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Research Article

ANTIFUNGAL ACTIVITY OF LANTADENEWHICH DEVELOPED ON *EMBICA OFFICINALIS*

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Abstract

A noxious weed; *Lantana camara* (verbenanceae) existing pentacyclictriterpenoid; Lantadene. Its antitumor activity is well known in therapeutic field. Attempt was made to assess its fungicide effect on fungus of *Embica officinalis* (amla fruit).Ethyl extract of lantadene in various parts of lantana were assessed on the cytotoxicity, showed no remarkable effect on fungus *Aspargillus fumigates*. Antibiotic discKetoconazole KT^{30}, Nystatin NS100, antibiotic disc showed excellent antifungal effect. Combination of antibiotic chemicals along with lantadene generates new fangled field in antifungal activity.

Keywords: noxious weed,cytotoxicity,antibiotic disc.

Introduction

Embica officinalis (amla fruit) has various healthful properties and nutritional values. Among these anti-inflammatory and anticoagulant are the important ones.It contains vital chemicals such as vitamin C, polyphenols and antioxidant. As per the recent research Amla's nutrient ability influences blood flow to antiaging processes (Kaye E Brock, 2011). Twelve different types of fungal species were isolated *Emblica officinal*. The Aspergillus, Penicillium, Rhizopus, Altenaria, Syncephalastrum are five different fungus was present in the form of contamination on *Embica officinalis*. Aspergillus and *Penicillium rubrum* were the most dominant fungal species isolated from Amla fruit. The presence of fungus Alternaria toxigenic, Penicillium and Aspergillus in the stored Amla fruits are alarming signal to customize for the formulation of herbal drugs. (Vartica Rai et al, 2005, Bungo A. et al.2006, E.funtoye M. O.2004).Hence species were selected for the study.

Medicinal plants have been considered to be the rich resource of curing various life threatening diseases. All the plants have very complicated structure of bioactive molecules. Most of the active bioactive molecules are organic compounds which are generally non polar and consisting of covalent bonds. As per the principle of solubility extraction of a specific bioactive compound dependent on the solubility of the bioactive compound in the nature of extraction medium (water or organic solvent or a mixture of organic solvents).

The processes for extraction system are totally differs from molecule to molecule which may be polar or non-polar. It's very difficult to identify a using uniform process to extract and isolate bioactive compounds from different plants.

A series of different physical as well as chemical processes are involved. To isolate bioactive compounds, the process begins with the extraction process in which enrich compound(s) can be extracted in a (generalized or different manner) with an extraction medium. Khaing, T.A. (**2011**). Since,*Lantana Camara*L. is among top ten uncontrolled weeds on earth (Sharma S. K. et al; 2011). It is belongs to verbenaceae family also is known as

Vervain family or Verbena family. It contain wide array of compounds exhibiting diverse range of bioactivity (Sharma M. et al; **2011).** Lantana contains number allelopathic compounds that are the triterpenes. These compounds contribute to the majorly of lantana in several ecosystems and responsible to loss of biodiversity. The triterpenes shown to be associated with hepatotoxicity in the field are lantadene A (primarily), lantadene C and icterogenin (Sharma, M 2008). Oleananetriterpenoids are versatile drugs that may be synthesized for the prevention and cure of an assortment of prolonged diseases driven by inflammation and oxidative stress. (Karen T. Liby**;** 2014). Since fungicides are very expensive and cause serious environmental pollution; control strategies are today directed towards replacing the use of hazardous chemical fungicides by environmentally friendly natural products. The antifungal property of the extracted essential oil obtained from the flowers of *lantana camara* towards the three pathogenic fungi was studied in vitro. It also has the capacity of becoming powerful and considerable safe alternative means of disease control instead of the hazardous pesticides. (Eweis M.et al; 2011; Goswam-Giri A.et al; 2011) According to revelations, antifungal compounds present in the plants are active at different stages of germination growth. The isolated basic proteins from Lantana especially the high molecular weight tractions of protein showed the novel antifungal properties which can be used in crop improvement program of sugarcane. (Hiremath L.et al; 2011).Hence lantadene was isolated for its antifungal activity of lantadenewhich developed on embica*officinalis*

Materials and Methods

Materials

Forceps, cotton plugs, paper, disc, double distilled water, micropipette, Nutrient malt agar, fungus culture, antibiotics, Absolute ethanol, Petri dish.

Collection of plant source

Leaves of *Lantana camara* Linn were identified and collected from Vidya Prasark Mandal's College campus, Thane (MS), India. It is situated very close to Thane/Kalwa creek. For the study, lantana plants growing near the creek soil was specially selected for study. Leaves were segregated, cleaned, washed and dried under shed for 10 days. Dried parts are powdered, packed and stored at room temperature.

Methods

Extraction of Pentacyclictriterpenoids (Lantadene) by methanolic reflux method

Lantana camara (100 gm)leaf powder was treated with (500 ml) methanol and refluxed for 3 hours. Solvent was removed under vaccum (13-14 mm/Hg and distillation temperature up to 58^{o}C) to get concentrated residue. Dark brown residue was suspended in 500 ml distilled water. The extract was separated by filtration through Whatman paper No.1 and the residue was added in a methanol–water (1:7) mixture and extracted with ethyl acetate (2 X 25 mL) and with n-butanol ((2 X 25 mL). After shaking well the layers were separated and collected in different flask. The ethyl acetate layer was concentrated under reduced pressure to get crude lantadenethat was loaded over silica gel column (60–120 mesh) and (30g) using chloroform and chloroform:methanol (9:1) as eluting solvent. The second lantadene fractions further chromatographed on a silica gel column using n-Hexane with successive increasing amount of acetone.(Keh-Feng Huang et al 2004,Juang F. et al; 2005) Different fractions were collected. Among these grayish elutant was checked with TLC using system (9.8:0.2) $CHCl_3$–CH_3OH.

Qualitative test for triterpenoids

Enriches fractions from silica gel column (50 µl) was treated with 0.5 ml chloroform and heated for about 30 minutes. Two to three drops of concentrated sulphuric acid was added and mixed well. The presence of triterpenoids was observed by change in colour to red.

The process started by screening and extraction medicinal plants for antifungal activity The extraction process includes various steps, such as cleaning, drying of plant materials pulverized to obtain uniform sample and proper care must be taken to ensure that potential active constituents are not vanished, distorted or destroyed while the preparation of the extract from plant samples.

Extraction and Isolation of Lantadenes was carried out as per experiment 1 from *Lantana camaralinn*leaves powder, stem powder, flower powder and fruits powder were taken separately for the antifungal activity.

Preparation of nutrient agar plates

Malt extract Broth (0.75 gms) 1.5 gm of Agar and 50 ml distilled water was taken in a 100 ml volumetric flask. Solution turns brown colour. Saline solution was prepared by adding 0.9 gm NaCl in 100 ml distilled water and taken in 100 ml volumetric flask. Both flask were covered by cotton plug, wrapped with paper and tied with thread. Solutions, test tubes and borer were sterilized for 45 minutes in pressure cooker**.** Amla fruits were identified and kept for 10 days in the open atmosphere and then wrapped and kept in the fridge. After 10 days approximately 1 gm fungus was removed carefully through sterilized spatula and added in the saline solution.

Preparation of inoculums

Amla fruits were kept for 10 days in the open atmosphere. After 10 days approximately 1 gm fungus was removed carefully through sterilized spatula and added in the saline solution and sterilized disc were kept ready.

For the experiment hot malt agar broth sterilised solution were poured in sterilized plates in the sterilized atmosphere. After pouring it solidified into transparent solid. To begin with the disc test method, three an inoculated plate that contains the broth ; Insert a cotton swab into the sample. Tubes were stirred the excess fluid. Used the sample to streak the surface of the prepared plate in all four directions at 90 degree angles so all the surfaces are covered. Then dried the disc plates at 35-37 degree celceius and incubate the plates for 48 hours. One disc plate was kept as controlled (containing malt agar). After 48 hours white, black, yellow fungus were observed. Photographs were taken using Debust Microvision (digital eye) using Binocular light microscope with microphotography attachment (Lynx).

Placement of the antibiotic discs

Ketoconazole KT30-
It is imidazole derivative with chloro group in the side ring .
Itraconazole IT30-
This is triazole antifungal drug. It consists of chloro group as that ofKetoconazole.
Nystatin NS100-
Structure consists of conjugation system with large rings of atoms. Structure consists of amino as well as carboxylic group.
Clotrimazole cc10 –
This is Imidazole derivative with ortho-chlorotrityl chloride. This is antifungal drug.
Ampiciline A10 –
Ampiciline is belonging to penicillin family and spectctrum against some gram negative and gram positive bacteria. The important feature of Ampiciline molecule is the presence of amino group. This amino group is responsible for it's activity**.**

Ketoconazole KT^{30}, Itraconazole IT^{30}, Nystatin NS100, Clotrimazole cc^{10}, AmpicilineA10antibiotic discs were placed on the surface of the agar (using flame-sterile forceps) to placed each antibiotic disc one at a time. The distributions of the discs were sufficient apart from each other and not close to the edges of the disc plate. By using sterilised borer three wells were created on the plate. By using micropipette drops of ethyl acetate extract (leaves, stems, flowers and fruits) were poured in the three wells in the sterilized medium.

Incubation of the plates

The temperature range was maintained about 35°C and incubation time was 48 hours.

Results and Discussion

Considerable change in composition was observed with samples obtained from different locations in India. Chemical composition of the whole plant and plant parts and essential oils are reported to be highly influenced by genetic, geographical, and seasonal factors as well as the stages of the development of the concerned plant, its parts/tissue(Ofeogbu, et al 2013). A number of different prominent extraction parameters were used for the lantadene isolations from *Lantana camara*. But exact concentration present in each aerial part is totally depends upon the optimum conditions maintain or methods used. Researcher generally used salt and solvent methods including microwave power soxhlet method or conventional method; extraction time, solvent type, and volume, PH were studied in a systematic methods for the determination of optimum extraction conditions.

Organic solvents contributed exceptionally low yield showing very little difference in physicochemical properties of pentacyclictriterpenoids. It revealed that interferon of triumphing a pure form of Lantadene. Ethyl acetate does not able to extract Lantadene completely while from the methanol–Water mixture extracted reproducible yield. The ethylacetate layer was concentrated under reduced pressure; the crude lantadene is 0.083grams from 100 gm of dried leaves powder.100 g of lantana leaves powder procured 25 mg of pure Lantadene. Its melting point do not gives sharp melting point. After reduction of solvent in active /enriched elution was appear as white solid having Rf 0.73.Lantadene, was changes its physical property during processing.Change in colour in different organic solvents aerial parts of lantana extract in leaves brownish shade to greyish while processing and in pure form of it is in white colour.Enriches fractions from silica gel column (50 µl) was mixed with 0.5 ml chloroform and warmed for about 30 minutes. Two to three drops of concentrated sulphuric acid was added and mixed well. The appearance of red colour indicates the presence of triterpenoids.(Hasan 2014).

Observation and Measurement of zone diameter

After the overnight incubation, there were observed a noticeable "clearing zone" around each of the antibiotic discs. The diameter of the each disc is measured and recorded in (mm). For measure, the plates are carefully examined for well-developed fungus colonies within the zone of inhibition. Thus the effectiveness of compound

extracts and antibiotic discs was determined by analysing the size of the zone of inhibition.

Amla fruit fungus was developed after 10 days in the open atmosphere. Approximately 1 gm fungus (figure 4.1) was removed carefully through sterilized spatula and added in the saline solution it was used for disc test. Three an inoculated plate and controlled disc plate incubated the plates for 48 hours at 35^{o}C. After 48 hours white, black, yellow fungus were observed. Photographs were taken using Debust Micro vision (digital eye) using Binocular light microscopewith microphotography attachment (Lynx) it was compared with crude lantadene (0.083 grams) dissolved in saline treated to plate).After overnight incubation, there observed a noticeable “clearing zone” around each of the antibiotic discs. The diameter of clearing zone is measured in (mm). For measurement, the plates are carefully examined for well-developed fungus colonies within the zone of inhibition. The size of the zone of inhibition directly measures the effectiveness of compound.

Disc diffusion test have been used as a preliminary screen for susceptibility testing of fungus on Amla fruit. Test is simple, easy to perform and a reliable method for determination of resistance. The malt agar dilution method can be used reliably in routine susceptibility testing of fungus on Amla fruit by using antibiotic test KetoconazoleKT30, Iitraconazole IT30, Nystatin NS100, Clotrimazole CC10, leaves (ethyl acetate fraction), Ampicillin A10, Fruits (ethyl acetate fraction), Flower (ethyl acetate fraction) .

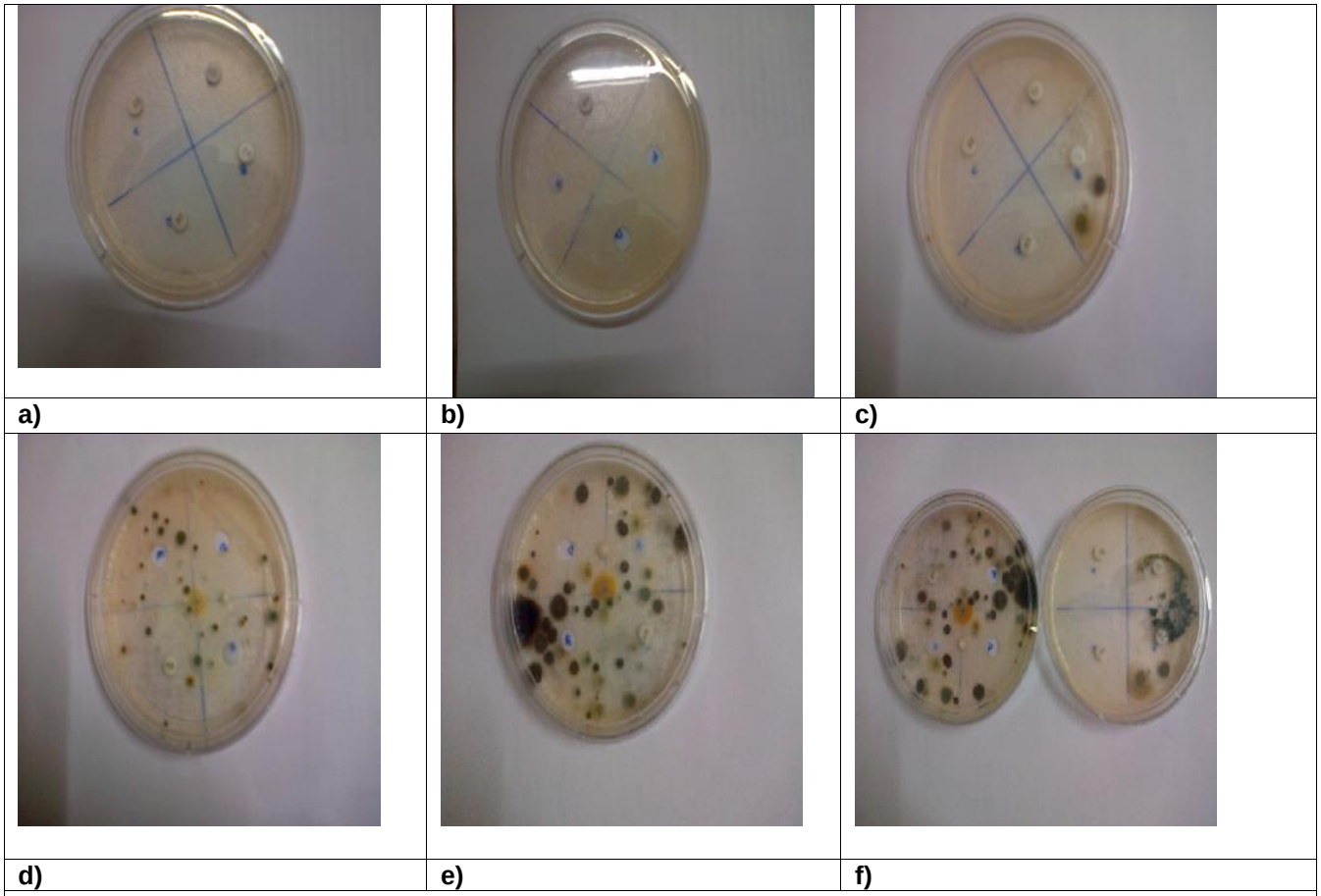
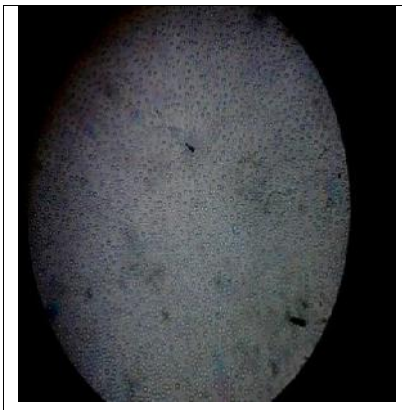
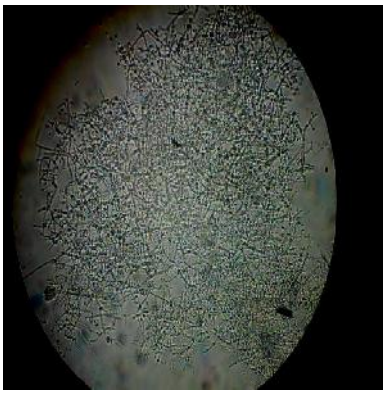
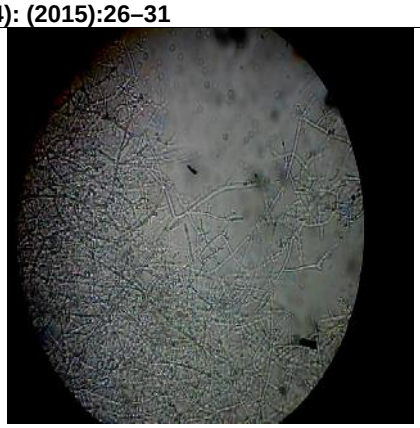
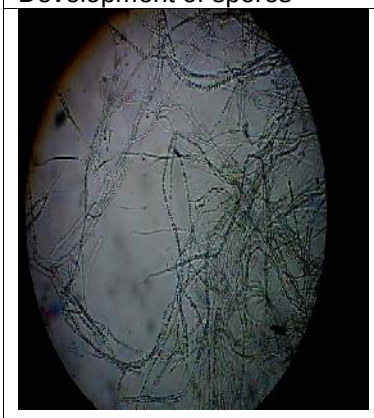
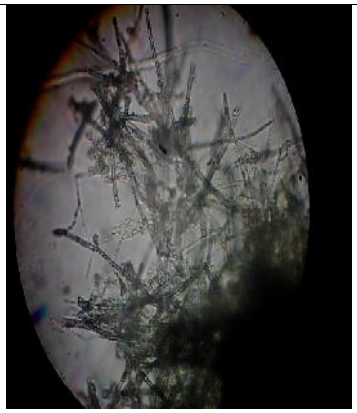


Figure 1 Fungus developed on discs (Incubation period 48 hours).
a) Disc1(A- Ketoconazole KT^{30} ,B- Itraconazole IT^{30},C- Nystatin NS100, D-Clotrimazole cc^{10}
b) Disc 2 (E- Ethyl acetate leaves, F- AmpicillinA^{10}, G- Ethyl acetate fruits extract, H- Ethyl acetate flower extract. **c)** After 48 hours, Disc 1(Ketoconazole KT^{30,} Nystatin NS100, antibiotic discs showed antifungal property while Clotrimazole cc^{10}Itraconazole IT^{30} antibiotic disc showed non antifungal property.**d)** After 48 hours , Disc 2(Ethyl acetate leaves showed more non fungicidal property as compared to the rest drugs.). **e)** After 72 hours showed growth of fungal colonies on the disc 2. **f)** Comparison of Disc 1 and Disc 2 for fungicidal property of different antibiotic drugs and lantadene ethyl acetate extracts of different aerial parts on *Phyllanthusemblica* fungus.

Development of spores | Generation of fine filaments | Fine filaments





Filaments bears spores

Figure 2 Microscopicimages of Fungus developed on*Phyllanthusemblica.*

Above Photographs were taken using Debust Microvision (digital eye) using Binocular light microscope with microphotography attachment (Lynx). Microscopic **i**mages of Fungus developed on*Phyllanthusemblica* showed the formation of conidiophores, Sporangium, spores, multinucleated highly branched filaments (hypae). As per the literature survey fungus resembled like *Aspargillus fumigates*. Fungus is white, yellow, green and brown in colour. Conidiophores are green in colour and are the main path of dispersal of the fungi.

Following observation were noted from table 4.1 Ketoconazole KT30 ,Nystatin NS100 shows complete inhibition as there was no fungal growth developed, Ketoconazole KT30 ,Nystatin NS100 are excellent fungus inhibitor of fungus which are developed on Amla fruit. Ampiciline A10 shows development of Greenish Black fungus. Ethyl acetate fruits extract and Ethyl acetate flower extract shows development of black, white and yellow fungus. Clotrimazole cc10 shows only white fungus. While ethyl acetate extract (leaves) shows white and black fungus. Itraconazole IT30 shows white, brown and black fungus. Ethyl acetate extract of fruits, flowers shows three different colours of fungus while ethyl acetate extract of leaves shows two different colours fungus.

The drug disc method evaluated the great potential in the development of antifungal or fungicidal properties of pentacyclictriterpenoids (Lantadene) from *Lantana camara* Linn for *Phyllanthusemblica* fruits. It demonstrates that the use drug disc method revealed the easy identification of fungicidal effect of drugs and Lantadeneethylacetate extract. Minimum concentration of Lantadene in ethyl acetate extract do no showed fungicidal effect on the fungus of *Phyllanthusemblica*fruits .Greater concentration of Lantadene solution may show fungicidal property. Ketoconazole KT^{30}, Nystatin NS100, antibiotic disc showed excellent antifungal property against on the fungus of *Phyllanthusemblica*.Greater concentration of Lantadene along with Ketoconazole KT^{30}, Nystatin NS100 drugs may show fruitful result against fungus of *Phyllanthusemblica*fruits. No inhibitory activity was observed; however, the ethylacetate extract of different aerial parts was found the zone of inhibition showing it's in the cytotoxicity experiment which may be due to the presence of Lantadene in very low amount in these extract.

Ketoconazole KT30, Itraconazole IT30 showed complete inhibition of fungus developed on the Amla fruit. Extract consist of penta cyclic triterpenoids (Lantadene) do not showed inhibition on fungus developed on Amla fruit. So in future, lantadene along with Ketoconazole KT30 or Itraconazole IT30 gives appreciable not only the fungus developed on Amla fruit but also against various commercial profits threatening fungus. It was determined by physical constant UV HPLC Infra-Red spectrum XRD and SEM. Lantadene silver nano particles with greater concentration in ppm may give excellent antifungal activity

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