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## Antimicrobial Activity of the crude extracts and fractions of *Ficus thonningii* (Blume) on Isolates from Urinary Tract Infections

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### ABSTRACT

*Ficus thonningii* extract is known for its ethnobotanical uses in treatment of various infections, including those of the urinogenital tract. This study investigated the antimicrobial activity of extracts of *F. thonningii* on thirty four bacterial isolates from symptomatic urinary tract infections and four *Candida* isolates from vagina. Qualitative phytochemical screening and successive gradient extraction of dried, pulverized leaves was carried out with hexane, ethyl acetate and methanol. Bioactivity-guided fractionation of the ethyl acetate extract was done using vacuum liquid chromatography. Antibiotic susceptibility testing of bacterial isolates was determined using standard antibiotic discs. Antimicrobial activity of the crude extracts and fractions was carried out on isolates using the agar well diffusion method. Minimum inhibitory concentrations (MIC) and minimum biocidal concentrations (MBC) were determined by agar-dilution and broth dilution respectively. Bactericidal kinetics of the ethyl acetate extracts of both plants was conducted using the viable count technique. Phytochemicals present include terpenoids, saponins, tannins and flavonoids. The majority of clinical isolates were multi-drug resistant. The extracts and fractions of *F. thonningii* showed broad spectrum antimicrobial activity on susceptible as well as multi-antibiotic resistant isolates. The MIC ranged from 6.25 mg/mL to 12.5 mg/mL while MBC was between

6.25 mg/mL and > 50mg/mL. The ethyl acetate extract showed potent bactericidal activity in a concentration-dependent manner on the microorganisms, with 100% microbicidal activity at 25 mg/mL within six hours for *Staphylococcus aureus* and three hours for *Candida albicans*. Leaf extracts of *F. thonningii* possess potent antimicrobial activity and may be useful in developing chemotherapeutic agents for the treatment of microbial infections.

### INTRODUCTION

Plants and plant products, exemplified by ancient Chinese, African and Indian herbs, have been used in the treatment and management of infectious diseases since antiquity. The effectiveness of plants in the treatment of infections can be inferred from the number of drugs derived from them (Gupta et al., 2012). Although standard antibiotics are useful in treatment of infectious diseases, the continuing emergence of resistant organisms, undesired effects and exorbitant cost of drugs pose a significant challenge (Neuhauser et al., 2003; Saga and Yamaguchi, 2009; Willey et al., 2008). Hence there is continued search for molecules with antimicrobial activity.

*Ficus thonningii* (Blume) is a tree in the Moraceae family (Orwa et al., 2009). *F. thonningii* is commonly referred to as common wild fig while the Yoruba tribe of Nigeria refers to it as 'odan' (Orwa et al., 2009), and used ethnobotanically in the treatment of urinary tract infections, venereal diseases, diarrhoea, dermatophyte infections

amongst others (Dangarembizi et al., 2013; Egharevba et al., 2015; Muthu et al., 2006; Nwachukwu et al., 2010; Orwa et al., 2009; Shanavaskhan et al., 2012; Teklehaymanot and Giday, 2007). *F. thonningii* has been shown to be non-toxic to mammals at high doses in a number of *in-vivo* studies (Aniagu et al., 2008; Coker et al., 2009); explaining why it has not been associated with toxicity in ethnomedicine. Extracts of *Ficus thonningii* have been shown to be active against clinical bacteria isolated from various sources (Koné et al., 2004; Ndukwe et al., 2007; Usman et al., 2009) as well as fungi (Coker et al., 2016; Oyelana et al., 2011) and protozoa (Dangarembizi et al., 2014; Falade et al., 2014) but there is little information on its effect on pathogens of urinary tract and sexually transmitted infections for which it is used in local traditional medicine. Hence, this study investigated the antimicrobial activity of *F. thonningii* extracts on pathogens isolated from vagina and symptomatic urinary tract infections. We hypothesized that extracts of *F. thonningii* is effective against selected isolates from urinary tract infections.

## MATERIALS AND METHODS

*Plant Collection.* Leaves of *F. thonningii* were collected from Ojoo in Ibadan and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria where a herbarium sample with voucher number FHI 1106898 was deposited. The leaves were then dried at room temperature ( $25 \pm 3$  °C) and thereafter pulverized with an industrial grinder.

*Phytochemical Screening and Extraction of Metabolites.* Qualitative phytochemical screening was conducted on the pulverized leaves following standard methods described by Vinoth et al., (2012).

*Test for tannins.* Pulverized plant sample (0.5 g) was stirred with 10ml of distilled water in a test tube. This was filtered and a few drops of 0.1% ferric chloride were added. A brownish- green, blue-black or blue-green precipitate indicates a positive result.

*Test for saponins.* Saponins were detected using the froth test in which 0.5 g of sample was weighed into a test tube containing 5 ml of distilled water. This was shaken and filtered. The filtrate was shaken vigorously for a few minutes and observed for persistent frothing which is indicative of a positive result. To further confirm the presence of saponins, three drops of olive oil was added to tubes with persistent froth. The formation of emulsion confirms the presence of saponins.

*Test for terpenoids.* Plant sample (0.5 g) was weighed into a clean test tube containing 2 ml of chloroform. This was then shaken and thereafter filtered after which concentrated H<sub>2</sub>SO<sub>4</sub> was added to the filtrate. A reddish-brown color at the interphase indicates the presence of terpenoids.

*Test for alkaloids.* Pulverized plant leaves (0.5 g) were acidified with a mixture of 1% hydrochloric acid and ethanol for 2 minutes. This was shaken after which it was filtered through a filter paper. Ammonium hydroxide was added to the filtrate in a clean test tube after which chloroform was added. The chloroform layer was removed with the aid of a Pasteur pipette and then a few drops of Dragendorff's reagent were added. The formation of orange brown precipitate indicates the presence of alkaloids.

*Test for cardiac glycosides.* To detect cardiac glycosides, 0.5 g of sample was weighed into a clean test tube containing 5 ml of water. This was then filtered and 2 ml of glacial acetic acid containing a drop of ferric chloride was added to the filtrate after which concentrated hydrogen tetra oxo sulphate (VI) acid was added. A reddish-brown color at the interphase indicates the presence of cardiac glycosides.

*Test for steroids.* Dried pulverized plant sample (0.5 g) was weighed into a clean test tube containing 2 ml of chloroform. This was then shaken and filtered. Acetic anhydride and concentrated hydrogen tetra oxo sulphate (VI) acid were then added. A positive test was indicated by a greenish color at the upper part of the liquid.

*Test for flavonoids.* To query for flavonoids, 0.5

g of plant sample was weighed into a test tube containing 5 ml of distilled water. This was then filtered and dilute ammonia was added to the filtrate. A yellow color, persistent with the addition of concentrated hydrogen tetra oxo sulphate (VI) acid indicates a positive result

*Test for phenols.* Ethyl acetate was used to extract 1g of plant sample. Extract was then filtered with Whatman filter paper. The development of blue black or brown coloration on the addition of ferric chloride reagent to the filtrate indicates the presence of phenol.

*Test for anthraquinones.* Concentrated H<sub>2</sub>SO<sub>4</sub> was added to a clean test tube containing 0.5 g of plant sample. This was then filtered and chloroform was added to the filtrate. A color change upon the addition of ammonium hydroxide indicates a positive result.

The dried, pulverized leaves of both plants were extracted successively with the soxhlet apparatus using n-hexane, ethyl acetate and methanol as solvents. The plant material was loaded into the thimble of the soxhlet which was then connected to a round bottom flask containing the desired solvent. The flask was mounted on a heating mantle while the condenser was fixed to the thimble for the purpose of condensing evaporated solvent. The condensed solvent drops into the thimble, soaks the plant sample and thus extracts the components of the plant. The extracts were then concentrated to dryness with the aid of a shaker water bath (Lab Tech shaker, model; LSI-3016R, Korea) set at 60°C. The weight of the extracts was taken after which they were stored in the refrigerator at -20°C.

*Microorganisms.* Microbial strains of *Escherichia coli* (6), *Pseudomonas aeruginosa* (6), *Klebsiella oxytoca* (4), *Klebsiella pneumoniae* (4), *Proteus vulgaris* (3), *Proteus mirabilis* (3), *Staphylococcus aureus* (6) and *Staphylococcus saprophyticus* (2) were obtained from the Department of Medical Microbiology, University College Hospital, Ibadan, Nigeria and Lancet Laboratories, Ibadan. Control strains (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC

29213) were obtained from Molecular Microbiology Laboratory of the Department of Pharmaceutical Microbiology, University of Ibadan. Bacterial strains were isolated from patients with symptomatic urinary tract infections. The strains were identified with the Microbact and API systems by the respective clinical laboratories and their identities were confirmed using a panel of biochemical tests including; Gram stain, growth on appropriate selective media, oxidase test, catalase test, coagulase test, motility test, DNase test, methyl red-voges proskauer test, citrate utilization, urease test, hydrogen sulfide production, fermentation of glucose, mannitol, maltose, lactose and sucrose. (Cheesbrough, 2006) after which they were archived in agar slants at 4°C.

*Antibiotic Susceptibility Testing.* Antibiotic susceptibility testing was conducted on all bacterial isolates using the disc diffusion technique (CLSI 2016). A panel of standard antibiotic discs which includes Ceftazidime 30 µg, Cefuroxime 30 µg, Gentamicin 10 µg, Ofloxacin 5 µg, Augmentin 30 µg, Nitrofurantoin 300 µg, Ciprofloxacin 5 µg, Cloramphenicol 30 µg, Augmentin 30 µg, Amoxicillin 25 µg, Erythromycin 5 µg, Tetracycline 10 µg, and Cloxacillin 5 µg were used for the screening. Isolate suspension equivalent to 0.5 McFarland equivalence turbidity standard for each isolate was used to inoculate Mueller Hinton Agar plates with the aid of sterile cotton tipped applicators. Antibiotic discs were then aseptically placed on the surface of the agar after which plates were incubated at 37 °C for 24 hours. Zones of inhibition were recorded and the results were interpreted based on CLSI standards and analyzed with WHONET application.

*Antimicrobial Screening of Extracts.* Antimicrobial screening of plant extracts was conducted using the agar well diffusion method. Mueller Hinton and Saboraud Dextrose Agar plates for bacteria and fungi respectively were inoculated with suspension of each isolate adjusted to 0.5 McFarland Equivalent Turbidity Standard. Inoculum was evenly spread on the plates with the aid of sterile cotton tipped applicators. Equidistant wells were bored on seeded plates with the aid of

sterile 8 mm diameter cork borer and 0.2 mL of respective extract dilutions and controls were aseptically dispensed into corresponding wells and the plates were incubated at 37 °C for 24 hours for bacteria and 25 °C for 48 hours for fungi. Ciprofloxacin and fluconazole were used as the standard drug control for bacteria and fungi respectively, while methanol was used as negative control. Tests were performed in triplicates and mean diameter of zones of inhibition were recorded after period of incubation.

*Determination of Minimum Inhibitory Concentration of Extracts.* Minimum inhibitory concentration (MIC) was determined using the agar dilution method (Klancnik et al., 2010; Wiegand et al., 2008). Different dilutions of extracts were made in Mueller Hinton Agar in petri dishes to final concentrations ranging from 0.39 mg/mL – 50 mg/mL. An overnight broth culture of each isolate on Tryptone Soy Broth (TSB) was diluted and inoculated, with the aid of sterile swabs, in agar plates containing respective extract dilutions. Plates were incubated at 37°C for 24 hours for bacteria and 25°C for 48 hours for fungi. The lowest concentration that prevented growth of isolates was recorded as the MIC.

*Determination of Minimum Biocidal Concentration of Extracts.* The Minimum Biocidal Concentration (MBC) of the bioactive extracts was determined using concentrations equivalent to the MIC values, twice, four times and eight times the MIC values. Overnight broth cultures of isolates were diluted and 0.5 mL of the 10<sup>-2</sup> dilution was inoculated into 3.5 mL of sterile TSB. Thereafter, 1 mL of each extract dilution which would yield the desired concentration was dispensed into appropriately labeled tubes. The tubes were then incubated at 37°C for 24 hours and 25°C for 48 hours for bacteria and fungi respectively. Streaking on fresh Tryptone Soy Agar (TSA) plates was done from each tube after incubation and the lowest concentration showing no growth after appropriate period of incubation was recorded as the MBC.

*Time-Kill Assay.* Bactericidal Kinetics was determined using the viable count technique. An

overnight broth culture of each isolate in 5 ml of TSB was obtained. The isolates used were *E. coli* ATCC 35218, *S. aureus* ATCC 29213 and *C. albicans*. Broth cultures of actively growing cells were diluted and 0.1 ml of the 10<sup>-2</sup> dilution was used to inoculate 3.9 ml TSB containing 1 ml of the extract at a final concentration equivalent to the MIC of each isolate. The resultant mixture, containing extract, culture and broth, was serially diluted and 100 µl appropriate dilutions were used to inoculate plates of TSA at different time intervals beginning at 0 minutes, 1 hour, 1 hour 30 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours and 24 hours. The inoculum was evenly spread with the aid of a sterile glass spreader after which the plates were left for a few minutes to dry. The plates were then incubated at 37 °C for 24 hours and 25°C for 48 hours for bacteria and fungi respectively. The entire procedure was repeated for extract concentrations containing 2 times the MIC and 4 times the MIC for each test isolate as well as controls without extract. After incubation period, the colony forming unit (CFU) was counted and a graph of the log of CFU per ml was plotted against time.

*Bioactivity Guided Fractionation.* Vacuum liquid chromatography (VLC) was conducted for the ethyl acetate extract of *Ficus thonningii*. Fifteen grams of the crude extract was adsorbed with silica gel (60-200 mesh size) and VLC was conducted using 100 mL ratios hexane (Hex), hexane-ethyl acetate (Hex-EtOAc) mixtures, ethyl acetate-methanol mixtures and methanol as eluents, all in increasing polarity. A thin layer chromatographic (TLC) plate was spotted with each fraction and this was chromatographed with a solvent mixture of ethyl acetate/methanol (9:1). Similar fractions on the basis of TLC profiles were pooled.

The pooled fractions from VLC were concentrated to dryness after which their antimicrobial activity on selected clinical isolates was conducted using agar well diffusion method as described previously.

## RESULTS AND DISCUSSION

The phytochemicals present in *F. thonningii*

include terpenoids, anthraquinones, saponins, tannins and alkaloids. Cardiac glycosides and steroids were absent (Figures 1 and 2) Clinical isolates used in this study were mostly multi-drug resistant. All Gram negative clinical bacteria used in this study were resistant to ampicillin and augmentin while Staphylococci were resistant to ceftazidime. The ethyl acetate extracts of *F. thonningii* produced larger zones of inhibition on clinical isolates than hexane and methanolic extracts as in Table 1. MIC and MBC values of ethyl acetate extract, as recorded in Table 2 are in the range of 6.25 mg/mL and  $\geq 50$  mg/mL. Pooled fractions of ethyl acetate extracts produced appreciable zones of inhibition, greater than the crude extract (Table 3). The ethyl acetate extract inhibited the growth of *E. coli* ATCC 35218, *S. aureus* ATCC 29213 and *C. albicans* by 100% at 1440 minutes, 390 minutes and 210 minutes respectively (Figures 3 – 5).

Some of the phytochemicals present in *F. thonningii*, as captured above have been reported previously. Egharevba et al., (2015) reported the presence of saponins, tannins and flavonoids as well as absence of cardiac glycosides and steroids on *F. thonningii*. However, anthraquinones and alkaloids which were found in this work were absent in their study. Similarly, alkaloids and flavonoids as well as cardiac glycosides (which was absent in this study) were detected in *F. thonningii* (Oyelana et al., 2011). Also, the main groups of phytochemicals present in *F. thonningii* as reported by (Dangarembizi et al., 2013) are alkaloids, terpenoids, flavonoids, tannins and essential oils. Worthy of note is the presence of anthraquinones observed in this study for *F. thonningii* as this was not reported in any literature encountered in the course of this work. The differences in phytochemicals detected in these studies could be as a result of difference in locations where the plants were collected as it has been shown that plant site affects its antimicrobial activity (Knief et al., 2010; Laforest-Lapointe et al., 2016). An array of phytochemicals isolated and identified from plants has been shown to confer varying degrees of antimicrobial properties to plants. It is believed that most plants produce such secondary metabolites in response to stress and invasion by microbes.

Flavonoids for instance are very effective against a wide range of organisms (Tsuchiya et al., 1996) and they probably do this by forming complexes with bacterial cell walls as well as extracellular and soluble proteins. Terpenoids are bactericidal as they distort the structure and integrity of the microbial cell membrane, leading to cell lysis (Cowan, 1999). These and other phytochemicals present in the plant might have resulted in the medicinal property of the plant. Indeed, it has been proposed and elucidated that different phytochemicals act synergistically to produce the overall antimicrobial effect associated with medicinal plants (Padmanabhan and Jangle, 2012).

Clinical isolates showed a high rate of resistance to standard antibiotics. The majority of strains were Multi Drug-Resistant (MDR), Possible Extensively Drug-Resistant (PXDR) or Possible Pan Drug-Resistant (PPDR) (CLSI, 2016; Magiorakos et al., 2011). There was a high rate of resistance among the *Pseudomonas aeruginosa* isolates as all were classified as PXDR. All these correlated with the studies of (Neuhauser et al., 2003; Okon et al., 2014) which reported increasing resistance amongst *P. aeruginosa* and other Gram negative bacilli. The resistance profile of *Proteus vulgaris* was remarkable as the isolates were resistant to almost all the antibiotics employed. Only one isolate was sensitive to nitrofurantoin. It is important to state that the resistance recorded in this study cannot be extrapolated to the general population of organisms in the communities or areas where the isolates were collected as the number of each species of organisms used in this study is small, with the aim of merely testing the activity of the extracts on them. However, antibiotic susceptibility testing was necessary to know the susceptibility pattern of the isolates.

The zones of inhibition shown by the hexane extract were small and as such it could be said that it had just minimal activity on test isolates or that the antimicrobial principles did not diffuse well. However the activity was consistent with concentration of the extract. The ethyl acetate extract was active against the isolates, producing appreciable zones of inhibition on both bacteria and fungi. Also, the ethyl acetate extract exhibited better

activity than hexane and methanol extracts, showing larger zones of inhibition. The methanol extract of *F. thonningii* was only moderately active against susceptible isolates showing lower zones of inhibition than the ethyl acetate extract but larger zones than the hexane. *F. thonningii* has been shown to be active against several bacterial pathogens (Usman et al., 2009; Koné et al., 2004; Ndukwe et al., 2007), similar to the observation in this study.

The standard drug control (ciprofloxacin 10 µg/mL) was active against only a few isolates. Although the activity of plant extracts, containing a lot of unknown and unstandardized substances, cannot be compared with standard drugs, the ethyl acetate extracts proved quite active. It produced appreciable zones of inhibition even on isolates that were resistant to the standard drug control and other drugs, including MDR, PXDR and PPDR bacteria. The activity of the plant extract on PPDR bacteria was observed keenly as this could be potentially useful in treating multi-drug resistant infections which continues to be a public health problem. This suggests that *F. thonningii* may be possible leads in the development of potential antimicrobial agents against UTI infections.

Minimum Inhibitory Concentrations of the extracts were quite high and this may be partly due to the fact that the extracts are crude, containing an array of unknown substances which made up the weight for concentrations, most of which may be inactive. Similarly, as expected based on the MIC values, the Minimum Biocidal Concentrations were high ranging from 6.25 mg/mL to above 50 mg/mL. MIC and MBC values for some isolates were the same; this suggests that the extract exerts its antimicrobial activity by killing rather than preventing the growth of susceptible bacteria.

VLC fractionation was conducted for the most active extract which was that of ethyl acetate. Antimicrobial screening of VLC fractions against representative isolates was conducted after pooling of fractions based on TLC profiles. The results of antimicrobial screening against test isolates showed the most active fraction to be fraction 1. This indicates that the active compounds of the plant are probably non-polar in nature. Nevertheless, all

fractions produced zones of inhibition indicating that there could be several compounds conferring antimicrobial activity to the plant. Zones produced by fractions were also larger than the crude, suggesting that further purification and isolation of active compounds would reduce the MICs and MBCs.

The extract at 4MIC produced 100% inhibition of *E. coli* strain at 24 hours. At the 2MIC and 1MIC, growth was moderately reduced but at the time 24 hours, there were still counts on plates. On *S. aureus*, the 4MIC and 2MIC resulted in 100% inhibition at 6 hours 30 minutes and 24 hours respectively. As observed with *E. coli*, growth was reduced at 1MIC but death was not recorded at 24 hours. In essence the extract was more effective in killing the Gram positive organism (*Staphylococcus aureus*) employed in this study than the Gram negative (*Escherichia coli*) as shown by the time-kill curves. The extract at 4MIC and 2MIC led to death of *C. albicans*. However, at 1 MIC growth reduced a bit up until after four hours before increasing until the end of the experiment, suggesting that that concentration is not sufficient to kill the fungus. The presence of growth at concentrations where only reductions but not kill was observed after the duration of the study does not definitely mean that death would not be achieved at such concentrations, especially as reduction was evident. Instead, it could indicate that had the experiment be conducted for longer duration, death would possibly have been observed.

This study has shown that *F. thonningii* is active on pathogens isolated from human urinary tract, thus corroborating its possible application in the treatment of urinogenital tract infections, treating even drug resistant infections if developed into standard drugs with further research.

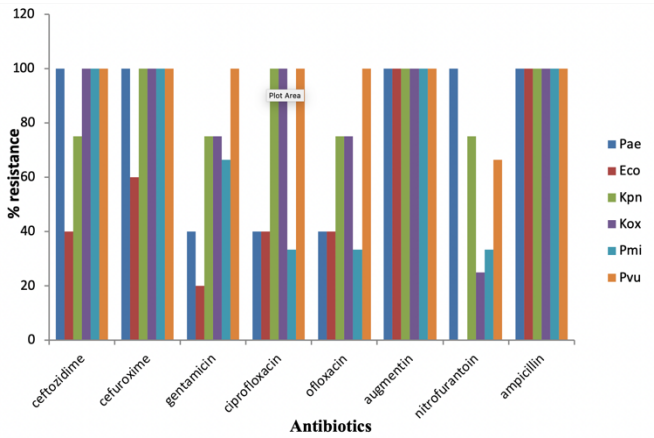


Figure 1: Percentage resistance of Gram negative isolates to select antibiotics. ‘Pae’ = *Pseudomonas aeruginosa*; ‘Eco’ = *Escherichia coli*; ‘Kpn’ = *Klebsiella pneumoniae*; ‘Kox’ = *Klebsiella oxytoca*; ‘Pmi’ = *Proteus mirabilis*; ‘Pvu’ = *Proteus vulgaris*

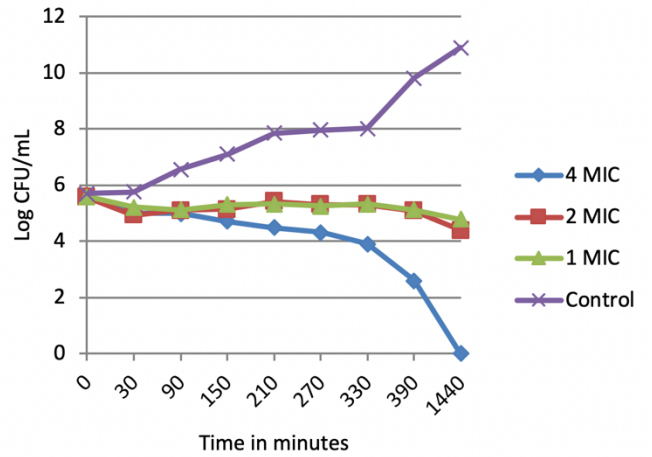


Figure 3: Kill Kinetics of the Ethyl Acetate Extract of *F. thoningii* on *Escherichia coli* ATCC 35218

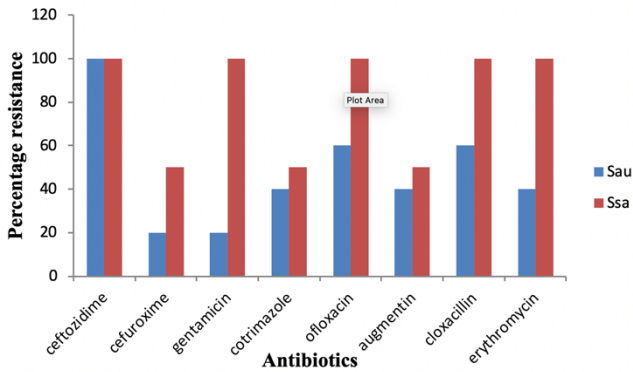


Figure 2: Percentage resistance of Gram positive isolates to select antibiotics. ‘Sau’ = *Staphylococcus aureus*; ‘Ssa’ = *Staphylococcus saprophyticus*

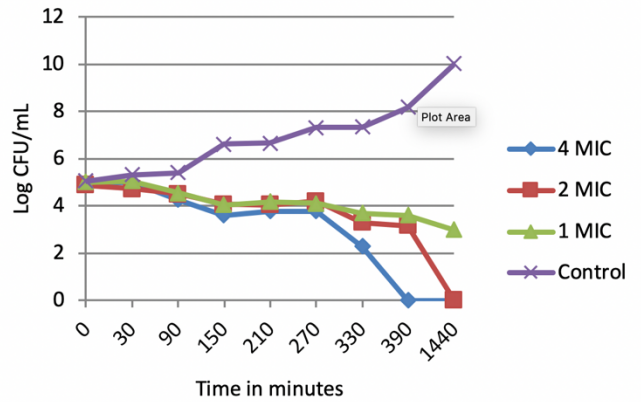


Figure 4: Kill Kinetics of the Ethyl Acetate Extract of *F. thoningii* on *Staphylococcus aureus* ATCC 29213



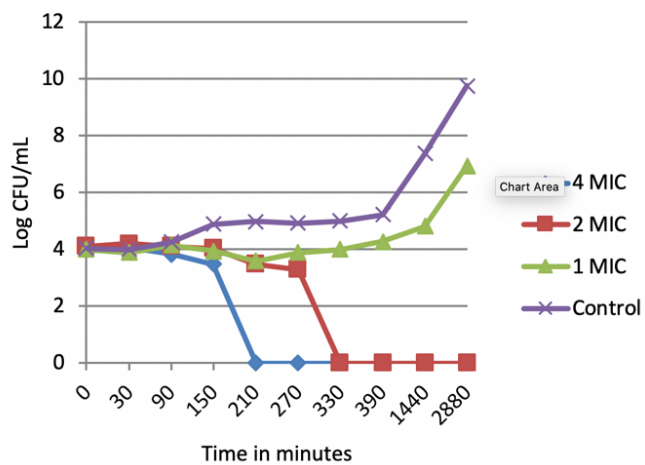


Figure 5: Kill Kinetics of the Ethyl Acetate Extract of *F. thonningii* on *Candida albicans* 4

Table 1: Antimicrobial Screening of Crude Extracts on Clinical Isolates

| Concentration             | Hexane extract           |             |             | Ethyl acetate extract |             |             | Methanol extract |             |             | Ciprofloxacin | Fluconazole |
|---------------------------|--------------------------|-------------|-------------|-----------------------|-------------|-------------|------------------|-------------|-------------|---------------|-------------|
|                           | 100<br>mg/mL             | 50<br>mg/mL | 25<br>mg/mL | 100<br>mg/mL          | 50<br>mg/mL | 25<br>mg/mL | 100<br>mg/mL     | 50<br>mg/mL | 25<br>mg/mL | 10<br>µg/mL   | 50<br>µg/mL |
| Isolate                   | Zones of Inhibition (mm) |             |             |                       |             |             |                  |             |             |               |             |
| <i>P. aeruginosa</i> 1    | NZI                      | NZI         | NZI         | 11.0 ± 0.8            | 14.0 ± 1.4  | 11.7 ± 0.5  | 12.3 ± 0.5       | 12.0 ± 0.0  | 11.3 ± 0.5  | NZI           | NT          |
| <i>P. aeruginosa</i> 2    | 9.3 ± 0.5                | NZI         | NZI         | 14.0 ± 0.8            | 12.7 ± 0.5  | 12.3 ± 0.5  | 16.3 ± 0.5       | 15.3 ± 0.5  | 12.0 ± 0.0  | 27.7 ± 1.3    | NT          |
| <i>P. aeruginosa</i> 3    | 12.3 ± 0.5               | 10.0 ± 0.0  | NZI         | 18.3 ± 1.2            | 13.3 ± 0.5  | 11.0 ± 0.0  | 14.3 ± 0.5       | 13.0 ± 0.0  | 12.3 ± 1.2  | NZI           | NT          |
| <i>P. aeruginosa</i> 4    | 10.7 ± 0.5               | 10.5 ± 0.5  | 10.7 ± 0.5  | 17.3 ± 0.5            | 14.3 ± 0.5  | 13.3 ± 0.5  | 14.7 ± 0.5       | 14.0 ± 0.8  | 12.0 ± 0.0  | 23.7 ± 1.3    | NT          |
| <i>P. aeruginosa</i> 5    | 13.3 ± 0.5               | 12.3 ± 1.2  | 10.3 ± 0.5  | 18.0 ± 0.8            | 15.0 ± 0.0  | 13.7 ± 1.2  | 14.0 ± 0.0       | 12.0 ± 0.8  | 10.7 ± 0.5  | 30.3 ± 1.3    | NT          |
| <i>E. coli</i> 1          | NZI                      | NZI         | NZI         | 13.0 ± 0.0            | 10.3 ± 0.5  | 9.3 ± 0.5   | NZI              | NZI         | NZI         | 27.7 ± 0.9    | NT          |
| <i>E. coli</i> 2          | NZI                      | NZI         | NZI         | 17.3 ± 0.9            | 12.3 ± 1.2  | 10.7 ± 0.5  | 12.3 ± 0.5       | 11.0 ± 0.8  | 10.3 ± 0.5  | 28.7 ± 1.3    | NT          |
| <i>E. coli</i> 3          | NZI                      | NZI         | NZI         | 13.3 ± 0.5            | 11.0 ± 0.0  | NZI         | 13.0 ± 0.8       | NZI         | NZI         | 27.3 ± 0.5    | NT          |
| <i>E. coli</i> 4          | NZI                      | NZI         | NZI         | 14.7 ± 0.9            | 12.3 ± 0.5  | 11.3 ± 0.5  | 12.0 ± 0.0       | 10.3 ± 0.5  | 10.0 ± 0.0  | NZI           | NT          |
| <i>E. coli</i> 5          | 10.3 ± 1.2               | NZI         | NZI         | 14.3 ± 0.5            | 11.3 ± 0.5  | 10.7 ± 0.5  | 10.7 ± 0.5       | 9.7 ± 0.5   | 11.3 ± 1.2  | NZI           | NT          |
| <i>K. pneumoniae</i> 1    | NZI                      | NZI         | NZI         | 12.0 ± 0.8            | 9.0 ± 0.8   | NZI         | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>K. pneumoniae</i> 2    | 11.3 ± 0.5               | NZI         | NZI         | 19.3 ± 1.2            | 12.3 ± 0.5  | 10.7 ± 0.5  | 12.3 ± 0.5       | 10.0 ± 0.0  | 9.5 ± 0.5   | NZI           | NT          |
| <i>K. pneumoniae</i> 3    | 10.3 ± 1.2               | 11.3 ± 1.2  | NZI         | 12.3 ± 0.5            | 10.3 ± 0.5  | NZI         | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>K. pneumoniae</i> 4    | 11.0 ± 0.8               | 9.0 ± 0.0   | NZI         | 14.7 ± 0.9            | NZI         | NZI         | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>K. oxytoca</i> 1       | NZI                      | NZI         | NZI         | 16.0 ± 0.8            | 14.0 ± 0.0  | 12.3 ± 0.5  | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>K. oxytoca</i> 2       | NZI                      | NZI         | NZI         | NZI                   | NZI         | NZI         | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>K. oxytoca</i> 3       | 12.3 ± 1.2               | 10.3 ± 0.5  | NZI         | 13.3 ± 0.5            | 10.3 ± 0.9  | NZI         | 17.5 ± 0.5       | 12.7 ± 0.5  | 12.0 ± 0.0  | 20.7 ± 2.5    | NT          |
| <i>K. oxytoca</i> 4       | NZI                      | NZI         | NZI         | NZI                   | NZI         | NZI         | 17.0 ± 0.8       | NZI         | NZI         | NZI           | NT          |
| <i>P. mirabilis</i> 1     | 14.3 ± 0.5               | 15.3 ± 0.5  | 13.7 ± 0.5  | 19.7 ± 0.9            | 18.3 ± 0.5  | 14.0 ± 0.0  | NZI              | NZI         | NZI         | NZI           | NT          |
| <i>P. mirabilis</i> 2     | NZI                      | NZI         | NZI         | 16.0 ± 0.8            | 14.7 ± 0.5  | 12.3 ± 2.1  | NZI              | NZI         | NZI         | 20.7 ± 0.9    | NT          |
| <i>P. mirabilis</i> 3     | 10.0 ± 0.8               | NZI         | NZI         | 15.7 ± 0.9            | 12.3 ± 0.5  | 11.7 ± 0.5  | 12.0 ± 0.0       | 10.7 ± 0.5  | NZI         | NZI           | NT          |
| <i>P. vulgaris</i> 1      | 12.0 ± 0.8               | 14.7 ± 1.2  | 11.7 ± 1.2  | 22.0 ± 1.4            | 13.0 ± 0.8  | 11.0 ± 0.0  | 14.7 ± 0.9       | 12.3 ± 0.5  | 10.0 ± 0.0  | NZI           | NT          |
| <i>P. vulgaris</i> 2      | 10.3 ± 0.5               | 10.0 ± 0.0  | NZI         | 20.7 ± 1.7            | 13.3 ± 0.5  | 12.0 ± 0.0  | 19.3 ± 0.5       | 10.3 ± 0.5  | NZI         | NZI           | NT          |
| <i>P. vulgaris</i> 3      | 12.0 ± 0.0               | 10.7 ± 1.7  | NZI         | 15.3 ± 0.5            | 12.0 ± 0.0  | 11.0 ± 0.8  | 11.0 ± 0.8       | 10.3 ± 0.5  | 9.0 ± 0.0   | NZI           | NT          |
| <i>S. aureus</i> 1        | 14.3 ± 0.5               | NZI         | NZI         | 17.0 ± 1.4            | 13.0 ± 0.8  | 12.7 ± 0.5  | 12.3 ± 0.5       | 12.0 ± 0.0  | 9.3 ± 0.5   | NZI           | NT          |
| <i>S. aureus</i> 2        | 11.7 ± 1.2               | NZI         | NZI         | 22.0 ± 0.8            | 15.3 ± 1.2  | 12.7 ± 0.5  | 12.3 ± 0.5       | 10.3 ± 0.5  | 10.0 ± 0.0  | 21.0 ± 0.8    | NT          |
| <i>S. aureus</i> 3        | NZI                      | NZI         | NZI         | 12.7 ± 1.2            | 14.3 ± 0.5  | 13.3 ± 0.5  | 14.0 ± 0.8       | 12.0 ± 0.0  | 10.7 ± 0.5  | 29.0 ± 0.8    | NT          |
| <i>S. aureus</i> 4        | 15.7 ± 0.9               | NZI         | NZI         | 17.3 ± 0.5            | 16.7 ± 0.5  | 12.0 ± 0.0  | 14.7 ± 1.2       | 11.7 ± 0.9  | 9.0 ± 0.0   | NZI           | NT          |
| <i>S. aureus</i> 5        | 14.3 ± 2.1               | NZI         | 10.0 ± 1.0  | 20.0 ± 0.0            | 15.0 ± 0.8  | 12.3 ± 0.5  | 12.0 ± 0.0       | 10.3 ± 0.5  | NZI         | NZI           | NT          |
| <i>S. saprophyticus</i> 1 | 10.3 ± 0.5               | NZI         | NZI         | 25.3 ± 0.5            | 18.3 ± 0.5  | 15.3 ± 0.5  | 14.3 ± 0.5       | 11.0 ± 0.0  | NZI         | NZI           | NT          |
| <i>S. saprophyticus</i> 2 | 13.7 ± 0.9               | NZI         | NZI         | 19.3 ± 0.5            | 15.0 ± 0.0  | 13.7 ± 0.5  | 12.0 ± 0.0       | 11.0 ± 0.0  | 10.7 ± 0.5  | NZI           | NT          |
| <i>C. albicans</i> 1      | 10.3 ± 0.5               | NZI         | NZI         | 15.0 ± 0.8            | 13.3 ± 0.5  | 10.0 ± 0.0  | 13.3 ± 0.5       | 9.3 ± 0.5   | 9.5 ± 0.5   | NT            | NZI         |
| <i>C. albicans</i> 2      | 10.7 ± 0.5               | 10.0 ± 0.0  | 9.0 ± 0.0   | 15.7 ± 1.7            | 13.0 ± 0.0  | 11.3 ± 0.5  | 10.7 ± 0.5       | 9.3 ± 0.5   | 9.7 ± 0.5   | NT            | NZI         |
| <i>C. albicans</i> 3      | 11.3 ± 0.5               | 10.3 ± 0.5  | 10.0 ± 0.0  | 16.3 ± 0.5            | 13.0 ± 0.8  | 12.7 ± 0.9  | 12.0 ± 0.0       | 11.7 ± 0.5  | 10.0 ± 0.8  | NT            | NZI         |
| <i>C. albicans</i> 4      | 10.0 ± 0.8               | 9.0 ± 0.0   | NZI         | 16.0 ± 0.8            | 13.7 ± 0.9  | 12.3 ± 0.5  | 10.0 ± 0.0       | 9.7 ± 0.5   | 9.0 ± 0.0   | NT            | 14.7 ± 0.9  |

Key: '*E. coli*' = *Escherichia coli*; '*P. aeruginosa*' = *Pseudomonas aeruginosa*; '*K. pneumoniae*' = *Klebsiella pneumoniae*; '*K. oxytoca*' = *Klebsiella oxytoca*; '*P. mirabilis*' = *Proteus mirabilis*; '*P. vulgaris*' = *Proteus vulgaris*; '*S. aureus*' = *Staphylococcus aureus*; '*S. saprophyticus*' = *Staphylococcus saprophyticus*; 'NZI' = no zone of inhibition 'NT' = not tested

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Biocidal Concentrations of the Ethyl Acetate Extracts Isolates

| Isolate                         | MIC (mg/mL) | MBC (mg/mL) |
|---------------------------------|-------------|-------------|
| <i>P. aeruginosa</i> ATCC 27853 | 12.5        | 12.5        |
| <i>P. aeruginosa</i> 1          | 6.25        | 12.5        |
| <i>P. aeruginosa</i> 2          | 12.5        | 25.0        |
| <i>P. aeruginosa</i> 3          | 12.5        | 12.5        |
| <i>P. aeruginosa</i> 4          | 12.5        | 25.0        |
| <i>P. aeruginosa</i> 5          | 12.5        | 25.0        |
| <i>E. coli</i> ATCC 35218       | 12.5        | 12.5        |
| <i>E. coli</i> 1                | 6.25        | 50.0        |
| <i>E. coli</i> 2                | 6.25        | 25.0        |
| <i>E. coli</i> 3                | 12.5        | 50.0        |
| <i>E. coli</i> 4                | 12.5        | 25.0        |
| <i>E. coli</i> 5                | 6.25        | 25.0        |
| <i>K. pneumoniae</i> 1          | 6.25        | 50.0        |
| <i>K. pneumoniae</i> 2          | 12.5        | 25.0        |
| <i>K. pneumoniae</i> 3          | 12.5        | 50.0        |
| <i>K. pneumoniae</i> 4          | 12.5        | 50.0        |
| <i>K. oxytoca</i> 1             | 12.5        | 25.0        |
| <i>K. oxytoca</i> 2             | 12.5        | ≥50         |
| <i>K. oxytoca</i> 3             | 12.5        | 25.0        |
| <i>K. oxytoca</i> 4             | 12.5        | ≥50         |
| <i>P. mirabilis</i> 1           | 12.5        | 12.5        |
| <i>P. mirabilis</i> 2           | 12.5        | 12.5        |
| <i>P. mirabilis</i> 3           | 6.25        | 12.5        |
| <i>P. vulgaris</i> 1            | 12.5        | 25.0        |
| <i>P. vulgaris</i> 2            | 12.5        | 25.0        |
| <i>P. vulgaris</i> 3            | 12.5        | 12.5        |
| <i>S. aureus</i> ATCC 29213     | 12.5        | 12.5        |
| <i>S. aureus</i> 1              | 12.5        | 12.5        |
| <i>S. aureus</i> 2              | 12.5        | 12.5        |
| <i>S. aureus</i> 3              | 12.5        | 12.5        |
| <i>S. aureus</i> 4              | 12.5        | 12.5        |
| <i>S. aureus</i> 5              | 12.5        | 25.0        |
| <i>S. saprophyticus</i> 1       | 6.25        | 12.5        |
| <i>S. saprophyticus</i> 2       | 12.5        | 12.5        |

Key: '*E. coli*' = *Escherichia coli*'*K. pneumoniae*' = *Klebsiella pneumoniae*'*P. mirabilis*' = *Proteus mirabilis*'*S. aureus*' = *Staphylococcus aureus*'*P. aeruginosa*' = *Pseudomonas aeruginosa*'*K. oxytoca*' = *Klebsiella oxytoca*'*P. vulgaris*' = *Proteus vulgaris*'*S. saprophyticus*' = *Staphylococcus saprophyticus*

Table 3: Antimicrobial Screening of VLC pooled fractions of *F. thonningii*

| Concentration             | Fraction 1               |          | Fraction 2 |          | Fraction 3 |          |
|---------------------------|--------------------------|----------|------------|----------|------------|----------|
|                           | 50 mg/mL                 | 25 mg/mL | 50 mg/mL   | 25 mg/mL | 50 mg/mL   | 25 mg/mL |
| Isolate                   | Zones of Inhibition (mm) |          |            |          |            |          |
| <i>P. aeruginosa</i> 2    | 31                       | 15       | 18         | 14       | 14         | 11       |
| <i>E. coli</i> 2          | 21                       | 15       | 12         | 11       | 10         | NZI      |
| <i>K. pneumoniae</i> 1    | 24                       | 10       | 13         | 10       | 10         | 9        |
| <i>K. oxytoca</i> 2       | 23                       | 14       | 14         | 11       | 10         | NZI      |
| <i>S. aureus</i> 2        | 30                       | 22       | 15         | 14       | 12         | NZI      |
| <i>S. saprophyticus</i> 2 | 29                       | 27       | 18         | 12       | 15         | 13       |

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