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## Research Article

### *In-vitro* sensitivity of bacterial and fungal pathogens to *Datura alba* - an antimicrobial approaches

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#### Abstract

The *in vitro* antimicrobial evaluation on *Datura alba* confirms that, the plant appears to contain substances that exhibit measurable *in vitro* antimicrobial activity against some bacteria and fungi used in this study. The results of the various screening tests indicate that all the parts of this plant viz., leaves and stem possess some measurable inhibitory action against both gram-positive and gram-negative bacteria used in this study. It is screening to note that *Pseudomonas aeruginosa*, which is known to be a very resistant bacterium even to synthetic drugs, and *Salmonella typhi*, a multi drug resistant strain to Amphotericin and Chloramphenicol, which infects only human and produce typhoid fever were found to be very susceptible to the leaf extract of this plant. Fungal strains such as *Aspergillus niger* and *Aspergillus flavus* which usually parasitize man and animal and cause pulmonary Aspergillosis and *Candida albicans* an ubiquitous fungus associated with the pathogenesis of skin diseases, urinary tract infections, vulva vaginitis and oral thrush were also found to be susceptible to the *Datura alba* extracts.

**Keywords:** *Datura alba* ; antimicrobial activity; leaves and stem; *Pseudomonas aeruginosa*; *Aspergillus niger*.

## Introduction

The use of antibiotics is losing the effectiveness since many of the pathogenic microbes especially bacteria and fungi have developed Substantial resistance to the antimicrobial drugs (Jones, 1998; Sushilkumar, 1998; Austin et al., 1999). This may be due to the widespread use of antibiotics which has led to the decimation of sensitive organisms from the population with the consequent increase in the number of resistant Organisms. Antibiotics began to increase which ultimately led to the evolution of drug resistance microorganisms. There

are many microorganisms which have developed resistance against antimicrobial drugs, such as *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Shigella dysenteriae*, *Mycobacterium tuberculosis*, *Enterobacter aerogens*, *Neisseria gonorrhoea*, *Enterococcus sp.*, *Haemophilus sp.*, *Candida albicans*, *Aspergillus sp.*, and *Trichophyton mentagrophytes*. These microbes are resistant to well known antibiotics such as chloromphenicol, erythromycin, penicillin G, tetracycline, vancomycin, sulfonamides, amphotericin B and

flucytosine and hence known as multidrug resistant strains (Purohit, 1977; John, 2002).

The development of microbial resistance to antibiotics pose a serious threat to public and clinicians since most of the rampant killer diseases are of microbial origin and account for high proportion of mortality in under developed as well as developed countries (John, 1998). A decade ago in India, typhoid could be cured with three inexpensive drugs namely Cephalosporin, penicillin-G and Chloramphenicol. But today these drugs are largely ineffective against the life threatening typhoid fever. In Eastern Europe and parts of Russia, more than 10% of tuberculosis patient cannot be cured completely because of the drug resistant stains which are insensitive to the most powerful antibiotics viz..., Streptomycin and Rifampicin. In the US alone about 14,000 people die every year because of drug resistant microbes which infect people in hospitals (Nosocomial infections). In much of the South-East Asia 98% of gonorrhea causing strains have become resistant to penicillin, which had been the first line treatment for decades (Kaufman, 2000; John, 2002). Therefore there is an urgent need for the discovery of alternative, safer and more effective antimicrobial agents in order to control the life threatening pathogens.

Plants are ancient source of antimicrobial compounds and have provided many medically useful compounds (Cord et al., 1981). It is estimated that more than two thirds of the world's population relies on plant derived drugs. Some 7000 medicinal compounds used in the western pharmacopoeia are derived from plants (Caufied, 1991).

The Bioactive compounds in plants are produced as secondary metabolites. Examples include alkaloids, proteins (Chakraborty and Brantner, 1999) naphthoquinones (Cai et al, 2000), coumarins (Hamburger et al, 1985), chalcones (Inamori et al, 1991), flavonoids (Watchter et al 1999), phenolic acids (Fernandez et al 1985) and terpenoids (Osawa et al 1990) which may be stage specific or organ or tissue specific. In fact there are several studies

which revealed the presence of such compounds with antimicrobial properties (Cowan, 1999). The roots stems barks and leave of several plants have been widely evaluate for such bioactive compounds and the results obtained proved that these compounds are the sources of new drugs, antibiotics and agrochemicals. These bioactive compounds are known as phytochemicals and are exploited on a large scale because of their less toxic more systemic and easy biodegradability. Therefore during the last few decades there has been a resurgence of interest in plants as source of medicines and of novel molecules for the treatment of microbial infection (Kinghom, 1987).

Numerous solanaceae species were used in traditional system of medicine as a remedy for of ailments. However, the potential for most of the solanaceae have not been proved scientifically. Hence it is necessary to establish the scientific basis for the therapeutic action of solanaceae especially antimicrobial properties in one such orchid, i.e., *Datura alba*. Because of the multifold medicinal use, this plant has been selected for the present study with the following objectives. To screen the antimicrobial (both antifungal and antibacterial) activity plant extracts on some selected pathogenic bacteria and fungi. To determine the specific inhibition of microbes by the plant extracts. To compare the efficiency of the plant extract on the test organisms with some standard antibiotics.

## Materials and Methods

### Plant collection

The plants used in this study were collected from their natural habitats namely from kondayankuppam, tiruvannamalai district in Tamil nadu. The plants were shad dried at ambient temperature (31° C) and the dried materials were powdered using an electronic blender.

### Preparation of plant extracts

#### Aqueous extracts

Hundred grams of each of the dried powder plant materials (leaf and stem) were suspended in 600 ml

of sterile double distilled water (1:6). They were kept for seven days at room temperature (31° c) for complete extraction. After seven days, the extracts were filtered through Whatman No. 1 filter paper. This crude extract was poured in labeled sterile universal bottles and kept in refrigerator at 4 ° c.

### Solvent extracts

Hundred gram of each of the dried powdered materials (leaves, stems) were soaked separately in 600 ml of each of the solvents viz. Ethanol, Chloroform, Ethylacetate and Petroleum ether in Soxhlet apparatus for 72 hr at 31° c until complete exhaustion of the material. Each mixture was stirred at every 24 hrs using a sterile glass rod. At the end of 72 hr, each extract was passed through Whatman No 1 filter paper and the filtrates were concentrated in vacuum rotary evaporator at 60 ° C in order to reduce the volume. The paste like extracts were stored in labeled screw capped bottles and kept in refrigerator at 4 ° C. Each of the extracts was individually diluted using minimal amounts of the extracting solvent prior to use.

### Test microorganisms

The bacterial strains used for the screening were *E.coli* and its mutant *K 12*, *Proteus vulgaris*, *Enterobacter aerogens*, *Bacillus cereus*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Serratia marcescens* and *Pseudomonas aeruginosa*.

### Test Fungi

The fungal strains were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Fusarium oxysporum*, *Microsporum gypseum*, *Trichoderma viridae* and *Candida albicans*.

### Determination of antimicrobial activity

The antimicrobial activity of the aqueous and other solvent extracts of *Datura alba* (leaf, stem) was evaluated on the basis of different assays. The

assays consisted of both antibacterial and antifungal determinations.

### Assays for antibacterial testing

#### Disc- Diffusion Test (Maruzzella & Henry, 1958)

Circular discs mm diameter were prepared from Whatman No 1 filter paper and sterilized in an autoclave. These paper discs were impregnated with test compounds ( plant extract) in the respective solvents for overnight and placed on nutrient agar plates seeded with the test bacterium. The plates were incubated at 37 ° c for 24 hrs, After 24 hrs the zone of inhibition around each disc was measured and the diameter was recorded. Gentamycin (10 mcg/disc) was used as the reference. A negative control was prepared using only the solvent used for extraction and kept for comparison. The tests were repeated 4 times to ensure reliability of the result.

#### Agar well diffusion method (Perez et al., 1990)

Agar well diffusion method is also known as Hole plate diffusion method (Brantner et al., 1993). Nutrient agar was used as the culture medium for this assay. The molten nutrient agar was dispensed in pre-sterilized petridishes (25 ml each) and allowed to cool. These agar plates were homogeneously inoculated with the test bacterium previously suspended in tryptose broth (10<sup>3</sup> cells/ml). The plates were allowed to solidify. After solidification holes/wells (cups) of 6 mm diameter were punched into agar with the help of flamed cork borer. Five wells were prepared for each plate. Of these five, three holes were filled with 0.2 ml of the plant extract and the fourth hole was filled with 0.2 ml of standard antibiotic solution ( Gentamycin, 500 ug/ml) and the fifth hole was filled with blank ( extracting solvent alone ). The Petri dishes were incubated at 37 ° c for 24 hr. After this incubation period the diameter of the inhibition zone formed around each hole (well/cup) was measured and the values were recorded. The antimicrobial activity was expressed as the ratio by the inhibition zone produced by the plant extract and the inhibition zone caused by the standard. Two sets of control were used. One control was the organism control

where standard antibiotic solution was used and the other control was the blank where only the extracting solvent was used. This was just to ensure the validity of the test. Testing was carried out for each bacterium in Quadruplicates.

### **Stroke method (Orzechowski, 1972)**

Nutrient agar was prepared and 8 ml of the molten agar medium and 2 ml of the plant extract were mixed thoroughly and the mixture was poured into a sterile Petri dish under aseptic condition. It was cooled at room temperature. After cooling, the bacterial culture was taken (24 hrs old) and using in inoculation needle, the bacterial culture was applied on the surface of the agar medium in the form of parallel strokes (streaks). For each bacterium triplicates were used. The plates were incubated at 37 ° c for 24 hr. After 24 hr, the results were rated as strong inhibition (if no growth of the test bacterium) partial inhibition (if less growth of the bacterium than the normal) and no inhibition (if full growth of the test bacterium). Control plates without the plant extract were also maintained for reference.

### **Assays for antifungal testing**

#### **Paper disc-agar diffusion method (Barry and Thornsberry, 1991)**

Test plates (petridishes) were prepared with Sabouraud Dextrose Agar medium and inoculated on the surface with a spore suspension of 10<sup>3</sup> CFU/ml. Sterile paper discs of 6 mm diameter impregnated with the extract at the concentration of 100 mg/ml were placed over the test plates. Nystatin (Sigma) 10 mcg per disc was used as the standard. The plates were incubated at 30° C for 48 hr.

#### **Agar well diffusion method (Gobdi and Irobi, 1992)**

Potato Dextrose Agar was used to culture the fungal organism. The plates were inoculated with 24 hr culture of respective fungi. With the help of a flamed cork borer, 8 mm wells were cut out and to

each of the well 0.1 ml extract (of different concentration) were aseptically added with the help of sterile syringe. The plates were kept in cold for an hour to facilitate diffusion of the test solution (the extract). Later the plates were incubated at room temperature. Inhibition was recorded by measuring the diameter of the inhibition zone after 72 hr. Griseofulvin was used as a standard for comparison of antifungal activity.

### **Stroke method**

Eight milliliter of the molten agar PDA medium and 2 ml of sterile plant extract were mixed thoroughly and the mixture was poured into a pre-sterilized petridish under aseptic condition. After cooling at room temperature, small strokes (streaks) of the test fungi were made on the surface of the medium using an inoculation needle. The plates were prepared in triplicates for each fungus. The plates were incubated at 37° c for 24 hrs. After the incubation period, the results were recorded as strong inhibition (if no growth of the test fungi), partial inhibition (if less growth of the fungi than the normal) and no inhibition (if full growth of the test fungi). Control plates without the plant extract were also maintained for reference.

## **Results and Discussion**

### **Antibacterial assays**

The antibacterial effect of aqueous ethanol, ethyl acetate, chloroform, petroleum ether, ethanol (1: 1) extracts of *Datura alba* (Leaf and stem) were evaluated by various assays against ten pathogenic bacteria. The bacteria include both gram-positive and gram-negative. Based on the screening it showed that the ethanol, ethyl acetate, chloroform extracts of *Datura alba* ( leaf and stem ) exhibited significant antibacterial activity, while the aqueous and petroleum ether, ethanol (1:1) extracts of the plant parts did not show satisfactory results.

### **Disc diffusion assay**

The results showed that both the parts (leaf, stem) of plant exhibited activity against all the pathogenic

organisms. The antibacterial activity was expressed in the form of inhibition zone around each disc caused by diffusion of antibacterial properties from the paper disc into the surrounding medium. Among various solvent extract showed high degree of inhibition, followed by ethyl acetate and chloroform. The petroleum ether, ethanol (1:1) showed low degree of inhibition against all the test bacteria. In other words, the antibacterial sensitivity was maximum in ethanolic leaf and lowest inhibition was observed in petroleum ether, ethanol (1: 1). The aqueous extract did not show any antibacterial activity.

The diameter of inhibition zones for each of the samples were compared with standard antibiotics (Chloramphenicol 30 mcg/disc). It was noted that the inhibition zones of the samples to be either less than or greater than or equal to the inhibition zones of standard antibiotics. The leaf extracts exhibited higher degree of inhibition than the stem extracts. The diameter of inhibition zones were noted in the leaf extracts, showed that the ethanolic extract exhibited significant inhibition against the pathogenic bacteria. The diameter of the inhibition zone is higher in the case of *E.coli* (8.1 mm), *E.coli mutant k12* (7.9 mm), *Proteus vulgaris* (6.7 mm). Moderate antibacterial activity observed against *Enterobacter aerogens* (4.2 mm), *Serratia marcescens* (4.1 mm), *Bacillus cereus* (4.1 mm), *Streptococcus faecalis* (3.9 mm), *Pseudomonas aeruginosa* (3.7 mm) whereas low degree of inhibition zones were noted against *Klebsiella pneumoniae* and *Salmonella typhi* (Plates 4 & 5 ). It is surprising to record that *Pseudomonas aeruginosa* which is known to be a very resistant bacterium even to synthetic antibacterial drugs was found to be sensitive to the leaf (ethanol). Similarly *Salmonella typhi*, a multi drug resistant bacterium was also susceptible to the leaf extracts (ethanol). The results various solvent extracts of stem of *Datura alba* exhibited inhibitory action against all the bacteria, the inhibition zones were less than the standard antibiotics. Hence the activity is insignificant nil activity (absence of inhibition zones) was also noted in some of the extracts viz. aqueous and petroleum ether, ethanol (1:1) extract.

The ethanol extract of *Datura alba* effectively inhibited the growth of both Gram-positive and gram-negative bacteria. Similar results were also reported by several workers ( Rabe and Vanstaden, 1997; Grierson and Afolayan, 1999) whereby majority of the antibacterial activity was observed in the ethanol extracts. The activity of the aqueous extracts of the plants showed nil activity. This is because most workers (Chakrabarty and Branter, 1999; Aburaji et al. 2001) have generally reported that aqueous extracts of plant do not have much activity against pathogenic organisms.

### Agar well diffusion method

The results indicate that all the parts (leaf, stem) of plant exhibited activity against one or more bacterial. The antibacterial activity of the various extracts are observed as the zone of inhibition. Among various solvent extracts screened, ethanolic leaf extracts showed significant inhibition against all the bacteria. In other words, the inhibitory effect was maximum in ethanolic extract and lowest inhibition was observed in petroleum ether, ethanol (1:1) extract. The aqueous extract did not show any antibacterial activity. The diameter of inhibition zones for each of the samples were compared with standard antibiotics (Chloramphenicol 500 ug/ml). It was noted that inhibition zone of the samples to be either less than or greater than or equal to inhibition zones of the standard antibiotics. Among the parts tested, the leaf exhibited greater inhibition zones than stem extracts. The diameter of the inhibition zones were found in the leaf extracts (Table 3) showed that the ethanolic extract exhibited greater inhibition against all the test bacteria. The diameter of the inhibition zone is greater in the case of *E.coli* (6.4 mm), *E.coli mutant k 12* (5.2 mm). Moderate activity was associated with *Proteus vulgaris* (4.6 mm), *Enterobacter aerogens* (4-5 mm) and *Serratia marcescens* (3.8 mm) whereas low activity was observed against *Pseudomonas aeruginosa* (3.1 mm ), *Bacillus cereus* (2.9 mm) and *Streptococcus faecalis* (2.1 mm). Poor inhibition was associated with *Klebsiella pneumoniae* and *Salmonella typhi* .

The results of various solvent extracts of stem of *Datura alba* exhibited against one or more test bacteria but not to all. The inhibition zones were less than the standard antibiotics. Hence the activity is not satisfactory, some of them showed nil activity (absence of inhibition zones viz. Aqueous extract and Petroleum ether, ethanol (1:1) on *Klebsiella pneumoniae*, *Salmonella typhi* and other test bacteria. The ethanol extract of *Datura alba* showed greater inhibition on both gram-positive and gram-negative bacteria, These results in accordance with the previous reports of Oleno et al. (1996). Essawi and Srour (2000) who also have been reported that ethanolic extract of plant parts exhibit more activity than other extracts. In addition, the present results coincide with the previous reports of Perumalsamy and Ignacimuthu (1998) and Vikas Dhingra et al. (1999).

### Stroke method

The data showed that there is a strong inhibition with the percentage of concentration of the plant extracts. It is evident from the tables that the concentration of the plant extract increases the inhibition rate also increases. At higher concentrations viz. 75 and 100% the inhibition rate is also higher. The antibacterial sensitivity was determined by the magnitude of presence or absence of growth. In control plates of each solvent extracts, the growth of bacteria was noted to be excessive whereas in experimental plates the degree of inhibition increased with the gradual increase in the concentration of plant extracts. Hence the higher concentration viz. 75 and 100% strongly or completely inhibit the growth.

It is evident that almost all the pathogenic bacterial species (both gram-positive and gram-negative) were noted to be susceptible to the leaf extracts (ethanol, ethyl acetate, chloroform and petroleum ether, ethanol 1:1) at higher concentration viz. 75 and 100% where complete inhibition (absence of bacterial growth) was observed. Ethanolic extract was found to be the more active on test bacteria other solvent extracts.

The extracts do not show satisfactory inhibition against bacteria even at higher concentrations (75% and 100%) except *E.coli* and *K 12*. Therefore the bacterial strains were found to be resistant to the plant extracts even at higher concentrations. In stem extracts the petroleum ether, ethanol (1:1) extract did not inhibit the test bacteria. Similarly chloroform extracts did not exhibit the bacterial strains followed by ethylacetate. It inhibits some gram-negative bacteria others are resistant to that treatment.

The results indicate that the leaf and its solvent extracts showed a significant inhibition against all the test bacteria. The stem does not show significant inhibition against most of the strains. The significant inhibition may due to the concentration of extracts. The increasing concentration of plant extracts which decreases the growth of bacteria. The present study results contrasted with the previous studies done by Brantner and Grein (1994), Alonsa-Paz et al., (1995) and John (2002).

### Antifungal assays

The antifungal activity of various solvent extract (aqueous, ethanol, ethyl acetate, chloroform, petroleum ether, ethanol 1:1) of *Datura alba* leaves and stems were evaluated invitro by various assays. The test fungi include *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Fusarium oxysporum*, *F. solani*, *Trichophyton mentagrophytes*, *T.rubrum*, *Trichoderma viridae*, *Microsporium gypseum* and *Candida albicans*. The results of the screening showed that the antifungal activity was observed in ethanol, ethyl acetate, chloroform, petroleum ether, ethanol (1:1) extracts while the aqueous extract did not show any inhibition against the test fungi. The parts tested regarding antifungal activity, the leaf showed high degree of inhibition.

### Paper Disc Diffusion Assay

The results reported that all the solvent extracts of the leaf exhibited inhibitory action against the test fungi except aqueous extract. The diameter of the inhibition zone formed around the plant disc indicates the degree of inhibition. Based on the

Results, it is cleared that the extent of antifungal sensitivity of each solvent extract against each of the fungi follows a rank of order. The activity was higher at the ethanolic extract while low activity was observed in petroleum ether, ethanol (1:1) when compared with standard antibiotics whereas there was no activity associated ( Nystatin 30 mcg/disc) with aqueous extract. It is noted that the inhibition zone of *A. niger* is 4 mm followed by *A. flavus* (4.2 mm), *Trichophyton mentagrophytes* (4.2 mm), *Fusarium oxysporum* (4.1 mm), *Trichoderma viridae* (3.8 mm) and *Candida albicans* (3.9 mm) It was found to be greater. The other organisms showed less inhibition zones.

In ethanolic extracts *Aspergillus niger* (3.7 mm), *A. flavus* (3.1 mm), *Trichophyton mentagrophytes* (3 mm), *Fusarium oxysporum* (2.9 mm), *Trichoderma viridae* (2.8 mm), *Candida albicans* (2.4 mm) and *Trichophyton rubrum* (2.1 mm) showed both increased and moderate zones when compared with that of the standard ( Nystatin 30 mcg/disc). The ethyl acetate and chloroform extracts produced more or less similar results, whereas petroleum ether, ethanol (1:1) extract showed decreased inhibition zones. The aqueous extracts did not show any zones of inhibition against test fungi. The present study results is in accordance with results of previous experiments done by Irobi and Daramola (1993) who reported that ethanolic extracts of *Mitracarpus villosus* showed antifungal activity against *Fusarium solani*, *Candida albicans*, *Microsporum gypseum*, *Trichophyton rubrum* and *Aspergillus niger*. Similar results were reported by Made and Irobi (1993) in their studies.

### Agar well diffusion method

The antifungal activity of various solvent extracts of *Datura alba* (leaf, stem) on different fungi by Agar well diffusion method has been depicted in the Tables 9 and 10. The results cleared that all the solvent extracts (Aqueous, ethanol, ethyl acetate, chloroform and petroleum ether, ethanol 1:1) exhibited their activity against all the fungi. The activity is noted in the form of inhibition zone formed around each well whose diameter measures the degree of inhibition. From the Tables 9 and 10,

it is cleared that the extent of inhibition varies in each solvent extract against the fungus.

The diameter of inhibition zone in each extract against each fungus varied considerably. The antifungal sensitivity was higher in ethanolic extract while lower in petroleum ether, ethanol (1:1) extract, when compared with the standard Nystatin (500 ug/ml) antibiotic. The inhibition zone diameter formed in each extract against each fungus was found to be either less than or greater than or equal to that of standard antibiotics. In ethanolic leaf extracts *Aspergillus niger* (4.1 mm), *A. flavus* (4.2 mm), *T. mentagrophytes* (4.7 mm), *M. gypseum*, *F. oxysporum* and *Candida albicans* showed increased zones of inhibition (Plate-8) whereas others are showed decreased values against fungi. The ethyl acetate and chloroform extract produced more or less similar results. The petroleum ether, ethanol (1:1) extract showed only less inhibitory action than the other solvents, whereas the aqueous extract did not show any antifungal activity. The results of the agar well diffusion method reveals that the antifungal activity was higher in ethanolic extracts of leaves than the other solvent extracts used. This work confirms the previous report of Purohit et al. (1995), Amphawan et al. (1995), and Aida Portillo et al. (2001) who established that the ethanol extracts of many plants inhibited the growth of several fungal strains.

### Stroke method

Close examinations of the results clearly indicate that there is a sharp degree of correlation with the percentage of concentration of the extract and the sensitivity of the fungal strain. It is evident from the table that as the concentration of the extract increased the degree of activity of the microbes also increases. In other words, at higher concentrations (75 and 100%) the sensitivity was also higher as a result, complete inhibition of growth examined in higher concentrations. It is evident from the plates that the thickness of the streaking line at the initial areas of contact is comparatively denser and thicker while it is progressively becoming thinner along the streaking lines towards the end. The thickness decreased further and further as the concentration

increased. Therefore at higher concentrations only the streaking lines with very thin layer of fungal colony were seen. Almost all species of fungi expressed the same type of result. The sensitivity of the fungi to the various concentration of the extract has been denoted as complete inhibition (-), partial inhibition (2+) and no inhibition (4+). The control plates of most of the fungi were found to be 4+ and the experimental plates of higher concentrations were found to be (-). The absence of the growth indicates the complete or higher inhibitory action of the plant extract. Almost all fungi especially *Aspergillus niger*, *A. flavus*, *A.fumigatus* and *Fusarium solani*, *T. mentagrophytes* and *Candida albicans* (Plate 9 and 10) exhibit same type of results in this sensitivity test. In this case also ethanolic extract showed maximum inhibitory activity than the other solvent used. Alade and Irobi (1993) stated that increasing concentrations of various plant extracts inhibit the growth of several fungi. The dermatophyte fungi, *Trichophyton mentagrophytes*, *T. rubrum* and *M. gypseum* however showed slight variations.

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