Application of Hydro-ethanol Extract of *Tithonia diversifolia* (Hemsl) in the Treatment of Experimental Murine Oral Candidiasis

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OMD and MOA designed the study, authors OMD and ATA performed the statistical analysis. Authors OMD, MOA, TO, OOA and ATA wrote the protocol, and the first draft of the manuscript. Authors TO, OOA and ATA managed the analyses of the study. Authors OMD and MOA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Oral infection caused by *Candida* spp. is a major healthcare problem in dental and oral care. Treatment failure has been reported in cases of oral candidiasis as a result of resistance to common antifungals.
Aim and Objective: In this study, the *in vitro* and *in vivo* activities of extract of *Tithonia diversifolia* against virulence factor-borne and antifungal resistant-*Candida albicans* were investigated. *Candida albicans* was isolated from the saliva of patients attending a tertiary hospital in Ekiti State.

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Methodology: Standard methods were used to determine the presence of virulence factors in the isolates. *In vitro* and *in vivo* anti-candidal activities of the hydro-ethanolic extract of *T. diversifolia* were also tested on the test fungus.
Results: The virulence factors have varying percentage of occurrence in all the isolates with catalase having the highest. Itraconazole and nystatin were not effective against the isolates. Out of the six isolates selected (based on antifungal resistance) only three produced strong biofilm. The reduction in the population of the test organisms by the extract was time and concentration dependent. At the end of candidal challenge and treatment assays, extract of *T. diversifolia* has lower anti-candidal property compared to nystatin.
Conclusion: This study has shown that *C. albicans* associated with the mouth carries virulence factors and are resistant to common antifungals. In this work, we noticed antifungal effects of hydro-alcoholic extract of *T. diversifolia* on *C. albicans* associated with oral infections.

1. INTRODUCTION

Medicinal plants are gifts of nature to cure diseases of human beings and animals. They are utilized globally especially among rural dwellers. Medicinal plants have proven to be relatively cheap, safe and effective remedies for infectious diseases [1] (Nyamukuru et al. 2017). The recent increase in resistance of causative agents of mycotic infections to antifungals have subsequently increased the rate of morbidity, mortality and pressure on health care globally [2]. Widespread use of immunosuppressive therapies, abuse and misuse of broad-spectrum antimicrobials, common use of indwelling intravenous devices, high rate of HIV/AIDS and cancers have been identified as the major factors responsible for the high incidence of drug resistant fungi [3;4] Nyamukuru et al. 2017; Ududua et al. 2019).

Medicinal plants have been a source of varieties of relatively cheap biologically active phytochemicals used extensively for the treatment of different infectious diseases. These plants are rich sources of bioactive secondary metabolites hence they could be better alternatives to curbing the increased resistance of pathogenic fungi to antifungal agents [5,6] (Nyamukuru et al. 2017). The alarming rate of resistance necessitates the need for discovery of new classes of antifungal compounds for the treatment of fungal infections [7]. Phytochemicals consisting of both primary and secondary metabolites have been reported to have antifungal activities. In most cases the folkloric usage of these compounds has not been documented [8].

Tithonia diversifolia belongs to *Compositae* with abundant showy sunflower-like flowers (Lorenzi,

1995; Otoide et al. 2018). It has become a noxious weed that now constitutes a serious threat to natural ecosystems in southern Nigeria due to its serious invasions in agricultural areas (Chukwuka et al. 2007; [9]; Otoide et al. 2018). Due to the presence of relevant phytochemicals [10]; Douglas and Jeruto, 2016), the plant has been reported to have antimicrobial activities and a wide application in the treatment of infectious diseases [11]; Hiransai et al. 2016) especially mycotic infections. Phenolic compounds in *T. diversifolia* stimulate macrophage production on admission to animals and consequently prevent fungal infections by its antithrombotic, anti-inflammatory and vasodilatory activities [12,13], Hiransai et al. 2016; Ejelonu et al. 2017). In this study, the *in vivo* and *in vitro* anti-candidal activity of crude hydro-alcoholic extract of *T. diversifolia* was investigated against drug resistant and virulent *C. albicans*.

2. MATERIALS AND METHODS

2.1 Source of Organisms

Nine *Candida albicans* strains primarily isolated from diabetic patients with oral candidiasis was collected from the stock culture of Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria. The isolates were cultured on Potato Dextrose Agar (PDA) supplemented with chloramphenicol and incubated for 48 h at 37˚C. Creamy and pasty colonies seen after incubating for 48 h at 37˚C were cultured on Hichrome *Candida* agar (Himedia Laboratories Pvt. India) which was incubated for 24 h at 37˚C. The agar gives distinct colouration for various species of *Candida*. The isolates were biochemically identified by Gram reaction and sugar utilization test as described by Williams and Lewis (2000).

2.2 Catalase Production among the Isolates

A loopful of 18 h old culture of *C. albicans* was simply smeared on grease-free slide and a drop of 3% hydrogen peroxide was added. Visible production of bubbles indicated production of catalase activity and a lack of bubbles indicated the absence of catalase.

2.3 Phospholipase Formation among Isolates

The modified method of Samaranayake et al. [14] was used to detect the production of phospholipase among the isolates. About 1.17 g of salt (NaCl) was added into 100 ml of water containing Potato Dextrose Agar base medium and was sterilized at 121°C for 15 min. After sterilizing, the basal medium was allowed to cool to about 50°C. Egg surface was sterilized with 1% hypochloride followed by 75% ethanol prior to breaking the shell. The yolk was added to the basal medium. Five microliters of 18 h culture of *C. albicans* with 0.5 McFarland standard was inoculated onto the egg yolk agar plates and incubated at 37°C for 48 h. The production of white halo around the colony after incubation indicated phospholipase production.

2.4 Haemolysin Production among the Isolates

This was done by dissolving 3.7 g of blood agar base in 100 ml of distilled water. This was heated while stirring until it was completely solubilized, then, it was sterilized in an autoclave at 121°C for 15 min. The mixture was allowed to cool to about 50°C, then 7% human blood was added to the melted sterile agar medium, dispersed into petri dishes and allowed to solidify. The isolate was then streaked on the medium and incubated for 48 h at 37°C. Clear zone implies the production of beta (β) haemolysin, green colour, alpha (α) haemolysin while gamma (γ) haemolysin, produces no apparent changes.

2.5 Gelatinase Production among the Isolates

Ten percent (10%) gelatin was added to 3.9 g of potato dextrose agar into 100 ml of distilled water and it was heated gently to dissolve the gelatin. It was then sterilized at 121°C for 15 min in an autoclave. After sterilizing, it was then allowed to cool to about 45°C and then poured into petri dishes and allowed to solidify. The organisms were streaked on the agar and incubated for 48 h at 37°C, after that, the plate was flooded with Fraizer Solution (Mercuric chloride, 15.0 g in 20 ml of 37% v/v hydrochloric acid, made up to 100 ml by adding distilled water), and left for 5 to 10 mins. The development of the clear zone around the colonies was taken to be as a result of production of gelatinase that hydrolysis gelatin incorporated into the agar.

2.6 Coagulase Production among the Isolates

Modified method of Yigit and colleagues [15] was used to determine the production of coagulase by *C. albicans*. A drop of normal saline (0.9% NaCl) was placed onto the grease free slide, and emulsified with the test organism using a sterile wire loop. A drop of human plasma was placed beside the inoculated saline drop and mixed well with the organism. The slide was rocked gently for about 10 seconds. The observation of clumping was taken for positive result, if negative, no clumping will be observed in the plasma within 10 seconds. Distilled water was used instead of human serum in the control experiment.

2.7 Hydrolase Production among Isolates

One and a half gram of skimmed milk was incorporated into 100 mL of 3.9 g of PDA supplemented with chloramphenicol. It was then sterilized at 121°C for 15 min, prepared plates were streaked with test isolates before incubation at 37°C for 48 h. After incubation clear zone around the colonies was taken to be positive.

2.8 Antifungal Sensitivity Test

Each of the test organisms was grown at 37°C in Mueller-Hilton broth (Oxoid) for 18 h and adjusted to an optical density of 0.5 McFarland Standard. The standardized culture was seeded on the surface of sterile Mueller-Hilton Agar (Oxoid). Stock of different antifungals including fluconazole (10 µg), griseofluvin (5 µg), itraconazole (50 µg), ketoconazole (7.5 µg) and nystatin (10 µg) were separately diluted to achieve different concentrations as recommended by European Committee on Antimicrobial Susceptibility (EUCAST, 2015). Antifungal susceptibility testing was done using the disk diffusion method. Disc (5 mm) was prepared from Whatmann No.1 filter paper and was sterilized in an oven at 160°C for 2 h. The sterile disks were soaked in different

concentrations of the antifungals and carefully placed on seeded plates of the test isolates. The plates were incubated at 37°C for 24 h. Zone of inhibition was measured and interpreted according to EUCAST standard.

2.9 Detection of Biofilm Formation among the Isolates

Congo red (0.17 g) was added to 100 mL of PDA supplemented with chloramphenicol before sterilization at 121°C for 15 min. After sterilizing, the agar was poured into petri dishes and allowed to solidify. The organisms were streaked on the medium and incubated for 24 h at 37°C. The isolates that produce biofilm appeared black while the negative biofilm formers do not produce black colouration. The biofilm produced were quantified using the method of Siegfried et al. [16]. A 100 µL of standardized cell suspension $(1.0 \times 10^6$ cells/mL) was added into a tube containing 2 mL potato dextrose broth (PDB) supplemented with chloramphenicol. The tube was incubated for 48 h at 37°C, the suspensions were aspirated after incubation and the tube was washed twice with sterile saline to remove loosely adherent cells. Five millilitres of 0.5% crystal violet solution was added into each of the tubes and allowed to stay for 15 min at room temperature and later discarded and rinsed with sterile saline. Thereafter, 5 mL of 95% ethanol was added to the tubes, the optical density (OD) was measured using a spectrophotometer at 520 nm and the result was interpreted as described by Siegfried et al. [16].

2.10 Chlamydospore Production among the Isolates

Each of the *candida* isolates was picked up with a straight wire and streaked using deep cutting on Corn Meal Agar plate at an angle and was then covered with cover slip to produce a relative anaerobic condition. The plates were examined under microscope for the presence of chlamydospore at 24 h interval for three days. The formation of chlamydospore and pseudohyphae was observed.

2.11 Collection, Preparation and Extraction of Plant Material

The fresh leaves of *T. diversifolia* were collected at Iworoko-Ekiti, Ekiti State Nigeria. The leaves were obtained from apparently healthy plants found within 5 m^2 of the area and were taken to the Herbarium of the Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti for identification. The leaves were rinsed in clean water, allowed to air-dry and grinded. Fifty grams of the powdered leaves were soaked in 1 L of 70% hydroethanol at room temperature for approximately 24 h with sporadic shaking. The sample was then suction-filtered through Whatmann Number 1 filter paper and washed with another 200 mL solvent after which the filtrate was concentrated. The dried extract was reconstituted in 5% dimethyl sulphoxide (DMSO) to give 50 mg/mL stock solution. This was further diluted to the required concentrations for the bioassay. The reconstituted extracts were filtered by 0.45 µl pore size membrane filter for sterility.

2.12 Time Kill Assay

The broth macrodilution technique of National Committee for Clinical Laboratory Standards (2012) was used to determine the rate of killing of the test fungus by the extract. The assay was performed with a final inoculum of approximately 1.0×10^5 cfu/mL. Different concentrations of sterile extract were added into 50 ml of glucose supplemented YNB to give a final concentration between 0.50 and 15.00 mg/mL; and each tube was inoculated with the fungus. At each sample time of 0, 2, 4, 6, 8, 10 and 12 h, aliquots (0.2 mL) of the samples were withdrawn, diluted in ten-fold series in sterile normal saline and plated onto PDA supplemented with chloramphenicol and incubated at 37°C for 24 h. The controls were set up consisting of a negative without the plant extract but the diluents, while the positive plate was supplemented with 0.5 mg/mL nystatin. The experiment was conducted in duplicates and repeated twice. The candidal colonies developed were counted and compared with the control.

2.13 Determination of Minimum Inhibitory Concentration (MIC)

The minimum concentrations of the extracts that inhibited the test organism (*C. albicans* EKSU 029) were determined using macrodilution method of Eloff (1998). An 18 h broth culture of *C. albicans* EKSU 029 in PDB supplemented with chloramphenicol was standardized to 0.5 McFarland scale $(1.0 \times 10^6$ cfu/mL). This was further diluted to a density of 1.0×10^3 cfu/mL. The organism was exposed to different concentrations of the extract with nystatin serving as control. The experiment was conducted in duplicates and repeated twice. The minimum inhibitory concentration (MIC) was defined as

the least concentration with no visible sign of growth.

2.14 Determination of Minimum Fungicidal Concentration (MFC)

A loopful of culture from the first three tubes that showed no sign of growth in the MIC tests were inoculated on Mueller-Hinton Agar plates. The plate was incubated at 37˚C for 24 h and afterwards examined for growth. The least concentration of the extracts that showed no growth was taken to be the minimum fungicidal concentration (MFC). The MIC index (MICI) of extract was calculated as the ratio of MFC and MIC of each of the isolate. The result was interpreted as follows: MFC/MIC ≤ 2.0 was considered bactericidal, > 2 or ≤ 16 was considered bacteriostatic and if the ratio is ≥ 16.0, the extract was considered ineffective [17].

2.15 Determination of Minimum Biofilm Inhibition Concentration (MBIC)

The method of Wasfi et al. (2012) was used with modification to determine the effect of sub-MICs of the plant extracts on *C. albicans* EKSU 029 adherence. In order to make different concentrations, 100 µl of standardized cell suspension (1.0×10^6 cells/mL) and sterile extract of *T. diversifolia*, were pipetted into each tube containing 2 mL potato dextrose broth (PDB) supplemented with chloramphenicol. The control tube has only the broth with the inoculum. All the tubes were incubated for 48 h at 37˚C. The biofilm form was quantified as described above.

2.16 Determination of Minimum Biofilm Eradication Concentration (MBEC)

The method of Antunes et al. (2011) was used to determine the concentrations of the extracts that will clear the preformed biofilm of the organism. A 100 µL of standardized inoculum of *C. albicans* EKSU 029 was added into tubes containing 2 mL of PDB supplemented with chloramphenicol and incubated at 37˚C for 24 h. The contents of the tube were discarded and sterile normal saline used to gently wash of remaining planktonic and loosely bound cells. Another 2 mL of sterile PDB (supplemented with chloramphenicol) containing appropriate concentration of the extract was added into the tube and incubated at 37˚C for 24 h. The amount of biofilm still remaining on the wall of the test tube was quantified as described above.

2.17 Animal Care and Management

Twenty healthy male Wistar rats (*Rattus norvegicus*) weighing between 150-200 g were obtained from the Animal house of the College of Medicine, Ekiti State University, Ado-Ekiti and acclimatized therein for a week. The rats were housed under standard laboratory conditions of natural light/dark cycle at room temperature and humidity; fed on standard rat pellets (Ladokun Feeds, Ibadan, Nigeria) and given water *ad libitum*. The rats were assigned randomly into groups (A, B, C and D) of 5 rats each and housed in standard rat plastic cages. All animals were handled in accordance with the Guidelines for animal research as detailed in the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication, 2011).

2.18 Induction of Oral Candidiasis and Animal Treatment

The *C. albicans* EKSU 029 strain was cultured on PDB supplemented with chloramphenicol for 48 h at 37˚C and centrifuged at 1300 g for 10 min. The supernatant was discarded while the sediment was resuspended in 5 mL sterile normal saline (0.85% NaCl) solution and the procedure was repeated. The animals were observed to be negative to any visible sign of oral candidiasis. The animals were anesthetized with chloroform intramuscularly in a 1/0.5 mL proportion - dose of 0.1 mL/100 of body weight. Intraepithelial injections containing 25 µL of the *C. albicans* suspension from stock containing 10^7 cells/mL was administered by means of a 1 mL insulin sterile syringe and 13 x 3.8 sterile needle into the central region of the dorsal tongue immediately before the giant papilla and two additional injections were made in the intermolar tubercle region. The challenged animals were observed for seven days and the colonization confirmed by observing the animals. Animals with creamy white lesions or patches on the tongue with or without redness were taken to have developed candidiasis. The establishment of *C. albicans* in the tongue of the animals was determined according to the method of Hisajima et al. (2008). The *Candida*-challenged rats were sacrificed after 24 h of introduction of the yeast, and the tongues were aseptically removed, washed with a sterile normal saline. The surface of the tongue was swabbed and also scraped gently by sterile scalpel. The swab was then cultured on PDA supplemented with chloramphenicol to confirm the viability of the organism while the scrape was placed on a slide

for Gram staining. Recovery of *C. albicans* from the tongue of challenged experimental animals was done by homogenizing the tongue in a sterile normal saline and cultured as described above and the viable *C. albicans* incubated for 20 h at 37˚C. The animals were treated two times in a day with appropriate concentration of the extract for 7 days. The animals in Groups A, B and C were treated with 12.5 mg/mL, 25.00 mg/mL of the extracts and nystatin (10 µg/mL) respectively. Animals in Groups D were not treated (negative control).

2.19 Recovery of *Candida albicans* from the Tongue *and* Histological Examination

Under ether anaesthesia, the animals were sacrificed at the end of the experiment. The tongues of the animals were aseptically removed and half of it was cut, aseptically macerated and serially diluted in sterile normal saline and plated on PDA supplemented with chloramphenicol. The plate was incubated for 24 h at 37˚C. The number of colonies were counted and recorded. The remaining half was fixed in 10% Neutral buffered formalin, for slide preparation according to routine procedures. Slices of 5 µm were stained with Hematoxylin-Eosin for demonstration of general tongue architecture. The sections were examined under Leica DM750 research microscope with a digital camera attached. Digital photomicrographs of the tissue sections were taken at various magnifications.

3. RESULTS AND DISCUSSION

Among the nine isolates screened, catalase had the highest occurrence among the six virulence factors determined. *Candida albicans* EKSU 013 and *C. albicans* EKSU 056 had three out of the six virulence factors (Table 1). Catalase plays a pivotal role in the protection of *Candida* spp. against anti-candida agents and contributes to the resistance of the fungus to leukocyte-mediated killing. It contributes to growth and development of *C. albicans* and increases its pathogenicity [18,19,20]. All the virulence factors screened for were present in *C. albicans* EKSU 009 and *C. albicans* EKSU 029. The pathogenesis of *C. albicans* has been reported to be largely determined by the ability of the organism to produce extracellular hydrolytic enzymes. These extracellular enzymes play vital roles in invasion of the host tissue [21]. The virulence of *C. albicans* is due to synergistic combination of many virulent factors and not just the presence of a factor in the organism [22].

Coagulase reacts with plasma fibrinogen to produce a series of intermediates that finally induce clotting of plasma [23]. Haemolysin production and then iron acquisition facilitates tissue invasion by *Candida.* It also enables progression of infection after colonization. The presence of these putative virulence factors is a pointer to the pathogenicity of the isolates.

Isolate *C. albicans* EKSU 019 was resistant to all the antifungals as shown in Table 1. Three isolates were resistant to four of the antifungals while only one was resistant to three. Ketokonazole and griseofulvin had higher performance compared to other antifungals. Nystatin is a polyene antifungal agent which interacts with ergosterol in the plasma membrane of fungal cell [24]. The isolates must have developed a resistant mechanism by modifying the cell membrane.

Out of the six isolates selected (based on antifungal resistance), three produced strong biofilms while only one of the isolates was a non-biofilm producer (Table 2). Phospholipases promotes the invasion of epithelia cell of the host and its production could be an indicator in differentiating invasive pathogenic strain of *C. albicans* from non-invasive colonizing strains [25,21]. This enzyme has a significant role in colonization of epithelial surface of the buccal cavity especially the tongue.

Effects of the crude hydro-alcoholic extract of *T. diversifolia* on antifungal resistant *C. albicans* is shown in Table 3. The MBIC and MBEC were higher than the corresponding MIC and also MFC in most cases. On both abiotic and biotic surfaces, *C. albicans* forms biofilms. Biofilms provide a support and protection for the sessile cells. The upper part of the biofilm provides a very suitable environment for *C. albicans* to transit hyphal cells [26].

The reduction in the population of the test organisms was time and concentration dependent as shown in Table 4. Nystatin had better activities on the isolates than any of the tested extract concentrations. At 0.05 significant level, the extract at 5.0 mg/mL, 10.0 mg/mL and 15.0 mg/mL significantly reduced the population of the fungus compared to the negative control group. This further supports the finding of Yemele and colleagues [10] that *T. diversifolia* has pronounced antifungal properties and also boost immunity (Ejelonu et al. 2017).

The pathogenicity of *C. albicans* is mainly dictated by its varying virulence factors. The

morphology of *C. albicans* is largely affected by the pH of the environment, serum and physiological temperature. Healthy human saliva has a pH of 7.4. It has been reported that at pH slightly greater than 7, the organism tends to transit from unicellular form to pseudohyphae [27,28].

After the presence of the pathogen in the tongue of the challenged animal showed its establishment, low level of inoculum facilitates the yeast-hypha transition in the tongue of the animal. Mayer et al. [29] reported that low cell densities ($< 10^7$ cells ml^{-1}) favour hyphal formation in *C. albicans,* also this changeover has been reported to be an important aspect of pathogenicity of the organisms. After treatment,

clearance of the organism from the site of inoculation was observed. At the end of the treatment, the candida load in the tongue of the organism in the nystatin-treated group was the least (Table 5).

Plate 1, quadrant A shows a photomicrograph section of tongue of animals after establishment of *C. albicans* EKSU 029. At this point these is production of pseudohyphae. The formation of the germ tubes precedes the formation of the phospholipase. The hydrolysing phospholipase is concentrated at the advancing edge of the candidal pseudohyphae [30]. The result shows that there is infiltration of the tissue by inflammatory cells and occurrence of granuloma with foreign body giant cell.

Table 1. The occurrence of virulence factors and antifungal susceptibility of *C. albicans* associated with oral candidiasis

C. albicans isolates	Virulence factors						Antibiotic resistance pattern				
	Cat	Coa	Gel	Hae	Hyd	Phos	Flu	Itra	Nys	Keto	Gris
EKSU 008	+	+	+	-	+	+	R	R	R	R	S
EKSU 009	+	+	+	+	+	+	R	S	R	R	R
EKSU 013	+	+	-	-	+	-	R	S	R	S	R
EKSU 019	+	+	+	+	-	+	R	R	R	R	R
EKSU 022	+	-	+	+	-	+	R	R	S	R	S
EKSU 029	+	+	+	+	+	+	R	R	R	R	S
EKSU 043	+	-	-	+	+	+	S	R	S	S	R
EKSU 056	-	+	+	-	+	-	S	S	S	R	R
EKSU 102	+	+	-	+	+	+	R	S	S	S	R

Cat= catalase, Coa= coagulase, Gel= gelatinase, Hae=haemolysin, Hyd= hydrolase, Phos= phospholipase, Nys=nystatin, Ket= ketoconazole, Itra= itraconazole, Flu= fluconazole, Gris= griseofulvin, + = present, - = absent, R= resistance, S= susceptible

Table 2. Production of biofilm and pseudohyphae among antifungal resistance isolates

Fungi Isolates	Biofilm type	Pseudohyphae
C. albicans EKSU 008	weak	++
C. albicans EKSU 009	weak	-
C. albicans EKSU 013	strong	+
C. albicans EKSU 022	None	+++
C. albicans EKSU 029	Strong	+++
C. albicans EKSU 019	strong	+

+++=profuse, ++=many, +=few, -=none

Table 3. Effects of the crude hydro-alcoholic extract of *T. diversifolia* on antifungal resistant *C. albicans* (mg/mL)

C. albicans Isolates	MIC	MFC	MICI	MBIC	MBEC
EKSU 008	12.5.00	50.00	4.00**	50.00	50.00
EKSU 009	50.00	100.00	2.00*	150.00	>200.00
EKSU 013	25.00	50.00	2.00*	100.00	100.00
EKSU 022	12.50	12.50	1.00*	50.00	100.00
EKSU 029	12.50	50.00	2.00*	50.00	100.00
EKSU 019	50.00	50.00	1.00*	50.00	100.00

*Key: *bactericidal in action **bacteriostatic in action. MIC = Minimum inhibitory concentration, MFC= Minimum bactericidal concentration, MICI= Minimum inhibitory concentration index, MBIC =Minimum Biofilm Inhibition Concentration, MBEC=Minimum biofilm eradication concentration*

Table 4. Time-kill assay of extract of *T. diversifolia* on the growth of *C. albicans* EKSU 029 (Log$_{10}$ cfu/mL)

Conc. (mg/mL)	Time (h)					
	0	12	24	36	48	60
0.50	7.59± 2.12	6.22± 1.38	5.83± 1.82	4.24± 2.29	4.09± 1.84	4.48± 2.00
1.00	7.01± 2.33	6.55± 2.01	5.85± 0.89	3.88± 1.58	3.51± 1.39	3.13± 1.59
2.00	7.13± 1.02	6.88± 1.72	5.91± 1.31	3.66± 1.92	2.33± 0.34	2.18± 1.43
5.00	7.63± 1.82	4.21± 1.41	3.52± 0.38	2.83± 0.08	3.28± 1.74	1.29± 0.52
10.00	7.42± 1.39	3.69±1.82	3.24± 1.29	2.89± 0.99	2.24± 0.27	1.13± 0.07
15.00	7.17± 1.43	2.94± 1.85	1.26± 1.91	1.06± 0.17	1.06± 0.48	1.01± 0.06
Nystatin	7.14± 1.45	1.64± 0.01	1.05± 0.92	1.01± 0.52	0.94± 0.54	0.56± 0.03
Control	7.10± 1.93	7.23± 2.42	7.32± 2.91	8.34± 2.03	8.93± 3.84	9.23± 1.40

Table 5. Recovery of *C. albicans* EKSU 029 from the tongue of challenged experimental animals

Groups	Agents		Candidal load (Log$_{10}$ CFU/g)
A	*T. diversifolia*	12.5 mg/mL	2.87 ± 0.62
B		25.00 mg/mL	2.36 ± 0.39
C	Oral nystatin (10 µg/mL)		1.88 ± 0.17
D	Negative control		3.01 ± 1.62
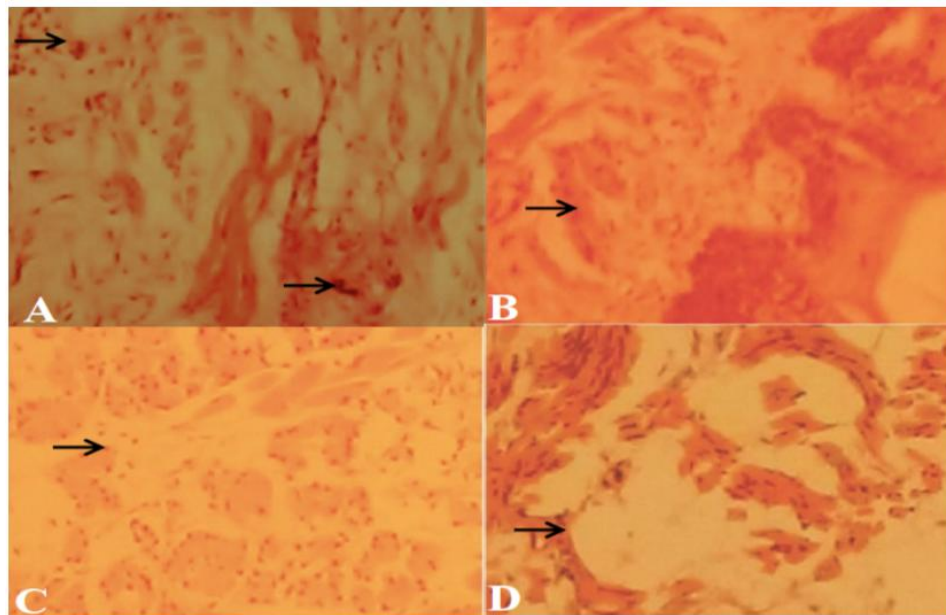
Plate 1. Representative photomicrograph showing a section of the tongue of rats after treatment with 12.5 mg/mL (A), 2.5 mg/mL (B), and nystatin (C). The untreated animal was represented with (D) using haematoxylin and eosin (H and E) stain Mag. (x400). Arrow showing the pseudohyphae of *C. albicans* EKSU 029

Quadrants A and B of Plate 1 are representative slides of samples collected from animals treated with 25 mg/mL and 2.5 mg/mL of *T. diversifolia* respectively, while quadrant C is the photomicrograph section of tongue of animal treated with oral nystatin. There was reduction in the fungal load though there appear to be sign of inflammation. *Tithonia diversifolia* has been reported to contain tithoniamarin, a potentially active antifungal [10]. Quadrant D was the tongue of the untreated animal (control) group. The extracts of the plant showed the reduced

population of the test fungus in the tongue of the experimental rats also the concentration. Plate C shows that the standard drug (oral nystatin) inhibited the growth of the test organism. In plate D which is the negative control, there was distortion in the cytoarchitecture and infiltration. There was distortion in the cytoarchitecture and infiltration by inflammatory cells. The ability of the extract to eradicate the biofilm of the *C. albicans* supports the findings of David and Afolayan [6] who reported the eradication of *C. albicans* biofilm by the extract of *Sansevieria aethiopica.*

4. CONCLUSION

From this study, it can be concluded that *C. albicans* from oral infection possessed diverse putative factors and are resistant to first line antifungal drugs. The strains of the organism were also susceptible to the extract of *T. diversifolia in vitro*. After the establishment of the pathogen, the plant extract reduced the *Candida* load in the tongue of the animal induced with oral candidiasis. The *in vitro* activities of the extract highlight its possible prophylactic activity against oral candidiasis. However, there is need for clinical studies to provide evidence of its efficacy and explore the potential of the extract in drug development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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