosmetic laws. According to the Rapid Alert System (RAPEX) of the European Commission (EC), 62 cosmetic products were recalled during the period between 2008 and 2014 due to contamination by microorganisms. The recalled products were notified by 14 different countries and their number was higher in 2013 and 2014 **[2]**.

In general, the cosmetic products spoilage is due to the presence of microorganisms or might result from the exposure to atmospheric oxygen. To prevent the products from these effects, two distinct groups of substances can be used, namely, antimicrobial preservatives, which act on microorganisms, and antioxidant preservatives capable of suppressing oxidation phenomena and the formation of free radicals **[3]**. In regulatory terms, a preservative is a substance of natural or synthetic origin intended to inhibit the development of microorganisms **[4]**. This inhibitor should be very effective over a broad activity spectrum and should have an expected shelf-life period with reference to the usage time. The cosmetic products are a nutrient-rich medium that favours the growth of microorganism, which, thereafter, influences the efficacy of the preservatives. The amount of antimicrobial agent to be used in a cosmetic is subject to use of other multifunctional components.

A cosmetic product is "any mixed formulations or emulsions intended to be applied on the surface of the human skin (*epidermis, hair, nails, lips and external genital organs)* with the purpose of Cleansing, Conditioning, smelling, Moisturizing, etc., **[5]**. These cosmetics products should be safe under normal or reasonably foreseeable conditions of use. While the regulation on cosmetic products (EC 1223/2009) (1), it requires that the cosmetic product safety report demonstrates the results of preservation challenge test to prove the microbiological stability. The first test method was established by ISO 11930 in first edition published in April 2012 under the title "Cosmetics - Microbiology - Evaluation of the antimicrobial protection of a cosmetic product" **[6]**.

Since, the use of chemical preservative in cosmetics are reported to be harmful side effects, are regarded as unsafe to human and environments **[7]**, the biological origin of preservatives especially plant sources are preferred by consumers. Medicinal Plants are

the important resource of drugs oftraditional systems of medicine, modern medicines, Nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs and finally the cosmetics applications **[8]**. In pharmaceutical intermediates, Jayarani, *et al* (2019) have identified the potential bioactivities of grape phytochemicals were examined in two racially different breast cancer cell lines **[9]**.

Recently, the cosmetic and personal care market has been more and more move towards natural / plant-based ingredients by raising consumers' awareness about personal health and safer cosmetics free of harmful chemicals. There are numerous synthetic preservatives are used in cosmetics preparation, suspicion they are produced toxicity, and hence the cosmetics industry forward to find natural alternatives and the availability of natural preservatives are very limited **[10]**. The best thing of the herbal personal care products is that it is purely made of the herbs and shrubs and therefore provides the body with nutrients and other useful minerals. However, growing scientific evidence that plants possess a massive and complicated collection of active components (phytochemical) in a position not only for sole purpose to calm or smoothen the skin but also, however, actively restoring, healing, skin protecting and self-preservative **[11]**.

Based on the above comments concerning the development of self-preserving cosmetics and to focus our research to evaluate the preservative efficacy of crude plant extracts of medicinally important plant species ***Acalypha indica Linn,*** in the cosmetics surfactant base preparations.

MATERIALS AND METHODS

Sample Collection and Preparation:

The plant species ***Acalypha indica Linn***. was identified by using The Flora of the Presidency of Madras by Gamble in our laboratory and further confirmation and authentication was done by reputed taxonomist, Dr. P. Jayaraman, Plant Anatomy Research Centre, Chennai, Tamil Nadu. The dried specimen in the form of herbarium is preserved in our Botany Laboratory for further reference. Fresh leaves of ***Acalypha indica Linn***, were collected from Pallipattu village of Thiruvallur district of Tamil Nadu, India, the leaves were air dried and powdered into fine material. The powdered final material is labelled and stored in an air tight container in our laboratory for further experiments. The morphology of ***Acalypha indica Linn*** is shown in **Figure 1**.

Figure 1: Morphology of *Acalypha indica Linn*

Extraction of plant Leaves:

The powdered plant material is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent of Methanol. The solvent is heated to reflux in soxhlet and solvent vapour is condensed and collected the plant extract. The chamber containing the solid material slowly fills with warm solvent and the plant material was repeatedly extracted and collected. This cycle was allowed to repeat for 5times. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After 5 cycles the desired compounds is concentrated in distillation flask. After extraction the solvent is removed with help of rotary evaporator, yielding the extracted compound. The extracted residues were re-dissolved in a small quantity of propylene glycol and take it to preservative efficacy test.

Cosmetic Formulations:

The aqueous formulations of cosmetics as shampoo were prepared with the ingredients of Aqua, Sodium Lauryl Ether Sulphate, Cocamidopropyl Betaine, Cocamide MEA, Ethylene Glycol Distearate, Perfume, Dimethicone, Sodium Chloride, Disodium EDTA, Sodium hydroxide. The ***Acalypha indica Linn*** leaf methanolic extract was added in the concentration of1% to 2% and labelled as sample A and sample B respectively for antimicrobial test.

Antimicrobial Study: Preservative effective:

Test Organisms:

The Anti-microbial activity of the present study is performed in gram negative bacteria such as *Pseudomonas aeruginosa (*ATCC 9027) as well as in the fungal stain Apergillusniger (ATCC 16404) respectively. The details of microbial cultures in given in the **Table 1 & Figure 2 & 3**.

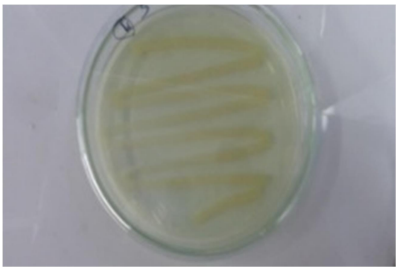


Figure 2: ***Pseudomonas aeruginosa***

Figure 3: ***Aspergillus niger***

Table 1: Details of Microbial Cultures Used in the Preservative Effect Test

Strain	Type	ATCC No.	Medium Used	Incubation temperature	Maximum duration
Pseudomonas aeruginosa	Bacteria	9027	TSA	30 to 35°C	3 days
Aspergillus niger	Fungi	16404	SDA	20 to 25°C	5 days

Preparation of Stock Inoculum:

Pure culture of bacteria and fungi were prepared by repeated sub culturing from the stock culture of agar slants in TSA and SDA media respectively. The inoculated bacterial culture was incubated at 37±1°C for 24 – 48hrs and the fungal cultures were incubated at 30±1°C for 3 to 5 days period. From the 24hrs bacterial broth culture, the serially diluted bacterial cells were made in normal saline solution up to 10^{-8} dilution. The alone diluted inoculum was kept at low temperature in refrigerator for further experimental study. The dilution contains 100 to 1000 cfu/mL for bacteria and 10 to 100 spores /mL were used in the experiments.

Validation Methodology for Test product sample:

The Validation parameters should establish the specificity of the test method, suitability of medium used, Bacteriostasis & Fungistasis of the bulk and recovery.

a. Preliminary microbial count on test samples:

Using a sterile pipette 5 g of product is weighted into a capped sterile container and serially diluted 1:10 and 1:100 by adding the validated diluent-neutralizing solution. 1mL – aliquots of each dilution of product are transferred into Petri dishes followed by 15 - 18mL of an appropriate molten agar at not more than 45°C (TSA for Bacteria and SDA for Fungi). Plates were rotated enough to evenly disperse the product and incubated at 37.0 ± 1°C for bacteria and 30.0 ± 1°C for fungi. The number of colony for each plate is counted in plates with containing <10 colonies for bacteria and <10 colonies for yeast and mould and observed for number of colonies grown and expressed as CFU/g (Colony Forming Unit per gram) of product.

b. Preparation of Diluent-Neutralizer:

On a glass container, Peptone – 1g/L, Tween 80 – 20g/L; water up to 1000ml were mixed well and sterilized in autoclave at 121°C for 15 minutes.

Inoculation of sample:

About 100g of the test products was weighted in sterile container that are individually inoculated with an aliquot of the different microbial inoculum suspension by using the ratio of 100:1 (Product: microorganism). Duplicate samples were maintained for avoiding the experimental error, control sample products also maintained without inoculating microorganism for comparison. Inoculated sample containers were incubated at 20 - 25°C sheltered from the light throughout the test. At regular interval period such as 24h, 48h, 5days, 7days, 14days, 21days and 28days, the samples were drowned and plated for growth of bacteria and fungi. The Quantitative pattern in terms of population (number) of bacteria and fungi were recorded and the individual species of organisms also were noted.

Sampling and Microbial analysis:

After incubation of respective sample products, exactly 1g of test product was transferred into the 9mL of diluent neutralizer and shaken thoroughly at regular sampling intervals. From each diluted sample, 1g was disbursed into a sterilized petri plate and 15 – 18mL of melted agar at warm condition was poured and swirled for uniform distribution of sample then the inoculated agar plates were incubated at 37±1°C for bacteria and 30±1°C for fungi. The number of bacterial colonies were recorded after 24 – 48hrs period and the fungal colonies were after 3 to 5days period. The bacteria and fungi were expressed in CFU/g of sample. Positive control plates were prepared by inoculating the microbial cultures in respective agar medium without test sample and maintained for comparison. The negative control plates without inoculating the respective microbial culture were also maintained in the study.

RESULTS AND DISCUSSION

Challenge test and Control sample:

Clear visible growth should be observed which have been challenged with 10 – 100 CFU in the samples. If clear visible growth is not observed within the specified period with the sample, then the medium should be modified with neutralizing agents such as Tween 20 and the validation should be repeated with the modified medium. The Positive control should show clear visible growth within respective incubation period for the presence of bacterial and fungi. Negative control should not show any growth within respective incubation period for the presence of bacterial and fungi. The recovery of the organisms inoculated to the sample should not be less than 70%.

Sample details:

The methanolic plant extract of ***Acalypha indica Linn*** in the form of concentrated solution 1% and 2% were subject to experimental study. The cosmetic preparation in a single formulation was used for the analysis for antimicrobial study and the bacteria namely *Pseudomonas aeruginosa* and the fungi *Aspergillus niger* were used in the present study.

The results of the antimicrobial study with 1% methanolic plant extract in cosmetic products shows and the bacterial growth after 24h was 500CFU/g and after 48h 100 CFU/g against 600CFU/g of initial count. However, after 5days the bacterial count was less than 10 CFU/g. The details were presented in **Table 1, Graph: 3 and Plate 4**.

The reports on antibacterial activity of ***Acalypha indica Linn*** leaf extracted with acetone and aqueous showed significant activity against most common pathogens like *Staphylococcus aureus*, *Bacillus subtilis, Echerichia coli* and *Klebsiella sp*.**[12]** similarly, the plant extracts of *Azadiracta indica* and *Azadiracta nilotica* showed antimicrobial effect against many common bacterial pathogens **[13]** and it was concluded that the plant phytochemicals have potential

antibacterial activities against sensitive and resistant pathogens through different mechanisms of action, which evidence the present study.

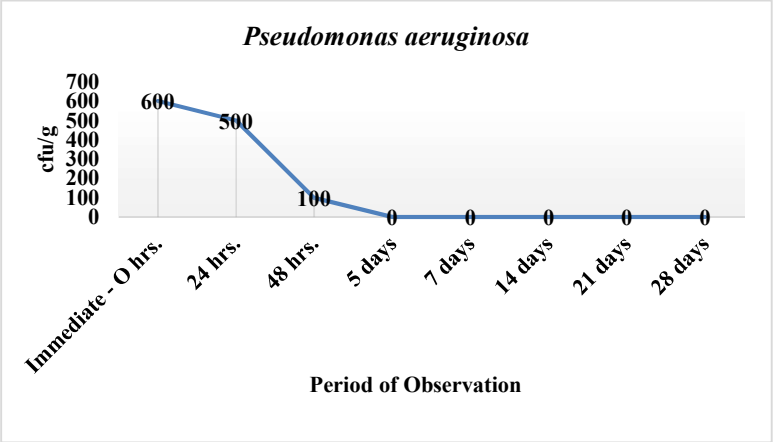
In the case of fungi tested in the present study, the initial count was 900 CFU/g for *Aspergillus niger* in the addition of 1% plant extract, the count decreased to 800 cfu/g after 24 hrs of sample preparation and further decreased to 200 cfu/g after 48 hrs period. Whereas the growth of *Aspergillus niger* has further reduced to less than 10 cfu/g after 5 days period. The details were presented in **Table 3, Graph 3 and plate 2**.

When the concentration of methanolic crude plant extract leaf of ***Acalypha indica*** was increased to 2.0%, the bacterial growth was drastically reduced after 24 hrs as 20CFU/g as and less than 10CFU/g after 48 hrs period. Thereafter, the bacterial growth was observed to be less than 10 CFU/g against the initial count of 600 CFU/g. The details were presented in **Table 5 and Graph 3**.

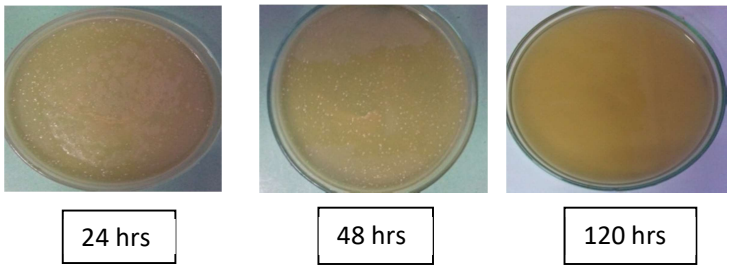
At the concentration of 2% extracts of ***Acalypha indica Linn*** amended in the medium, the growth of *Aspergillus niger* after 24hrs is found to be 700 CFU/g against initial count of 900cfu/g. However, after 48hrs, the inhibition of *Aspergillus niger* found to be less than 10cfu/g and observed up to 28 days period. The results were indicated in **Table 5, Graph 4 and Plate 4**.

Table 2: Preservative Challenge Test on Cosmetic Preparations With 1% of *Acalypha indica linn.* Leaf Extract Against *Pseudomonas aeruginosa*

Period of observation	UOM	*Pseudomonas aeruginosa*
Bacterial count at initial period	cfu/g	*600*
Bacterial growth at 24 hrs.	cfu/g	*500*
Bacterial growth at 48hrs	cfu/g	*100*
Bacterial growth at5 days	cfu/g	*<10*
Bacterial growth at 7 days	cfu/g	*<10*
Bacterial growth at 14 days	cfu/g	*<10*
Bacterial growth at 21 days	cfu/g	*<10*
Bacterial growth at 28 days	cfu/g	*<10*



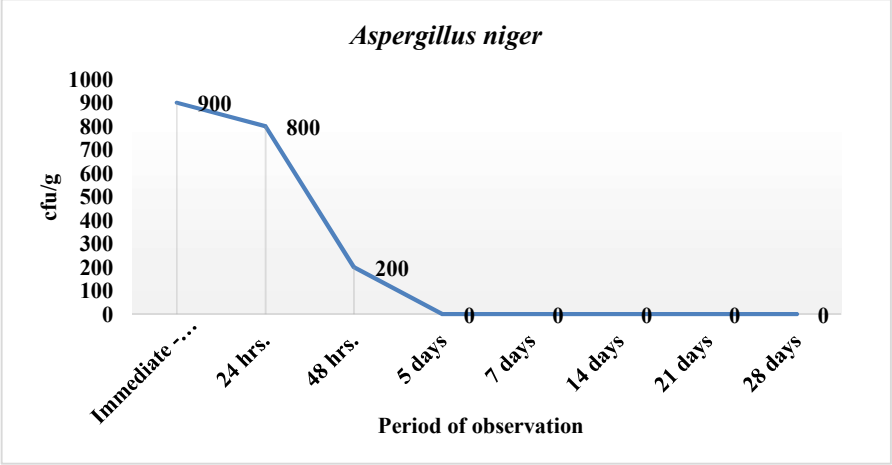
Graph 1: Preservative Challenge test on cosmetic preparations with 1% of *Acalypha indica Linn* leaf extract of against *Pseudomonas aeruginosa*



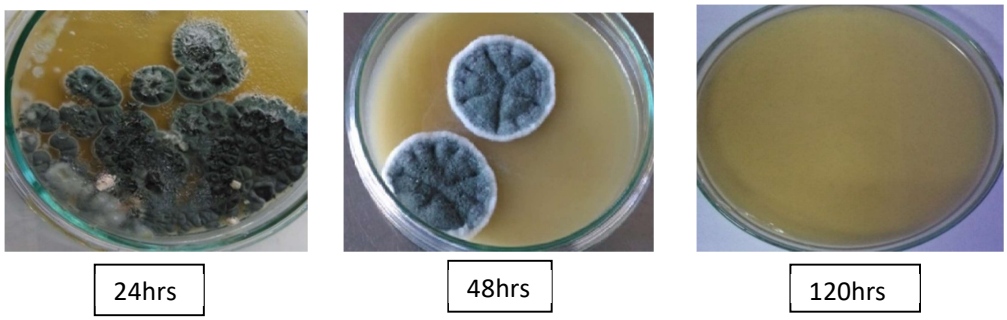
Plates 1: Growth of *Pseudomonas aeruginosa* in plant leaf extract amended medium at different incubation period

Table 3: Preservative Challenge Test On Cosmetic Preparations With 1% of *Acalypha indica Linn* Leaf Extract of Against *Aspergillums niger*

Period of observation	UOM	*Aspergillums niger*
Growth of *Aspergillus niger*at initial period (0 hrs)	cfu/g	*900*
Growth at 24 hrs	cfu/g	*800*
Growth at 48hrs	cfu/g	*200*
Growth at 5 days	cfu/g	*<10*
Growth at 7 days	cfu/g	*<10*
Growth at 14 days	cfu/g	*<10*
Growth at 21 days	cfu/g	*<10*
Growth at 28 days	cfu/g	*<10*



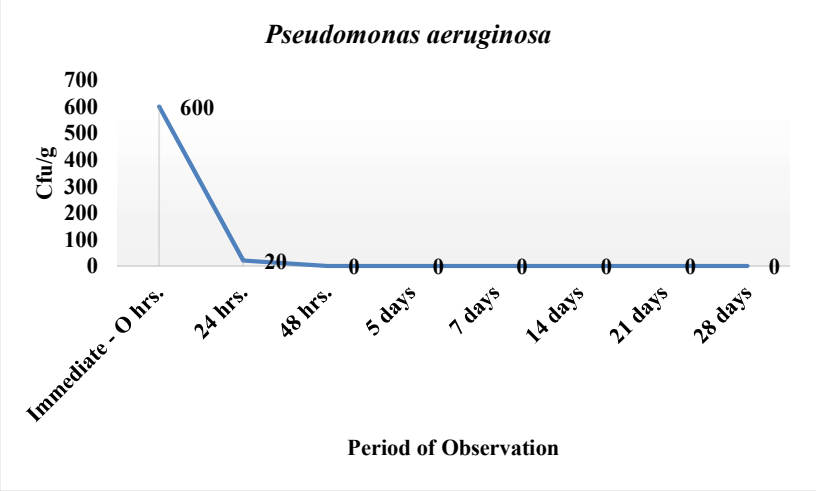
Graph 2: The population count of *Aspergillums niger* in the medium added with 1% *Acalypha indica Linn* leaf extract



Plates 2: Growth of *Aspergillums niger* in plant leaf extract amended medium at different incubation period

Table 4: Preservative Challenge Test on Cosmetic Preparations With 2% OF *Acalypha Indica Linn* Leaf Extract of Against Bacteria

Period of observation	UOM	*Pseudomonas aeruginosa*
Bacterial growth at initial period (0 hrs)	cfu/g	*600*
Bacterial growth at 24 hrs	cfu/g	*200*
Bacterial growth at 48hrs	cfu/g	*<10*
Bacterial growth at 5 days	cfu/g	*<10*
Bacterial growth at 7 days	cfu/g	*<10*
Bacterial growth at 14 days	cfu/g	*<10*
Bacterial growth at 21 days	cfu/g	*<10*
Bacterial growth at 28 days	cfu/g	*<10*



Graph 3: Preservative Challenge test on cosmetic preparations with 2% of *Acalypha indica Linn* leaf extractagainst bacteria *Pseudomonas aeruginosa*

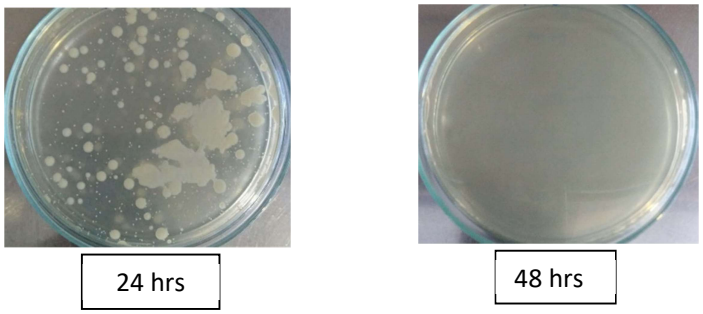
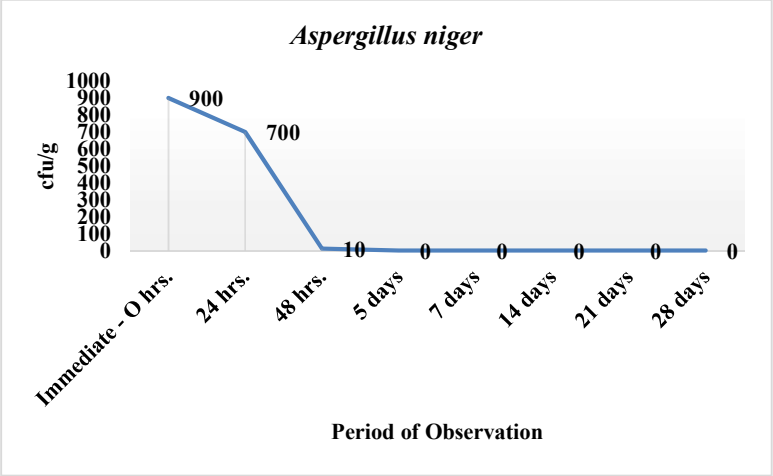


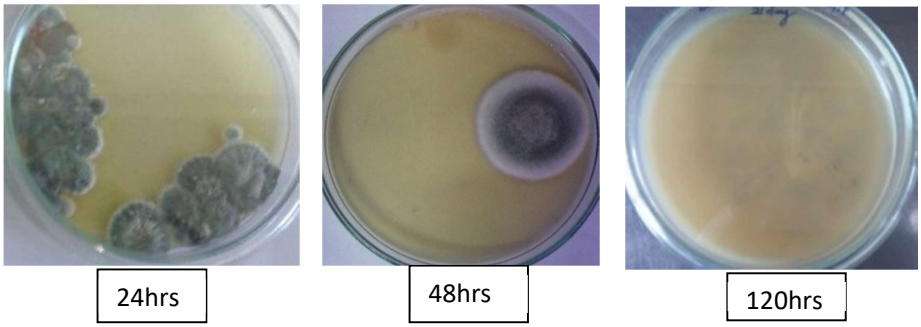
Plate 3: Growth of *Pseudomonas aeruginosa* was reduction gradually from 24 hrs to 48hrs

Table 5: Preservative Challenge Test on Cosmetic Preparations With 2% of *Acalypha indica Linn* Leaf Extract of Against *Aspergillus niger*

Period of observation	UOM	*Aspergillus niger*
Growth of *Aspergillus niger*at initial period (0 hrs)	cfu/g	*900*
Growth at 24 hrs	cfu/g	*700*
Growth at 48hrs	cfu/g	*10*
Growth at 5 days	cfu/g	*<10*
Growth at 7 days	cfu/g	*<10*
Growth at 14 days	cfu/g	*<10*
Growth at 21 days	cfu/g	*<10*
Growth at 28 days	cfu/g	*<10*



Graph 4: The population count of *Aspergillums niger* in the medium added with 2% *Acalypha indica Linn* leaf extract



Plates 4: Growth of *Aspergillums niger* in plant leaf extract amended medium at different incubation period

In earlier studies, the antifungal compound namely thymol found to be potent inhibition activity against the clinical isolates of *C. albicans*, *C. glabrata*, and *C.krusei*, in alone well as in combination of fluconazole **[14]**. Whereas thymol also exhibited synergistic antifungal activity against all the tested stains of Candida species in combination with fluconazole **[15]**. Abbaszadeh *et al* 2014. also suggested thymol and carvacrol **[16]** in addition to eugenol **[17]** and menthol **[18]** as good alternatives of synthetic fungicides in food industries.

Indeed, they reported that all of these compounds were effective in different extents against various food-decaying fungi including *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium spp.*, *Penicillium citrinum*, *Penicillium chrysogenum*, *Fusarium oxysporum*, and *Rhizopus oryzae* **[19]**. These observations were also confirmed by other research groups **[20]**. There are many evidence suggesting that medicinal plants are very effective in the treatment of infectious

diseases. Since the plants hold great promise as a source of novel antimicrobial agents, they are readily available, cheap, and not to have any side effects **[21]**.

Hence, people have been paying more attention to formulate herbal-based medicines due to their desirable properties to solve many clinical problems. However, studies still need to be conducted to ensure the mechanism of purity of bio compounds and clinical studies for their safety.

To conclude, from the present investigation, it was observed that the methaonalic plant extract of ***Acalypha indica Linn,*** exhibited the antimicrobial activity against the bacteria *Pseudomonas aeruginosa,* and fungus *Aspergillus niger* in the concentration level of 1% as well as 2% on the cosmetic preparation. Therefore, it is concluded that the plant species ***Acalypha indica Linn*** may be used as a natural source for preparation of effective preservatives that can be applied in various products such as cosmetics and health care therapeutic applications. Further the clinical studies for the above plant bio compounds to be concluded for the safety of the product before being the nature plant compound is taken for final formation.

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