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Full Length Research Article

INSILICO ANALYSIS OF BINDING INTERACTIONS AND EVALUATION OF MODE OF ACTION OF HYDROXY TYROSOL ON *CANDIDA ALBICANS* I, II AND *PARAPSILOSIS*

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ABSTRACT
This work was aimed at purifying the most potent antibiofilm principle from *Acalypha wilkesiana* leaves against *Candida* species, analyzing its binding interactions with the molecular targets and evaluating its mode of action. Bioassay guided fractionation was carried and purification of the most potent fraction was achieved by Preparative -TLC. Proton NMR -spectroscopy was used to elucidate the structure of the most potent fraction which was hydroxy tyrosol (HT). There was significant ($p < 0.05$) increase in the IC_{50} of HT and caspafungin in the presence of sorbitol. In the presence of ergosterol, there was no significant ($p > 0.05$) increase in the IC_{50} of HT but there was significant ($p < 0.05$) increase in the IC_{50} of voriconazole. *Insilico* molecular studies revealed a good docking score (-7.7 and 4 hydogen bonds) with glucan synthase and (7.0 and 1 hydrogen bond) with lanosterole-14α- demethylase. The mode of action of HT is most likely by inhibiting the activities of β-1,3-D glucan synthase. The Significant increase in IC_{50} of HT in the presence of sorbitol showed that its inhibition leads to depletion of cell wall glucan and subsequent lysis of fungal cells.

Keywords: Hydroxy tyrosol, voriconazole, caspofungin, *Candida,* Biofilms

1.0 INTRODUCTION

There is increased incidence of invasive opportunistic mycoses due to increased number of immune compromised patients including patients with acquired immune deficiency syndrome (AIDS), cancer, post-surgery recovery and neonates (Nathan and Connie, 2018). Candidiasis are commonly caused by *Candida albicans* (Mary *et al.,* 2016). This disease can be invasive in humans. About 1.5 million deaths are reported annually due to fungal infections (Nucci *et al*., 2010). Superficial and local mucosal infections are mostly caused by *Candida* species and the prominent of all is thrush. Such infectious diseases affect gastrointestinal, esophageal, vagina and oral phargeal mucosa. It was reported that most women suffer from vulvovaginal Candidiasis at least once in their life time (Sobel, 1997). There are three main classes of antifungal drugs which are polyenes, azoles and echinocandins. The polyenes and azoles act by binding to ergosterol on the cell membrane and lanosterol -14-α demethylase respectively and therefore, cause osmotic leakage. The echinocandins (caspofungin, micafungin and anidulafungin) are the only antifungal drugs that act as non-competitive inhibitor of 1,3--D-glucan synthase complex on fungal cell wall which is not present in mammalian (host) cells (Odds *et al*., 2003).
The main disadvantages of these antifugal drugs are the high toxic effects they have on the host tissues, high cost and decrease susceptibility of some *Candida* strains to these drugs (Rajeshkumar and Sundaraman, 2012). Recent studies have also shown that *Candida* species can easily form biofilms as major virulent factor (Maiojo *et al*.,2014: Gulati and Nobile, 2016). Biofilm is a community of microorganisms encased in an extracellular matrix. Biofilm formation by the Candida strains has effects on pathogenicity and resistance to known antifungal drugs (Staniszewska *et al*., 2015).
In view of this, it has become necessary to develop drugs from plants that target pathway other than the membrane ergosterol, plants that are abundant and has the ability to eradicate the biofilms formed by these *Candida* strains to circumvent resistance. *Acalypha wilkesiana* (English name: copper leave) has been commonly used in the northern and western part of Nigeria to treat fungal infections.

2.0 MATERIALS AND METHODS

2.1 Ethical Clearance: Ethical clearance certificate was granted by the Health Research Ethics Committee (HREC) of Ahmadu Bello University Teaching Hospital Shika with an assigned number of ABUTHZ/ HREC/AO1/2017 dated 30th April, 2018.

2.2 Collection and Identification of Clinical Isolates: Three Clinical isolates of *Candida* species were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika. The isolates were characterized with API 20 C AUX Reagent Strips.

2.3 Qualitative and Quantitative Phytochemical Screening of Leaves of *Acalypha Wilkesiana* Extracts

2.3.1 *Collection and Authentication of Plant Material*: Fresh leaves of *Acalypha wilkesiana* were collected from the garden of National Ear Center, Independence Way, Kaduna. Samples of *Acalypha wilkesiana* were authenticated in the herbarium section of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, and Zaria by Namadi Sanusi and the voucher sample number 02805 was deposited.

2.3.2 Extraction of Phytochemical Components from Leaves of *Acalypha wilkesiana*

The leaves of *A. wilkesiana* were air dried and pulverized into uniform powder using mortar and pestle. Four (4) solvents with increasing polarity from n- Hexane, ethyl acetate, methanol and lastly water were used for extracting the phytochemicals from the Leaves of *Acalypha wilkesiana.* Successive extraction using Cold maceration method was used as described by Handa *et al*. (2008). Two Hundred grams (200g) of the pulverized leaves of *Acalypha wilkesiana* was soaked in 1.5 L of n-Hexane for 48h in a conical flask after which the mixture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 40°C using rotary evaporator to get constant dry weight of the extract. The remaining plant residue from n-Hexane was air dried completely and soaked in 1500 mL of ethyl acetate, the same procedure for n-Hexane was applied for the ethyl acetate, methanol and aqueous extraction. The Percentage yield of each extract was calculated using the following formular described by Anokwuru *et al*. (2011).

Yield (%) = $W_2 – W_1 / W_0 \times 100$

Where: W_2 = weight of extract and the container, W_1 = weight of the container alone and W_0 = weight of the initial dried sample.

2.3.3 Qualitative Phytochemical Assays of Crude Extracts of *Acalypha wilkesiana* Leaves

(i) Test for Flavonoids

Test for flavonoids was carried out as described by Trease and Evans (2002). About 0.5g of each extract was dissolved in 10 mL water (tween 80 was used for n-hexane and Ethyl acetate extracts) and filtered; to this, 2 mL of 10 % aqueous sodium hydroxide was added to produce a yellow coloration. A change in colour from yellow to colorless on addition of dilute hydrochloric acid *indicates the presence of flavonoids*

(ii) Test for Tannins

About 0.5 g each extract was mixed with 10 mL of distilled water (tween 80 was used for n-hexane and Ethyl acetate extracts) and then filtered. About 2 drops of 1% ferric chloride solution was added to 2 mL of the filtrate. Appearance of a blue-black, green or blue-green precipitate indicates the presence of tannins (Trease and Evans, 2002).

(iii) Test for Alkaloids

About 0.5 g of each extract was mixed with 5 mL of 1 % aqueous HCl on water bath at 40 ºC and then filtered off the filtrate, 1 mL was then taken individually into 2 test tubes. To the first portion, a few drops of Dragendorff's reagent was added and occurrence of orange-red precipitate indicates the presence of alkaloids. To the second portion, 1 mL Mayer's reagent was added and appearance of buff-coloured precipitate indicates the presence of alkaloids (Sofowora, 1993).

2.3.4 Quantitative Assays for Phenolics, Flavonoids and Alkaloids

(i) Total Phenolic Content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent (Mallick and Singh, 1980). The plant extract (0.5 mL) was mixed with 0.5 mL of FC reagent (1:1 diluted with distilled water) followed by addition of 2 mL of 20 % Na_2CO_3 after five (5) minutes. After ninety (90) minutes, the absorbance of the mixture was measured at 650 nm. The total phenolic content (mg/mL) was calculated using gallic acid as standard.

(ii) Total Flavonoid Content

The total flavonoid content (mg/mL) was determined using a modified colorimetric method as described by Vabkova and Neugebauerova (2012). The assay mixture consisting of 0.5 ml of the plant extract, 0.5 mL distilled water, and 0.3 mL of 5 % $NaNO_2$ was incubated for 5 min at 25°C. This was followed by addition of 0.3 mL of 10 % $AlCl_3$. Two milliliters (2mL) of 1 M NaOH was then added to the reaction mixture, and the absorbance was measured at 510 nm. Quercetin was used as a standard.

(iii) Total Alkaloid Content

The total alkaloid content was determined according to UV-Spectrophotometric method described by Manjunath *et al*. (2012). This method was based on the reaction between alkaloid and bromocresol green. The plant extract (0.5 mL) was dissolved in 2 N HCl and then filtered. 1 mL of this solution was transferred to separatory funnel and washed with 10 mL chloroform. The pH of phosphate buffer solution was adjusted to neutral with 0.L N NaOH. One mL (1mL) of this solution was transferred to a separating funnel and then 5 mL of bromocresol solution along with 5 mL of phosphate buffer (7.0) was added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fraction was collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. Atropine was be used as standard.

2.4 Bioassay Guided Fractionation

The minimum inhibitory concentration and minimum fungicidal concentration of the crude extracts on planktonic cells of *Candida* species were carried using microdilution method as described by Takahagi *et al*. (2009). The concentration of the antifungal in a microdilution well that had absorbance nearly that of the control well is taken as the MIC. The Minimum Fungicidal Concentration (MFC) of the extract with the best inhibitory activity was determined using the method of Russell and Furr (1977). This method involves sub culturing portions of the agar from petridishes that showed no growth in the tests for determination of MICs. These agar portions were transferred into petridishes containing freshly prepared Sabouraud Dextrose Agar (SDA). The plates were incubated at 27°C $\pm$ 2 for 2 days and observed daily for growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MFC (Siddiqui *et al*., 2013). The extract with the least MIC was selected for fractionation using column chromatography –thin layer chromatography. Fractions that showed the same retention factor (RF) were pooled together. The pooled fractions collected were subjected to antifungal activities and then the most potent subfraction was selected for further bioassay. Purification and structural elucidation were carried out as described by Yueh-ksiuig *et al*. (2019). Preparative thin layer chromatography was carried out to purify the most potent subfraction. HNMR spectra were run with $CDCl_3$ as solvent on a Bruker Avance-400 MHz NMR (Bruker, Germany) at NMR lab school of Chemistry and Physics, University of KwanZulu-Natal, Westville campus, Durban, South Africa.

2.5.0 Biofilm Formation and Quantification of *Candida albicans* I, II and *Parapsilosis*

Biofilm quantification of the three (3) *Candida* strains were determined by microdilution method as described by Charles *et al*. (2017). For each strain, 100 µL fungal suspension of 0.5 McFarland standard (1 × 10^8 CFU / mL) serially diluted to 1×10^2 CFU/mL was inoculated into individual wells of polystyrene 96 well plates (flat bottom) containing 100 µL of SDA broth containing 0.05 M glucose and 1% bovine serum albumin. Three repeats were performed for each strain. The plates were incubated at 37 °C for 90 minutes (adhesion period). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with phosphate buffered saline (PBS) to get rid of any non-adherent cells. A 100 µL aliquot of 2% crystal violet was added to each well and incubated for 20 minutes at 37 °C. Then, 150 µL 95% ethanol was added to dissolve the dyed biofilm cells and 100 µL of each mixture was transferred to a new 96 well microtiter plate. The absorbance for each well was determined using microplate reader at 630nm. Wells containing only SDA broth without microbes were used as negative control.

2.5.1 Biofilm Eradication Concentration was determined by microdilution method as described by (Djordjevic *et al*., 2002). A 100 µl aliquot of SDA broth was added to each well, and 100 µL cell suspension (0.5 McFarland standard) of each of the three strains of *Candida* was added to the corresponding wells and incubated for 24 h at 37 °C for biofilm growth. After incubation supernatant was discarded and wells washed twice with 200 µL PB and incubated for 2 h at 37°C followed by addition of the extracts at concentration corresponding to the MIC, 2 MIC and 4 MIC. The plates were, allowed for initial yeast adherence. Subsequently, the wells were washed with phosphate-buffered saline (PBS) to remove loosely bound cells, fresh medium was added, and the plates were incubated for 48 h at 37°C. The wells were washed twice with PBS, air-dried for 45 min, and dyed with 0.4% crystal violet aqueous solution. Absorbance values were read at 600 nm using a micro plate reader. Amphotericin B, voriconazole and caspofungin were used as positive controls and untreated biofilm served as the growth control.

2.6 Evaluation of Mode of Action of the Purified Fraction of Ethylacetate Extract of *Acalypha wilkisiana* Leaves.

Mode of action of the Purified Fraction of Ethylacetate Extract of *Acalypha wilkisiana* Leaves was evaluated using sorbitol and ergosterole assays.

2.6.1 Sorbitol Assay

Microdilution technique was used to determine the mode of action of the purified fraction of *C. albicans* cell wall as described by Perlin (2011); Pierce *et al*. (2013) in the presence of sorbitol (D-sorbitol anhydrous), an osmotic protector. For this assay, the inoculum was prepared with sorbitol at a concentration of 0.8 M. The plates were incubated at 37 °C, and readings were performed 24 h and 48 h after incubation (Escalante *et al*., 2008; Lima *et al*., 2013; Freires *et al*., 2014). Caspofungin was used as a positive control in this assay at an initial concentration of 5 mg/ml due to its known activity on the yeast cell wall.

2.6.2 Ergosterol assay

The assay was performed using the micro dilution technique, as earlier described, in the presence of exogenous ergosterol at concentration 200 mg/mL. The plates were incubated at 37°C, and readings were carried out after (24–48) h (Escalante *et al*., 2008; Lima *et al*., 2013; Freires *et al*., 2014). Amphotericin B was used as a positive control due to its known activity on the yeast cell membranes by binding to membrane sterols and thus changing membrane permeability (Perlin, 2011; Pierce *et al*., 2013). A control with 96% ethanol and DMSO, which was used for preparation of ergosterol solutions, were also included.

IC_{50} was carried out as described by Jessica *et al*. (2013). The IC_{50} is defined as the concentration where the response is reduced by half.

The following equation was used for calculations of cell inhibition:

% cell inhibition = 100 – {(At- Ab)}/ (Ac- Ab) ×100

Where,

At=Absorbance value of test compound
Ab= Absorbance value of blank
Ac= Absorbance value of control.

2.7 Binding Interaction of Hydroxy Tyrosol using Insilico Molecular Docking Techniques

(i) Protein Preparation

The X-ray crystal structure of 14-alpha-demethylase with PDB ID: 4LXJ with resolutions of 1.9Å was downloaded from RCSB Database (http://www.rcsb.org/pdb). The 1,3-glucan synthase was not crystallised (not available in PDB data base), thus, its model was developed using FASTA sequence (ID: 074475) accessed from UniProt database and the model_01 was developed at swiss modeller webpage using respective FASTA sequence. Receptors were optimised, the energy was minimised and also, water was removed using discovery studio visualiser 3.5 respectively and save as PDB. The receptor binding site was identified using Discovery studio visualiser 3.5 through searching within receptor cavity. Four different binding sites were identified in 1,3-glucan synthase whereas three different binding sites were identified in 14-alpha-demethylase. All the identified binding sites were mapped and saved as PDB.

(ii) Ligand Preparation

The ligand used for molecular docking in this work were hydroxy tyrosol, caspofungin and voriconazole. Their molecular structures were obtained from pubchem site https://pubchem.ncbi.nhm.gov and then optimized by using Merck Molecular Force Field (MMFF) and the semi-empirical Austin Model (AM1) method, both of which were implemented in Discovery studio visualiser (version3.5, BIOVIA Software). http://www.3dsbiovia.com/product/collaborative-science/biovia-discovery-studio/in order to remove all strain from the molecular structure. This enhanced a well-defined conformer relationship among compounds of the study (Viswanadhan *et. al*., 1989). From the setup calculation option on Discovery studio visualiser 3.5, the calculation was set to equilibrium geometry at the ground state using density functional theory at B3LYP (Becke88 three-parameter hybrid exchange potentials with Lee-Yang-Parr correlation potential) level of theory and 6-311G (d) basis set for the geometrical optimization of the cleansed structures i.e. B3LYP/6-311G (d) level of theory. After optimization, Discovery studio visualizer descriptors were obtained from the display-output and display-properties option on Discovery studio visualizer 3.5.

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The fully optimized 3D structure without symmetry restrictions, were saved as PDB file through the file option on the Discovery studio visualiser 3.5.

2.8 STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation. All tests were carried out in triplicate. Significant differences were analyzed between groups using one-way analysis of variance (ANOVA) followed by Turkey- Kramer Multiple Test. All statistical analyses were carried out using the GraphPad Instat 3.10 software with statistical significance set at $p < 0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Some Phytochemicals and their Concentrations in Aqueous, Methanol, Ethylacetate and N- hexane Crude Extracts of *Acalypha Wilkesiana* Leaves

Table 1 shows that the n-hexane, ethylacetate, methanol and aqueous extracts of *Acalypha wilkesiana* leaves contains flavonoids, tannins alkaloids phobatannins, phenolics and saponins while all the extracts showed negative results for cardiac glycoside. Ethyl acetate extract of *Acalypha wilkesiana* leaves contains more phlobatannins than the remaining extracts. Table 2 shows that methanol extract has increased total phenolic content (255.5 ± 0.51mg / 100g of the powdered plant) which is significantly different ($p \leq 0.05$) from the n-hexane and ethylacetate but not significantly ($p \geq 0.05$) different from the aqueous extract. There's no significant ($p \geq 0.05$) difference between the flavonoid content of methanol and aqueous extract of *Acalypha wilkesiana* leaves. There is also no significant difference ($p \geq 0.05$) between the flavonoid content of n-hexane (169.0 ± 0.4051mg / 100g of the powdered plant) and ethyl acetate (175.5 ± 0. 2051mg / 100g of the powdered plant) extract of *Acalypha wilkesiana* leaves. The methanol extract was shown (Table 2) to have the highest flavonoid content (398 ± 0.2551 mg/100g of the powdered plant) not significantly ($p \geq 0.05$) different from the flavonoids content in the aqueous extract. This result is in conformity with the findings of Awe and Eme (2014) where the phytochemical screening of *Acalypha wilkesiana* leaves showed the presence of some bioactive components such as tannins, phenolics, saponins, alkaloids, phlobatannins and flavonoids. Several plants which are rich in phenolics compounds and alkaloids have been shown to possess anti-microbial activities against a number of microorganisms (Awe and Eme, 2014).

Table 1: Some Phytochemicals Detected in the Aqueous, Methanol, Ethyl Acetate and n- Hexane Extracts of *Acalypha wilkesiana* Leaves

Extract	Flavonoids	Tannins	Alkaloids	Phlobatannins	Phenolics	Saponins
n-Hexane	+	+	+	+	+	+
Ethyl acetate	+	+	+	++	+ +	+
Methanol	+	+	+ +	+	+ +	+
Aqueous	+	+	+ +	+	+	+

The signs + and++ shows the presence of the corresponding phytochemicals but ++ are in higher concentration by reacting faster.

Table 2: Concentrations of some Phytochemicals in Aqueous, Methanol, Ethyl Acetate and n- Hexane Extracts of *Acalypha wilkesiana* Leaves

Extract	% Yield	Total Phenol mg/ 100g of powdered plant	Total Flavonoids mg/ 100g of powdered plant	Total Alkaloids mg/ 100g of powdered plant
n-Hexane	2.01	$114.9^{a} \pm 0.31$	$169.0^{a} \pm 0.40$	$86.0^{a} \pm 0.21$
Ethyl acetate	1.70	$159.8^{b} \pm 0.02$	$175.5^{a} \pm 0.20$	$151.1^{b} \pm 0.03$
Methanol	9.10	$255.5^{c} \pm 0.51$	$398.0^{b} \pm 0.25$	$333.6^{d} \pm 0.20$
Aqueous	13.45	$186.5^{cb} \pm 0.68$	$300.5^{b} \pm 0.08$	$259.6^{d} \pm 0.03$

Different superscripts across the column are significantly different ($p \leq 0.05$).

3.2 Identification, Formation and Quantification of Three Clinical Isolates of *Candida*

The Three Candida samples were identified to be 88.1%, 98.1% and 98.2% *C. albicans* II, *C. albicans* I and *C. parapsiloosis*, respectively using API 20 stips for *Candida*. Figure 1 showed the quantity of biofilm formed by clinical isolates of *Candida albicans* (I and II) and *Candida parapsilosis* over a Period of 72 h. There was no significant ($p \geq 0.05$) difference in the quantity of biofilms (log CFU/mL) formed among the three strains of *candida* when incubated over a period of 72 h. This agrees with the report of Rita *et al*. (2011) which stated that biofilm growth reduces with time.

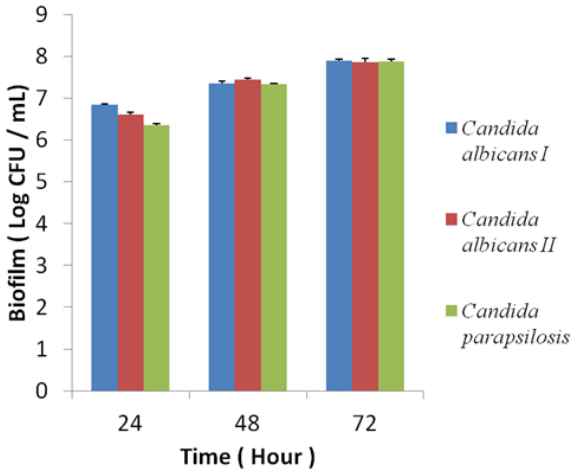

Figure 1: Biofilm Formed by Clinical Isolates of *Candida albicans (I* and *II)* and *Candida parapsilosis* over a Period of 72 h.

3.3 Minimum inhibitory and minimum Fungicidal Concentrations of Crude Extracts of *Acalypha wilkesiana* Leaves on Planktonic Cells of *Candida albicans I, II* and *parapsilosis*

Table 3 shows that ethylacetate has the lowest MIC of 25 mg/mL against all the three strains of Candida used for this work and MFC of 50 mg/mL against *Candida albicans* I and *Candida albicans* II but 100 mg/mL on *Candida parapsilosis*. There was no total cell death observed with the other extracts except methanol extract but at a higher concentration of 100 mg/mL against *Candida albicans* I and II. *In view* of this result, Ethylacetate was selected for further analysis using bioassay guided fractionation. The standard drugs (amphotericin B and caspofungin) showed fungistatic and fungicidal activities but voriconazole had only fungistatic activity

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against *Candida albicans I, II* and *parapsilosis* (Table 3) this conforms with the findings of Mohamed *et al*. (2015) which stated that voriconazole had only fungistatic activity on *candida* cells.
Column chromatographic separation using two solvent systems starting from absolute n-hexane (100%), n-hexane: Ethylacetate (4:1, 3:2, 2:3,1:4), and absolute ethylacetate (100%) gave 62 fractions where fractions with the same Retention factors were pooled together and gave seven fractions (A, B, C, D, E, F and G). The seven pooled fractions were then developed in 100% absolute ethyl acetate in a TLC tank. The TLC plates were removed, allowed to dry and then sprayed with vanillin sodium sulphate to develop the chromatogram.
Fractions A, B, C, D E, F and G were further subjected to bioassay and fraction G was found to be the most potent by showing fungistatic and fungicidal activities at the lowest concentration compared to other fractions. Preparative-TLC was carried out on Fraction G, in two solvent system (n-hexane: Ethyl acetate 2:8) which showed only one chromatogram that glowed when viewed with UV Lamp. The TLC glass was sprayed with vanillin sulphuric acid which showed purple colored spot and then scraped for proton NMR spectroscopy.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) mg/ml of the Crude Extracts of *Acalypha wilkesiana* Leaves and Some Standard Drugs against *Candida albicans* (I and II) *and Candida Parapsilosis*

Extract / Drugs	*C. albicans* I MIC / MFC	*C. albicans* II MIC / MFC	*C. parapsilosis* MIC / MFC
n-Hexane	100 / -	100 /-	100 / -
Ethylacetate	25 / 50	25 / 50	25 / 100
Methanol	100 / 100	100 / 100	50 / -
Aqueous	100/-	100 /-	100 / -
Voriconazole	12.5×10^{-6} /-	12.5×10^{-6} /-	12.5×10^{-6} /-
Amphotericin B	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}
Caspofungin	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}

Different superscripts across the column are significantly different ($p \leq 0.05$).

3.3 Percentage Biofilm Eradicated by the Purified Fraction of *Acalypha wilkesiana* Leaves and the Reference Drugs

Candida biofilms enable the organisms to escape antifungal drug activity (Bouchcherit *et al.*, 2011). Figure 2 shows the Percentage Biofilm Eradication (%BE) by the purified fraction of ethylacetate extract, voriconazole, amphotericin B and caspofungin. There was significant ($p \leq 0.05$) increase in the biofilm eradicated by the reference drugs compared to the purified fraction at 1.0 mg/mL on *C. albicans* I, II and *C. parapsilosis*. There was no significant ($p \geq 0.05$) difference between the biofilms eradicated by the voriconazole, amphotericin B and caspafungin on *C. albicans* I, II and *C. parapsilosis*. This agrees with the findings of Mohamed *et al.* (2015) which stated that amphotericin B. and voriconazole reduced the biofilms of *Candida* in a comparable manner.

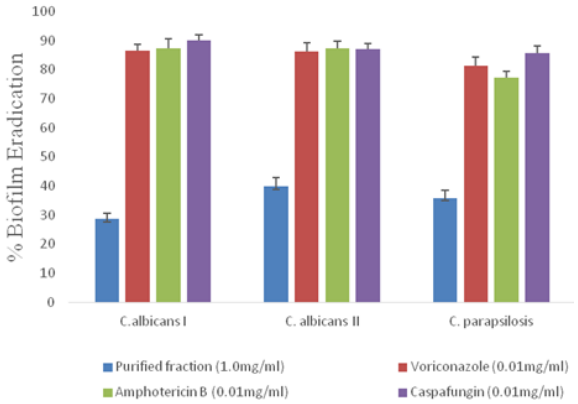


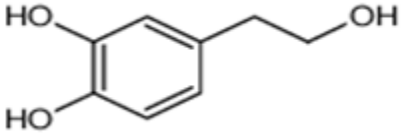
Figure 2: Percentage Biofilm Eradicated by Hydroxy Tyrosol, Voriconazole, Amphotericin B and Caspafungin on *C. albicans* I, II and *parapsilosis.*

3.4 Identification and Structural Elucidation of the Purified Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves

Figure 3 showed proton NMR spectra of the purified fraction of ethyl acetate extract of *Acalypha wilkesiana* leaves. The ^{1}HNMR (400MHZ, $CDCl_3$) analysis gave δ: 7.53(1H, doublet (d), 8.8Hz), 7.35 (1H, singlet), 7.13 (1H, doublet of doublet (dd), 8.8, 2.28Hz), 3.65 (2H, CH_2OH), 2.09 (2H, CH_2CH_2OH). This result is in conformity with the findings of Amalia *et al.* (2019) who reported that H1 NMR (600 MHZ CD_3OD) for Hydroxy tyrosol gave δ: 6.63(doublet and a coupling constant (J) of 8.2 Hz), 6.61(d, J= 2.2 Hz), 6.48 (dd, J= 8.2, 2.2 Hz), 3.63 (J=2H, CH_2OH), 2.62 (2H, CH_2CH_2OH). The chemical shifts for the two results were within the range for aromatic hydrocarbon which accounts for the aromatic ring in hydroxy tyrosol.

3.5 Evaluation of Mode of Action of Hydroxy Tyrosol using IC_{50}

Table 4 shows the IC_{50} of hydroxy tyrosol (HT) which was identified to be the purified fraction of ethylacetate extract of *Acalypha wilkesian* Leaves. Caspofungin was used as a standard because it has a known mode of action which is to obstruct the synthesis of fungal cell wall by inhibiting the enzyme (β) 1,3-glucan synthase. This leads to inhibiting the synthesis of D-glucan which is an essential component of the fungal cell wall, and its inhibition leads to depletion of cell wall glucan and lysis of fungal cells (Perlin, 2011). The IC_{50} increased significantly (≤ 0.05) when sorbitol (osmotic protectant) was added to hydroxy tyrosol and caspofungin compared to IC_{50} without sorbitol. Table 5 showed IC_{50} of HT and amphotericin B which was used as a control due to its known mode of action to create pores that interferes with the membrane integrity leading to leakage of cellular contents and death (Ostrosky *et al*., 2010). There was no significant increase in the IC_{50} of HT without ergosterol compared to the IC_{50} of HT with ergosterol in all the strains of *Candida* used for this research work.



Hydroxy Tyrosol

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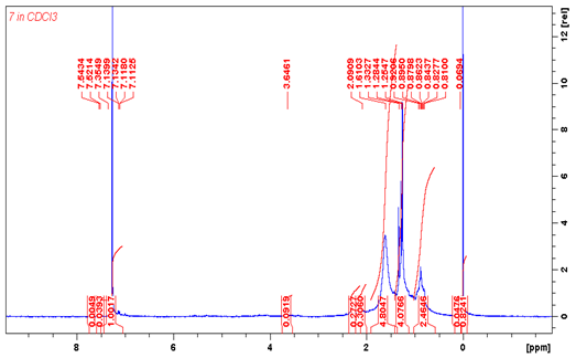

Figure 3: Proton- NMR Spectra of Purified Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves

Table 4: IC_{50} of Purified Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves in the presence and Absence of Sorbitol as Osmotic Protectant.

	Without sorbitol			With Sorbitol		
Antigungal Agent	***C. albicans* I**	***C.albicans* II**	***C. parapsilosis***	***C. albicans* I**	***C.albicans* II**	***C. parapsilo***
HT (mg/ml)	5.75 ± 0.52[a]	4.75 ± 0.38[a]	7.26 ± 1.51[b]	8.64 ± 0.22[b]	17.2 ± 2.47[c]	11.2 ± 0.69[d]
Casp. (mg/ml)	72.0×10^{-5} ± 2.09×10^{-6e}	72.0×10^{-5} ± 3.3×10^{-6e}	72.0×10^{-5} ± 9.1×10^{-6e}	12.6×10^{-4} ± 1.17×10^{-5f}	10.1×10^{-4} ± 1.8×10^{-5f}	10.0×10 ± 7.8×10

Values having different superscripts across the column (a- e) and row (1- 6) are significantly ($p \leq 0.05$) different.

Key:
HT = Hydroxy tyrosol
Casp= caspofungin

Table 5: IC_{50} of Purified Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves in the presence and Absence of Ergosterol.

	Without Ergosterole			With Ergosterole		
Antifungal ag1ent	***C. albicans* I**	***C. albicans* II**	***C. parapsilosis***	***C. albicans* I**	***C. albicans* II**	***C. parapsil***
HT	5.75 ± 0.52[a]	4.75 ± 0.38[a]	7.26 ± 1.51[b]	5.97 ± 0.56[a]	5.01 ± 0.54[a]	8.19 ± 0.47[b]
Amp. B (0.01mg/ml)	69.0×10^{-5} ± 7.8×10^{-6c}	71.0×10^{-5} ± 8.9×10^{-6c}	70.4×10^{-5} ± 6.3×10^{-6c}	3.1×10^{-3} ± 4.0×10^{-4d}	2.9×10^{-3} ± 2.9×10^{-4d}	3.4×10^{-3} ± 5.0×10^{-4}

Values having different superscripts across a column (a- d) and row (1-6) are significantly ($p \leq 0.05$) different.

Key:
HT= hydroxy tyrosol
Amp. B= amphotericin B

4.6 Docking Scores from the Interactions of Hydroxy Tyrosol, Caspafungin and Voriconazole with their Respective Molecular Targets

Table 6 shows the docking scores generated due to binding interaction between hydroxy tyrosol and caspafungin with β-1,3-D glucan synthase, respectively**.** Hydroxy tyrosol shows an optimum binding energy (-7.7 Kcal/mol) and formation of four (4) hydrogen bonds with β-1,3-D glucan synthase compared to caspofungin which had binding energy of -6.7Kcal/mol and three hydrogen bonds formed between the hydroxy tyrosol and amino acids residues of the β-1,3-D glucan synthase. This agrees with the findings of Joseph *et al*. (2017) which stated that the number of hydrogen bonds formed is related to the free energy of binding found for each compound. The value of free energy showed the bond strength of ligand and receptor. The negative sign in the free energy value shows that a spontaneous interaction is achieved between a ligand and the molecular target (Muchtaridi *et al*., 2015). Figure 4 shows the hydrogen bonding between alanine (A:1711), alanine (A: 1713), Histidine (A:1714) and isoleucine (A:1712) residues of β-1,3-D- glucan synthase with hydroxy tyrosol. Three (3) hydrogen bonds were formed between glycine (A:1717), lysin (A:1755) and leucine (A:1674) residues of glucan synthase with caspofungin (Figure 5).
Table 7 shows that the binding energy between hydroxy tyrosol and lanosterol-14α-demethylase was -7.0Kcal/mol and, -7.8Kcal/mol between voriconzole and lanosterol-14α-demethylase. Hydroxy tyrosol formed Pi-Pi stacking interaction with phenylalanine residue of Lanosterol-14α-demethylase and one (1) hydrogen bond with arginine (A: 98) (Figure 6). Non-covalent binding interactions such as pi-pi stacking is relevant for exhibiting necessary biological functions during ligand-protein interactions (Michal, 2018). Figure 7 showed one hydrogen bond interaction between aspartate residue of lanosterol-14α-demethylase with voriconazole.

Table 6: Binding Energy and Hydrogen Bond Produced by Interaction of Hydroxy Tyrosol and Caspofungin with β-1,3-D Glucan Synthase

	Binding Energy (Kcal/mol)	Hydrogen bond	Hydrophobicity	In charge	SAS
Hydroxytyrosol	-7.7	4.0	-1.00	0.00	20.0
Caspofungin	-6.7	3	-1.00	0.033	20.0

Table 7: Binding Energy and Hydrogen Bond Produced by Interaction of Hydroxy Tyrosol and Voriconazole with Lanosterol-14α-Demethylase

	Binding energy (Kcal/mol)	Hydrogen bond	Hydrophobicity	In charge	SAS
Hydroxytyrosol	-7.0	2	-1.00	0.00	22.5
Voriconazole	-7.8	1	-1.00	-0.33	20.0

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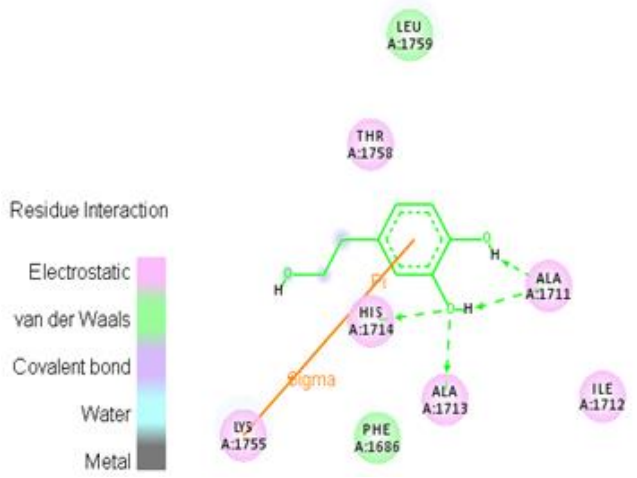

Figure 4: 2D Image showing Interaction of Hydroxy Tyrosol with Amino Acid Residues of Glucan Synthase

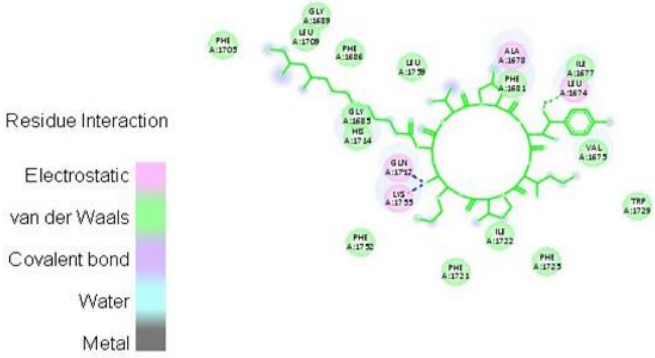

Figure 5: 2D Image showing Bond Interaction of Caspofungin with Amino Acid Residues of Glucan Synthase

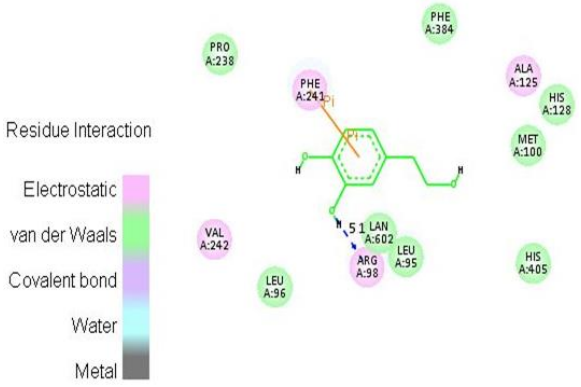

Figure 6: Structure of Hydroxytyrosol and its Interactions with Amino Acid Residues in Lanosterol-14α- Demethylase

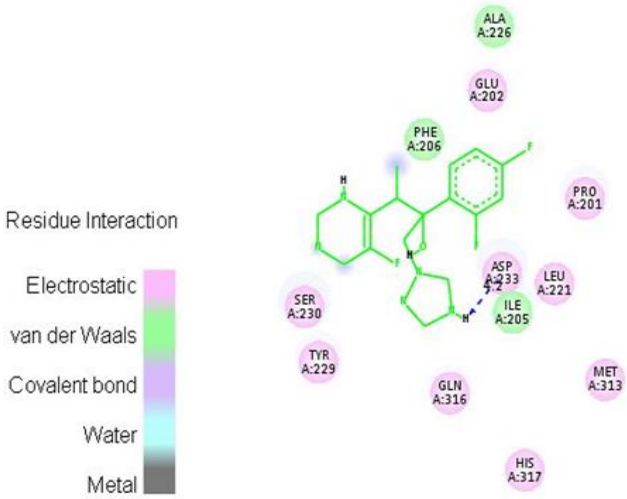

Figure 7: Hydrogen Bonding between Voriconazole and Amino Acids Residues of Lanosterole -14α-Demethylase

Conclusion

Hydroxy tyrosol is a potent antifungal agent against *Candida albicans* I, II and *parapsilosis*. The mode of action of hydroxy tyrosol may involve inhibiting the activities of both β-1,3-D glucan synthase and lanosterol-14α-demethylase as it was shown to have a good docking score with both enzymes. The Significant increase in IC_{50} of hydroxy tyrosol in the presence of sorbitol showed that its inhibition leads to depletion of cell wall glucan and subsequent lysis of fungal cells.

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