EVALUATION OF THE ANTIFUNGAL ACTIVITY, OF *Datura metel* LINN ETHANOLIC LEAF EXTRACT ON *Candida albicans*

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Emosairue Catherine Orevaoghene, Enwa Felix Oghenemaro*, Anie Clement Oliseloke and Michael Oghenejobo
Department of Pharmaceutical Microbiology, Delta State University, Abraka, Nigeria
*Corresponding author: felixenwa@yahoo.com, felixenwa@delsu.edu.ng

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Abstract: The antimicrobial and antioxidant activity of *Datura metel* has been studied by different researchers in the north, west and east of Nigeria except the south-south precisely Delta State. The study was carried out to evaluate the antifungal activity of the crude ethanolic extract of the leaves of *Datura metel* Linn against *Candida albicans*. The agar well diffusion method was used in the study and all determinations were done in triplicates. A modified agar dilution method was used in the determination of Minimum inhibitory concentration (MIC). The treatments used were 50, 100 and 150 mg/ml of plant extract for the Antifungal susceptibility test (AST) and 20–200 mg/ml of plant extract for the MIC determination. The antifungal activity of the plant extract was compared with those of Clotrimazole, a standard antifungal agent. The results of the antifungal screening revealed that the plant was most sensitive at 150 mg/ml with inhibition zones of 11–14 mm and least sensitive at 50 mg/ml with inhibition zones of 10-11 mm. Clotrimazole was significantly ($P<0.05$) more effective than the plant extract in inhibiting the yeast. The MIC and Minimum fungicidal concentration (MFC) were at 20 and 160 mgml, respectively. The results revealed that the plant extract if formulated to avoid certain metabolic pathways that enhances resistance to antimicrobial agents can be considered as a potential antifungal agent against *C. albicans*.

Keywords: Antifungal activity, agar well diffusion technique, *Datura metel*, sensitivity

Introduction

The use of medicinal plant extracts as antimicrobial agents has increased over the years and represents a rapidly expanding area of health science (Chopra *et al.*, 1956). It was found that large populations of people in developing countries particularly in the rural areas use traditional medicine especially plants in the treatment of various medical illnesses (Saranraj *et al*., 2011). Plants are reservoirs of a wide variety of secondary plant metabolites which have been shown to have antimicrobial properties (Lewis and Ausubel, 2006). These secondary plant metabolites are mainly tannins, alkaloids, and flavonoids. Thus, it is anticipated that plant phytochemicals with adequate antibacterial efficacy will be used for the treatment of infections caused by microorganisms (Balandrin *et al.*, 1983). Antimicrobial agents have been available for a broad range of pathogenic organisms for the past two decades. However, due to their widespread use, new forms of antimicrobial resistance have emerged to these agents (Cockerill, 1999). The increasing prevalence of multidrug resistant strains of bacteria and also strains with reduced sensitivity to existing conventional antibiotics which has led to the emergence of untreated bacterial infections and recurrent infections are the major reasons why plants are being screened for bioactive components and this has necessitated the search for new antimicrobial agents (Sieradzki *et al*., 1999; Tomoka *et al.*, 2002; Bandow *et al*., 2003). In addition, the increased research carried out on drugs from natural sources has led to the extraction and development of several drugs and chemotherapeutic agents from medicinal herbs present in abundance in the tropics (Falodun *et al*., 2006). The resistance of clinical pathogens to antibiotics particularly those used for first line treatment is becoming an issue of global concern. This is quite common in Nigeria where much of the antibiotic therapy is not laboratory-individualized or are even laboratory extrapolated leading to empirical prescription (Ozumba, 2005). The various treatment failures attributed to resistance of disease pathogens to commonly used antimicrobial agents has prompted many researchers to focus their investigations on natural products as new sources of medicines.Thus, higher plants have been randomly screened as potential sources of new biologically active molecules which will be useful as therapeutic agents. A number of methods are used for this purpose and the results obtained are influenced by both the method and microorganisms used in the test (Okwu and Igara, 2009).

One unique characteristic of plants is their limitless ability to synthesize aromatic secondary metabolites, which comprise mostly of phenolic compounds or their oxygen-substituted derivatives (Geissman 1963). The findings of a study carried out over two decades ago revealed that the deleterious effect of phenolic compounds on micro-organisms is due to the site(s) and number of hydroxyl groups present (Scalbert, 1991). Flavones, flavonoids, and flavonols on the other hand are phenolic structures with one carbonyl group synthesized by plants in response to bacterial infection (Dixon, 1983) and are often found to be active *in vitro* as antimicrobial substances against a wide range of micro-organisms (Bennet and Wallsgrove, 1994). Tannins are polymeric phenolic compounds and have astringent properties (Basiri and Fan, 2005). The findings from a recent study revealed that the external surfaces of plants are often protected by biopolymers such as; waxes, fatty acids, and esters such as cutin and suberin. The external tissues were also found to be rich in phenolic compounds, alkaloids, diterpenoids, steroids, and other compounds which possess antimicrobial activity against fungi and bacteria. In addition, the cell walls of some monocotyledons also contain antimicrobial proteins, referred to as thionins (Angeh, 2006). The basic principle involved in pharmaceutical extraction of bioactive substances from plants is to mill the plant material (dry or wet) into final particles which increases the surface area for effective interaction between the plant material and extraction solvent thereby increasing the rate of extraction (Das *et al*., 2010). It involves separation of medicinally active components of plant tissues from inert ones by using selective solvents and suitable extraction technology. These solvents enter the solid plant tissues by diffusion and solubilize compounds of similar polarity (Green, 2004). Therefore, the factors that determine the quality of the extract are the plant material, choice of solvents, and the methods of extraction (Das *et al* 2010).

Datura metel (also called Devil trumpet, Hairy thorn-apple) belongs to the Solanaceae family which comprises of about 85 genera and 2800 species distributed all over the world and the genus *Datura* (Jamdhade *et al.*, 2010). It is a common medicinal plant found in many states in Nigeria and some of the common names are: *Gegemu*, *Zakami*, *Ajegun-eegun*,

Apikan and *Furenjuu* (Medicinal Plants Database from Nigeria, 2013). *D. metel* is widely used in phytomedicine in the treatment of diseases such as asthma, cough, convulsion, insanity and management of dysmenorrhea. The leaves and the seeds are therapeutically active and are used as antispasmodics, anesthetics, antiasthma, antitussives, bronchodilators, and as hallucinogens. The major phytochemical constituents of the plant comprise majorly of alkaloids (tropane alkaloids), flavonoids, phenols, tannins, saponins, and sterols (Tomoka *et al.,* 2002; Bandow *et al*., 2003). Also, *D metel* is presently an active ingredient in the decoction used by herbalists in Eastern Nigeria for the treatment of gonorrhea, asthma, cough, skin ulcers, burns, and wounds (Tomoka *et al.,* 2002). The interest in the scientific investigation of medicinal plants in Nigeria is based on the claims by traditional practitioners of their efficacy in the treatment of many diseases. Therefore, research into the antimicrobial effects of these medicinal plants is expected to establish the rationale for their use in the treatment of diseases caused by different organisms (Shagal *et al.,* 2012). The findings from these studies are also expected to enhance their use in antimicrobial chemotherapy.

This study was done to determine the antifungal activity of *Datura metel* ethanolic leaf extract against *Candida albicans* and compared with those of a standard antifungal drug (Clotrimazole). The prime objective of the study was to investigate the antifungal potential of *D. metel* and determine the MIC and MFC of the ethanolic leaf extract of *D. metel*.

Materials and Methods

Study area

The study was carried out in 2012 at the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, Delta State University, Abraka.

Datura metel leaves, Ethanol (BDH, England), Mueller-Hinton agar (Titan Biotech Ltd Delhi, India), Whatman filter paper No.1 (Whatman Limited, England) and Clotrimazole (Duben Laboratories Pvt. Ltd, India) which were purchased from a registered retail pharmacy in Abraka, Delta State, Nigeria were used in the study. Other reagents used in the study were of analytical grade.

Collection of plant materials

Fresh leaves of *D. metel* free from disease were collected from the Teaching and Research Farm of the Department of Agronomy, Faculty of Agriculture, Delta State University, Asaba Campus, Delta State and was identified Dr Ilondu Martida of the Department of Botany Delta state University. The fresh leaves were shade-dried, pulverized to powder using an electric blender (Binatone, China; Model No. BLG- 450), and stored in a polythene bag prior to extraction.

Preparation of plant extract

Plant extract preparation was carried out as described by Enwa *et al.* (2019). The powdered leaves were poured into a clean pre-sterilized wide mouth bottle containing ethanol and for seven days extracted with constant agitation. The extract was concentrated at 40ºC in an oven and filtered with Whatman filter paper No. 1 using a suction pump. The crude extract obtained was dark brown to green in colour, viscous in nature and it was stored in an air tight glass jar and refrigerated prior to use. The weight of the extract (yield) was 11.84 g and the percentage yield (23.68%$^{W}/_{W}$) was calculated using the formular below:

$$\text{Percentage yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of sample}} \text{X} \frac{100}{1}$$

Collection of test microorganisms

C. albicans isolated from the high vaginal swab of an infected female was obtained from Echo Scan Laboratory Asaba, Delta State. It was subcultured and pure culture obtained was used for the study.

Identification and confirmation of yeast

The collected microorganism was identified by cultural and biochemical methods, characterized microscopically as described by Monica (2018), and then maintained on freshly prepared Sabouraud Agar slants for further use.

Preparation of inoculum standard

Themethod used by Sakthi *et al*. (2011) was adopted in preparing 0.5 McFarland Standard (Saranraj *et al.,* 2011). The turbidity of the 0.5 McFarland Standard which is equivalent to a cell density of 1.5 X 10^8 CFU/ml was used to standardize the inoculum.

Preparation of fungi inoculum

0.9 g of Sodium chloride was dissolved in 100 ml of distilled water and sterilized in an autoclave. Five colonies from a 48 h culture were collected with a flame-sterilized wire loop and were inoculated into a test tube containing 5 ml of the sterilized normal saline. The test tubes were incubated at 35ºC for 1 h till a moderate turbidity was obtained. Thereafter, the turbidity was adjusted to 0.5 McFarland Standard by visual comparison.

Preparation of agar wells and inoculation of test plates

The agar well diffusion method was adopted with slight modifications (Oluduro *et al.,* 2011; Saranraj 2010). A flame-sterilized cork-borer was used to make three wells of 5 mm in diameter and approximately 1.5 cm apart on freshly prepared Mueller Hinton agar plates. The dried agar surfaces were inoculated with the yeast using the streak plate method.

Plant extract solution preparation and application on test plates

50, 100, and 150 mg of plant extract were individually weighed using an analytical balance and dissolved in 1 ml of Dimethylsulphoxide (DMSO). The purpose was to enhance the penetration of the antimicrobial constituents of the extract into the cell wall of the yeast. After homogenizing, the solution was added into the wells on the test plates by means of a 1 ml syringe.

Preparation of control plates

Clotrimazole 100 mg tablet was crushed and dissolved in the 250 ml of absolute ethanol to obtain a concentration of 20 µg/ml. 1 ml of this solution was added into the wells on the control plates which served as the positive control. Also, 1 ml of DMSO was added into 5 mm wells on the three control plateswhich served as negative control.

Incubation of test plates

The plates were allowed to stand for 15 min after inoculation and addition of plant extract solutions to allow proper penetration of the extract into the media before incubation in a pre-sterilized incubator set at 35ºC for 48 h. The plates were observed for microbial growth after 24 and 48 h of incubation.

Minimum inhibitory concentration (MIC)

Plant extract solutions were prepared to obtain concentrations of 20–200 mg, dissolved in 1 ml of DMSO and aseptically dispensed into a Petri dish by means of a pipette. 19 ml of molten Mueller-Hinton agar was added to 1 ml of each concentration of plant extract and mixed thoroughly to allow uniform distribution of the plant extract. The plates were allowed to set at room temperature prior to inoculation. A suspension of yeast was prepared using pre-sterilized normal saline and incubated for 1 h until moderate turbidity was observed. The suspension was then adjusted to 0.5 McFarland Standard by visual comparison. The culture plates were then inoculated using the streak plate method. The plates were incubated at 35ºC and observed for growth after 24 and 48 h. The MIC was determined as the lowest concentration of plant extract in the agar medium that inhibited the visible growth of the yeast. A control plate containing only Mueller Hinton agar was prepared, incubated with the test plates and also observed

for growth after the period of incubation. The purpose was to ensure that absolute sterility was maintained throughout the inoculation process.

Minimum fungicidal concentration (MFC)

The surface of the MIC plates on which no growth was observed were streaked and the swab sticks were used to inoculate fresh Mueller-Hinton agar plates containing no plant extract. The plates were incubated at 35ºC and observed after 24 h. The MFC was recorded as the lowest concentration of antimicrobial that did not permit any visible growth of the organism after subculture on antimicrobial-free media and overnight incubation.

Statistical analysis

Results are reported as Mean ± Standard deviation. The data obtained from the study were subjected to Analysis of variance (ANOVA) and significant means separated using least significant difference (LSD) test.

Results and Discussion

The results of the antifungal activity of the plant extract on *C. albicans* are presented in the pie chart and the percentage inhibition in Figs. 1 and 2. The mean zones of inhibition and standard deviations from the mean were, 7.0 ± 3.53, 7.3 ± 3.70, and 8.3 ± 2.05 for concentrations of 50, 100 and 150 mg/ml, respectively. The mean zone of inhibition and standard deviation of Clotrimazole was 15 mm ± 0.00 and the percentage inhibition was 81.9%. There was no significant difference between the antifungal effect at 50 and 100 mg/ml. However, there was a significant difference between the effect at 50 and at 150 mg/ml.

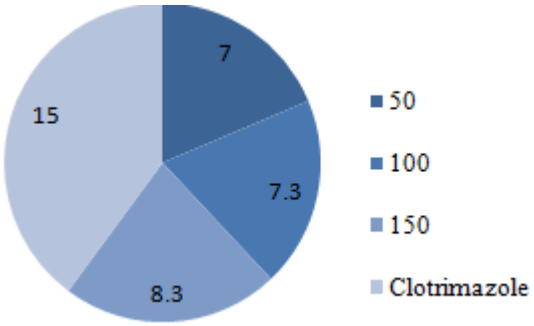

Fig. 1: Mean zone of inhibition of *C. albicans* at different concentrations of *D. metel* extract

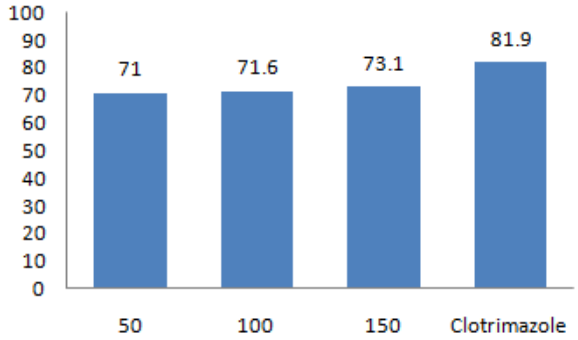

Fig. 2: Percentage inhibition of *C. albicans* by the different treatments used in the study

The percentage inhibitions of the plant extract were 71.0% at 50 mg/ml, 71.6% at 100 mg/ml, and 73.1% at 150 mg/ml. The results indicated that the inhibitory effect of *D. metel* extract was concentration dependent. There was no significant ($P<0.05$) difference between the percentage antifungal activity of the plant extract at 50 mg/ml and at 100 mg/ml. There was also no significant difference between the antifungal activity observed at 100 mg/ml and at 150 mg/ml but a significant difference was observed between the antifungal effect of *D. metel* leaf extract at 50 and 150 mg/ml. This impiled that an increase in concentration may trigger a corresponding significant increase in antifungal activity. However, the antifungal effect of Clotrimazole was significantly ($P<0.05$) greater than those of the plant extract at all concentrations. The minimum inhibitory concentration (MIC) results after 24 h showed no microbial growth at 20, 40, 60, 140 and 160 mg/ml while minimal growth was observed at 120 and 200 mg/ml and dense microbial growth at 80, 100 and180 mg/ml. After 48 h, minimal microbial growth was observed at 60 mg/ml (Table 1). The MIC was taken as 20 mg/ml which was the lowest concentration to inhibit the visible growth of the yeast. The minimum fungicidal concentration (MFC) results after incubation showed absence of growth at 160 mg/ml, hence 160 mg/ml was taken as the MFC.

Table 1: MIC of *D. metel* ethanolic extract on *C. albicans* at 24 and 48 h of incubation

Concentrations of *D. metel* extract (mg/ml)	Incubation Period			
	24 hours		48 hours	
20	NG	No growth	NG	No growth
40	NG	No growth	NG	No growth
60	NG	No growth	G	Sparse microbial growth
80	G^+	Dense microbial growth	G^+	Dense microbial growth
100	G^+	Dense microbial growth	G^+	Dense microbial growth
120	G	Sparse microbial growth	G	Sparse microbial growth
140	NG	No growth	NG	No growth
160	NG	No growth	NG	No growth
180	G^+	Dense microbial growth	G^+	Dense microbial growth
200	G	Sparse microbial growth	G	Sparse microbial growth

For several decades, micro-organisms particularly opportunistic organisms such as *C. albicans* have played a significant role in human health. This organism is commonly implicated in nosocomial infections and community-acquired infections (Eryilmaz *et al.*, 2010). It is the common causative agent of *Candidiasis* especially among females and about 45% of clinical fungal infections are caused by *C. albicans* (Gupta *et al.*, 2004).

The antifungal activity of the plant extract against *C. albicans* was similar to the report of previous researchers. The zones of inhibition obtained ranged from 10 – 14 mm. Saranraj *et al*. (2010) obtained zones of inhibition 11 – 15 mm in his study which were within the same range as those obtained in the study (Saranraj *et al.,* 2011). However, Sakthi and Geetha (2011) obtained lower zones of inhibition at similar concentrations than those obtained in the study as their findings showed that at 300 mg/ml the zone of inhibition for *C. albicans* was 15 mm (Sakthi *et al.,* 2011). The activity of the standard antifungal drug (Clotrimazole) against the yeast was consistent and differed significantly from the activity of the plant extract. Thus, the standard antifungal agent was more effective than the plant extract against the yeast. The overall better activity shown by the conventional antimicrobial agent could be hinged on the fact that they have been properly formulated to inhibit particular metabolic pathways and also destroy certain features of the organisms that are essential for their survival compared with the crude extracts of medicinal plants which are easily degraded and decomposed on storage (El-Mahmood and Amey, 2007). MIC is the most basic laboratory measurement used to confirm resistance of clinical pathogens to new antimicrobial agents and monitor activity of these agents (Sen and Batra, 2012). The MIC on the yeast was read at 24 and 48 h as most *Candida spp*. have suitable growth for MIC determination at 24 h and a shorter incubation time is more efficient and practical for use in the clinical laboratory (Espinel-Ingroff *et al.,* 2009). The MIC of *C. albicans* was 60 mg/ml after 24 h. Similarly, after 48 h of incubation the result remained the same with slight changes seen at 160 mg/ml (Table 1). Thus, the results indicate that a higher concentration of the plant extract was more effective in inhibiting the yeast. This result agreed with the findings of Hendi *et al*. (2011) that higher concentrations of plant extract were required to inhibit the growth of *C. albicans* (Hendi *et al.,* 2011). The MFC of *C. albicans* was at 160 mg/ml which was 8 times higher than the MIC value. This emphasized the findings of earlier researchers that inhibition of fungi required higher concentration of plant extract (Espinel-Ingroff *et al.,* 2009).

Recommendations

Further work should be done on *D. metel* leaves. The leaf extract should be fractionated into their individual phytochemical components and tested individually on different types of clinically implicated fungi or the phytochemicals that have antimicrobial properties such as tannins, alkaloids and flavonoids should be individually separated, mixed together and tested on a wider range of fungi pathogens.

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Conflict of Interest

Authors declare that there is no conflict of interest in this study.

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