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Antibacterial and Antiadherence Properties of the Leaves of *Ficus Thoninngii* Blume on A*cinetobacter Baumannii*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: The ability of *Acinetobacter baumannii* to adhere and persist in any environment has aided its persistence in hospital environments. *Ficus thonningii* Blume has antimicrobial abilities and may halt the attachment of microorganisms to air-liquid surfaces.

Objective: To investigate the antibacterial and antiadherence properties of the leaves of *Ficus thonningii* on *A. baumannii*.

Materials and Methods: Antibiogram of twenty-three clinical isolates of *A. baumannii* including a typed strain was determined using disc-diffusion method. Antimicrobial activities of the leaf extracts and fractions of *Ficus thonningii* were screened by agar-well diffusion. The minimum inhibitory concentration (MIC) was determined by agar-dilution. The ability of the organism to adhere to air-liquid surface and form pellicle, and the ability of the extracts to inhibit the pellicles formed were investigated. Pellicle forming strains of *E. coli* ATCC 35218 and *A. baumannii* 111A were used as controls. One-way ANOVA was used to compare the optical densities of the pellicles formed.

Results: At 50 mg/mL, the inhibition zone diameter produced by ethyl acetate, methanol and *n*-hexane extracts ranged from 0-15 mm with ethyl acetate extract having the highest activity. The MIC ranged from 0.039-1.25 mg/mL, 0.039-20 mg/mL and 1.25-20 mg/mL for ethyl acetate, methanol and hexane extracts respectively while the MBC ranged from 5-20 mg/mL, 1.25-20 mg/ml and ≥20 mg/mL also respectively. Pellicle formation was observed in 62.0 % of the bacterial isolates. There was a slight reduction in the pellicles formed at 12.5 mg/mL concentration of the extracts.

Conclusions: The study has shown that *Ficus thonningii* can be used in the management and treatment of infections caused by *A. baumannii*. It also has the ability to inhibit pellicles formed by the organism at increased concentrations.

Keywords: *Acinetobacter baumannii, Ficus thonningii,* Antibacterial, Antiadherence, Pellicle

INTRODUCTION

Bacterial pathogens that are resistant to multiple drugs represent a growing public health threat (Chang *et al*., 2015; Brunel and Guery 2017, Bassetti *et al*., 2017). This is because multiple drug resistant (MDR) infections are difficult and expensive to treat. The obstruction in disease control by MDR infections increases the possibility of spread of resistant pathogens, which results in prolonged time of infection in patients, high cost of treatment and transfer of these resistant pathogens (Falagas *et al*., 2006; Chang *et al*., 2015).

Acinetobacter baumannii forms part of the most common and serious MDR pathogens (Ballantine *et al*., 2019; Skariyachan *et al*., 2019). The aerobic, non-motile, Gram-negative coccobacilli, *A. baumannii*, has a high incidence rate among the immuno-compromised individuals particularly those that have stayed for more than ninety days in the hospital. It colonizes the skin and can be isolated in high numbers from the respiratory and oropharynx secretions of infected individuals. It colonizes irrigating solutions

and intravenous fluids. (Machanda *et al*., 2010). Infections caused by *A. baumannii* include pneumonia, bloodstream infections (bacteremia and sepsis), meningitis, wound and surgical site infections, including the "flesh-eating" bacterium necrotizing fasciitis and urinary tract infections (UTI) (Cerquieira and Peleg 2011).

The organism's ability to quickly acclimatize to selective changes in environmental pressures contributes to the rapid emergence of the multi and pandrug resistant strains (Peleg *et al*., 2012). Its characteristic feature of withstanding desiccation in the environment has been associated with biofilm formation (Nait *et al*., 2014). The solid-liquid interface is one of the best-studied biofilms in which bacteria adhere to biotic and abiotic surfaces (Kentache *et al*., 2016). *A baumannii* forms biofilms that adhere to the solid-liquid interface and also forms another specialised type of biofilms known as pellicle or air-liquid biofilms (ALB) that adhere to air-liquid interfaces. Pellicles, just like biofilms, are a structure of connected cells surrounded by a matrix of extracellular polymeric substances. The air-liquid interface often requires complex organization than the solid-liquid biofilm due to lack of a solid surface for the initiation of microbial growth. Also at this interface, strict aerobic bacteria obtain nutrients from the liquid media and oxygen from the air (Marti *et al*., 2011). Suggestion has been made of a correlation between this characteristic feature and the virulence of *A. baumannii* that causes nosocomial infections (Kentache *et al*., 2016).

Pellicle formation is an emerging area of research in Gram-negative bacteria especially in *A. baumannii*. Studies have been carried out on the investigation, characterisation and quantification of pellicles formed by *A. baumannii* (Nait *et al*., 2014) but there are scarce reports on the inhibition of pellicles. Plants produce compounds that inhibit multidrug resistant pathogens in addition to the production of antimicrobial compounds (Stermitz *et al*., 2000a). Screening of crude plant extracts for antimicrobial and antiadherence activity could go a long way in reducing the menace of multidrug resistant organisms. *Ficus thonningii* contains bioactive substances such as alkaloids, tannins, flavonoids, terpenoids, saponins, steroids which forms part of the antimicrobial constituents. They are present in the leaves, roots, fruits and flowers of *F. thonningii*. The plant parts can be used alone or in combination with other plants for the treatment of diseases like diarrhea, epilepsy and mental illness. The ethanolic leaf extract has shown good activity against *Streptococcus pyrogenes and Enterococcus faecalis* in the treatment of septicemia and urinary tract infections (Kone *et al*., 2004). The plant has also been discovered to have antidiarrheal properties by stimulating water absorption, reducing intestinal motility and electrolyte secretion (Njoronge and Bussmann 2006). The stem bark has been found useful in the treatment of diarrhoea, dysentery and wound infections (Dangarembizi *et al*., 2013).

Therefore, this study aimed at investigating the antimicrobial and antiadherence properties of leaf extracts of *Ficus thonningii* on *A. baumannii.*

METHODOLOGY

Collection and Preparation of Plant Samples

The leaf samples were collected at Arulogun Road, Ojoo, Ibadan, Nigeria. They were identified, authenticated and deposited at Forestry Research Institute of Nigeria (FRIN) with the voucher number FHI 110160. The leaves were air-dried, pulverized and weighed.

Phytochemical analysis

The powdered leaves of *F. thonningii* were screened for secondary metabolites using standard procedures (Vinoth *et al*., 2011).

Steroids: The plant sample (0.5g) was weighed into a clean test tube containing 2 ml of chloroform. This was then shaken and filtered. Acetic anhydride and concentrated hydrogen tetraoxosulphate (VI) acid were then added. A positive test was indicated by a greenish colour at the upper part of the liquid.

Cardiac glycosides: The sample (0.5g) was weighed into a clean test tube containing 5 ml of water. This was then filtered. Two (2) ml of glacial acetic acid containing a drop of ferric chloride was added to the filtrate after which concentrated hydrogen tetraoxosulphate (VI) acid was added. A reddish-brown colour at the interphase indicates the presence of cardiac glycosides.

Flavonoids: To 0.5 g of plant sample weighed into a clean test tube was added 5 ml of distilled water. This was then filtered and dilute ammonia was added to the filtrate. A yellow colour, persistent with the addition of concentrated hydrogen tetraoxosulphate (VI) acid indicates a positive result.

Alkaloids: The plant sample (0.5g) was acidified with a mixture of 1% hydro chloric 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.

Terpenoids: To 0.5 g of plant sample weighed into a clean test tube was added 2 ml of chloroform. This was then shaken and thereafter filtered after which concentrated H_2SO_4 was added to the filtrate. A reddish-brown colour at the interphase indicates the presence of terpenoids.

Phenol: One gram of the sample was first extracted with ethyl-acetate and then filtered with Whatman filter paper. The development of blue black or brown colouration on the addition of ferric chloride reagent to the filtrate indicates the presence of phenol.

Anthraquinone: Concentrated H_2SO_4 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 colour change upon the addition of ammonium hydroxide indicates a positive result.

Saponins: To 0.5 g of sample weighed into a test tube was added 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.

Tannins: The plant sample (0.5g) was stirred with 10 ml 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.

Plant extraction and fractionation

Successive gradient extraction was carried out with *n*-hexane, ethyl acetate and methanol using soxhlet apparatus. The extracts were afterwards concentrated to dryness using a shaker set at 60 ^oC. The extracts were weighed and then stored in the refrigerator at 4 °C for subsequent use. Ethyl acetate extract which had better antimicrobial activity was subjected to fractionation using Vacuum Layer Chromatography (VLC) with solvents of different polarities (*n*-hexane, ethyl acetate and methanol). Different fractions were obtained.

A thin layer chromatographic (TLC) plate was spotted with each fraction and this was chromatographed with solvent mixtures of *n*-hexane/ethylacetate (4:1), ethylacetate/methanol (1:1) and ethylacetate/methanol (3:2). The fractions were then pooled based on their TLC profiles. A total of three pooled fractions were obtained.

Microorganisms used

Twenty-three clinical isolates of *Acinetobacter baumannii* were used in this study. They were obtained from the Medical Microbiology Department of the University College Hospital, Ibadan, Nigeria. Typed strains of *Escherichia coli* (*E. coli*) ATCC 35218, *E. coli* ATCC 11175, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, and two pellicle forming isolates of *E. coli* were collected from Molecular Biology Laboratory, Department of Pharmaceutical Microbiology, University of Ibadan. The isolates were all maintained on nutrient agar slants at 4°C prior to use.

Antibiogram profile of test isolates

Overnight broth cultures of clinical *A. baumannii* isolates were made in nutrient broth and diluted in normal saline. Dilutions corresponding to 0.5 McFarland standards were used. Inoculum from each isolate was spread evenly on the surface of each Mueller Hinton agar plate with the aid of sterile glass spreader. An antibiotic multi-disc (RapidLabs, UK) was placed gently and firmly on the surface of the inoculated plates with the aid of sterile forceps. The antibiotic multidisc contained the following; Ampicillin 10 µg, Nitrofurantoin 300 µg, Cefuroxime 30 µg, Ciprofloxacin 5 µg, Ofloxacin 5 µg, Gentamicin 10 µg, Augmentin 30 µg, Ceftazidime 30 µg. The plates were then incubated at 37 °C for 24 hours. Zones of growth inhibition were measured and interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI) 2016 edition.

In vitro* antimicrobial screening of plant extracts and fractions against *A. baumannii

A 0.5 McFarland standard equivalent suspension of each isolate of *A. baumannii* was made in 0.85 % normal saline and 0.1 mL of it was used to inoculate plates of Mueller Hinton Agar. Equidistant wells were bored with the aid of a standard sterile 8mm cork borer and 0.1 ml of different concentrations of ethyl acetate extract and controls were placed into the corresponding wells. Ciprofloxacin (5 µg/ml) was used as the standard drug. The screening was done in duplicates. The plates were allowed to stay at room temperature for an hour to allow for pre-diffusion of the extracts into the agar medium. The plates were then incubated at 37 °C for 24 hours. The same procedure was repeated using *n*-hexane and methanol extracts of the plant.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts

Concentrations ranging from 0.039 mg/mL to 20 mg/mL were prepared. Eighteen milliliters of Mueller Hinton Agar (MHA) was prepared and sterilized in McCartney bottles. Two milliliters of each extract concentration was gently mixed with the MHA and poured into Petri dishes. Thereafter, a 0.5 McFarland standard equivalent suspension of each isolate was made in 0.85 % normal saline from which 0.1 mL was used to inoculate the plates. The plates were then incubated at 37 °C for 24 hrs. The MIC was recorded as the lowest concentration of the extract that inhibited the growth of the test organisms. For the MBC, sterile inoculating loop was swabbed on the MIC plates that did not show growth and used to inoculate freshly prepared nutrient broth. The inoculated broths were incubated at 37 °C for 24 hrs. The lowest concentration in which extracts did not allow growth of the test organisms was recorded as MBC (Adeniyi *et al*., 2016).

Time-Kill Assay

The time-kill assay was carried out using the method described by Lajubutu *et al*., (1995). Overnight broth culture of *A. baumannii* isolate was made in 5mL of nutrient broth. Subsequently, 1mL of the broth culture was inoculated into another 4mL of nutrient broth and incubated for 18 hours at 37°C. Thereafter, 0.1 mL of the culture was used to inoculate 2.9 mL of nutrient broth containing 1mL of fraction 1 that was set at a final concentration equal to the MIC (0.625 mg/mL). Serial dilutions were made from this mixture and 0.1 mL each from 10^{-3} and 10^{-5} dilutions was used to inoculate a freshly prepared nutrient agar at predetermined time intervals specifically 0 minute, 2 hours, 4 hours, 6 hours , 8 hours, 10 hours, 12 hours and 24 hours. A sterile glass spreader was used to evenly spread the inoculum on the nutrient agar plate. The plates were allowed to dry for few minutes at room temperature before incubation at 37 °C for 24 hrs. This procedure was repeated for fraction 1 containing concentrations 2 times the MIC and 4 times the MIC. Also, a control broth was set up but with no added extract. The entire procedure was also repeated for fractions 2 and 3. After the incubation, microbial colony count was done and a graph of log of CFU/mL was plotted against time.

Pellicle formation analysis

Pellicle-forming assay was performed in 22 mL glass tubes with a diameter of 14 mm. Overnight broth cultures of the test isolates were made in Mueller Hinton Broth. Freshly prepared 3 mL Mueller Hinton Broth was adjusted to an initial Optical Density (OD_{600}) of 0.01 using each of the overnight broth cultures and then incubated at 25 °C statically in the dark for 72 hours. A white structure of connected cells surrounded by a matrix of extracellular polymeric substance indicated pellicle formation. The isolates were considered positive when pellicle covers the entire broth surface and also when it formed round the walls of the glass tube (Marti *et al*., 2011, Giles *et al*., 2015).

Inhibition of pellicle formation by the plant extracts

The same procedure for the pellicle formation was used. After setting the initial OD_{600} of 0.01, 100 µl of 12.5 mg/mL of ethyl acetate extract was added to the broth cultures. It was repeated with methanol and hexane extracts in separate broth cultures. Cranberry was used as the positive control while the ethyl acetate was used as the negative control. The same procedure was also done with 3.125 mg/mL of the extracts. The tubes were then incubated at 25 °C statically in the dark for 72 hours.

Quantification of pellicles

After 72 hours incubation period, 5 mL pipette was used to withdraw the culture from the bottom of the tube without touching the sides of the tube. Four milliliters of sterile Phosphate buffered saline (PBS) was added to each tube and rocked briefly from side to side and then withdrawn. PBS is isotonic and non-toxic to cells. It helped to attach the cells to the tubes. This was repeated two more times. Four milliliters of 0.5% crystal violet was added to each tube and left standing for 10 minutes. Crystal violet stained the attached cells and further enhanced their quantification. The crystal violet was then withdrawn and washed off three times with sterile water. The tubes were then left to dry inverted. Thereafter, 1.5 mL of ethanol and 1.5 mL of acetone (in that order) were added to the tubes and rocked for about 20 minutes to dissolve the attached cells. One hundred microliter (100 µL) of each was dispensed into 96-well microtiter plate in triplicates. Ethanol-acetone (1:1) was used as the blank. The absorbance was then read at 570 nm.

Statistical analysis

The optical densities of the pellicles of each extract concentration were compared using One-way ANOVA and results expressed as Mean ± Standard deviation

RESULTS

Acinetobacter baumannii forms part of the most common and serious MDR pathogens which has been classified into the group ESKAPE standing for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp* (Pendleton *et al*., 2013; Peneş *et al*., 2017; Gulick 2017). These are multidrug resistant pathogens with high level of resistance to antibiotics. With respect to the CLSI guideline on zone diameters for detecting sensitive and resistant organisms in Table 1, this study identified the organism to be resistant to more than three different classes of antibiotics as seen in Fig 1 thus confirming the multidrug resistant nature of the organism (Ike *et al*., 2014, Jean *et al*., 2016). More than half of the isolates were resistant to ciprofloxacin and gentamicin. The ability of *A. baumannii* to effectively resist these antibiotics may be linked in part to the organism's permeability defects, possession of antimicrobial inactivating agents and efflux pump systems and alteration of the antibiotics target sites (Harmanjit *et al*., 2013).

Qualitative phytochemical screening revealed the presence of alkaloids, tannins, flavonoids, terpenoids, saponins and steroids in the plant. This is in agreement with the reports seen in the determination of phytochemicals in *F. thonningii* (Ndukwe *et al*., 2007, Coker *et al*., 2015). These phytochemical constituents contribute significantly to the antimicrobial effects of this plant. According to reports, tannins are known to inactivate cell envelope, microbial adhesion and enzymes and also complex with cell polysaccharides to inhibit the growth of microorganisms (Cowan 1999). The presence of tannins in the extract is likely to be a contributory factor to the antimicrobial activity observed with the plant extract. Alkaloids, based on their physiological effects on microorganisms, contribute to the antimicrobial activities of plants by intercalating with DNA, thereby rendering them potential antimicrobial agents (Raaman 2006). Its presence in *Ficus thonningii* as observed in this study contributed to the inhibitory activities of the plant against *A. baumannii* isolates. Flavonoids which have been found in various parts of the plant partially inhibit the activities of bacterial DNA gyrase, inhibit energy metabolism and sometimes inhibit cytoplasmic membrane functions was also detected in this study further suggesting its antimicrobial activities. Terpenoids have a reputation for disrupting microbial cell membranes resulting in the death of the cells thereby increasing the bactericidal effects of the plant (Cowan 1999, Usman *et al*., 2009). There might be possible synergistic and additive effects of the phytochemical compounds on *A. baumannii.*

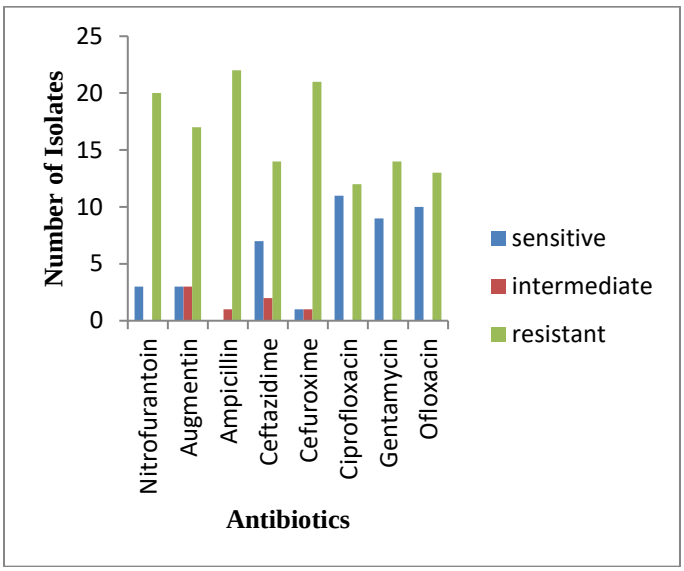

Figure 1: Antibiotics susceptibility test of *A. baumannii*

Table 1: CLSI disc diffusion standard for *A. baumannii*

	Antimicrobial agent	Disk content	Zone diameter (interpretative criteria) (nearest whole mm)		
			Sensitive	Intermediate	Resistance
1	Nitrofurantoin	300 µg	≥17	15-16	≤14
2	Augmentin	30 µg	≥18	14-17	≤13
3	Ampicillin	10 µg	≥17	14-18	≤13
4	Ceftazidime	30 µg	≥18	15-17	≤14
5	Cefuroxime	30 µg	≥18	15-17	≤14
6	Ciprofloxacin	5 µg	≥ 21	16-20	<15
7	Gentamicin	10 µg	≥15	13-14	<12
8	Ofloxacin	5 µg	≥16	13-15	<12

Plant-based alternatives are one of the solutions that are being harnessed in the fight against infections caused by resistant organisms. *Ficus thonningii* has been reported to have antimicrobial activities against both Gram-negative and Gram-positive bacteria (Kone *et al*., 2004, Usman *et al.,* 2009). Table 2 showed that ethyl acetate extract of *F. thonningii* produced the highest zone of growth inhibition. Methanol and *n*-hexane extracts produced minimal zones of inhibition on *A. baumannii* as seen in Tables 3 and 4, respectively, while the standard drug used had limited activity on the organism. This gives credence to the potential of the plant as a reliable alternative to antibiotics that the organism has developed resistance to.

The MIC of *n*-hexane extracts of *Ficus thonningii* determined in this study was high. Most of the isolates had an MIC value ≥20 mg/mL with only one isolate having an MIC of 1.25 mg/mL as observed in Table 5.

The MBC of the hexane extract was ≥20 mg/mL for all the isolates. This was expected as it produced insignificant inhibition zone diameters. The methanol extract had better activity considering the MIC values which were as low as 0.039 mg/mL for most of the isolates. This was in contrast to the result of the inhibition zone diameters. Its activity on the agar medium may have been halted. This may be connected to the rate of diffusion of this extract in the agar medium.

The MIC of *Ficus thonningii* extracts against most Gram-negative bacteria ranged from 1.25-12.5 mg/Ml (Dangarembizi *et al*., 2013). An MIC of 50 mg/mL has been observed when the methanolic extracts of *F.thonningii* were tested on *Klebsiella pneumoniae* (Ndukwe *et al*., 2007).The present study found an MIC of the methanol and ethyl acetate extracts to be within the range of 0.039mg/mL and 1.25mg/mL. However, an MIC of 20 mg/mL was observed in some isolates. This study suggests that the extracts of the plant are likely to have more activity against *A. baumannii* when compared with the reports of Ndukwe *et al*., (2007) on *Klebsiella pneumoniae*. Bioactive guided fractionation was carried out with the ethyl acetate extract. Table 6 showed that the zone diameters of the pooled fractions were not different from that of the crude extracts. The Time-Kill analysis in Figures 2-4 showed a gradual reduction in the number of surviving colonies per hour. In all the fractions, there was no surviving organism at the end of 24 hours of incubation. The rate of kill of the fractions was concentration dependent with respect to time.

Table 2: **Antimicrobial Screening of Ethyl Acetate Crude Extract of *F. thonningii* on *Acinetobacter baumannii* Isolates**

Concentrations	***50 mg/ml***	***25 mg/ml***	***12.5 mg/ml***	***6.125 mg/ml***	***3.125 mg/ml***	***Ciprofloxacin (5 µg/ml)***
Isolates	***Zones of Inhibition (mm)***					
A. baumannii 1	12	11	11	10	10	NZI
A. baumannii 2	12	11	10	10	10	NZI
A. baumannii 3	11	10	10	10	10	10
A. baumannii 4	15	11	11	10	9	27
A. baumannii 5	12	10	10	10	10	36
A. baumannii 6	12	11	10	10	NZI	12
A. baumannii 7	15	12	12	12	11	NZI
A. baumannii 8	11	11	11	11	NZI	37
A. baumannii 9	11	10	10	10	10	NZI
A. baumannii 10	11	11	11	10	9	33
A. baumannii 11	10	10	10	10	9	33
A. baumannii 12	10	10	10	10	NZI	35
A. baumannii 13	10	10	10	10	10	NZI
A. baumannii 14	12	10	10	10	10	30
A. baumannii 15	10	10	10	10	NZI	10
A. baumannii 16	12	11	11	11	10	NZI
A. baumannii 17	12	12	12	10	10	30
A. baumannii 18	13	11	11	11	10	33
A. baumannii 19	10	10	10	NZI	10	35
A. baumannii 20	11	11	11	11	10	11
A. baumannii 21	11	11	10	NZI	NZI	NZI
A. baumannii 22	13	NZI	10	10	10	33
A.baumannii NCTC 7363	11	11	11	10	10	33

Key: "NZI" - NO ZONE OF INHIBITION; "A. baumannii" – Acinetobacter baumannii

Table 3: Antimicrobial Screening of Methanol Crude Extract of *F. thonningii* on Acinetobacter baumannii Isolates

Concentrations	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**6.125 mg/ml**	**3.125 mg/ml**	**Ciprofloxacin (5 µg/ml)**
Isolates	**Zones of Inhibition (mm)**					
***A. baumannii* 1**	9	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 2**	10	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 3**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 4**	NZI	NZI	NZI	NZI	NZI	27
***A. baumannii* 5**	NZI	NZI	NZI	NZI	NZI	35
***A. baumannii* 6**	NZI	NZI	NZI	NZI	NZI	12
***A. baumannii* 7**	11	9	9	9	NZI	NZI
***A. baumannii* 8**	NZI	NZI	NZI	NZI	NZI	37
***A. baumannii* 9**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 10**	10	NZI	NZI	NZI	NZI	32
***A. baumannii* 11**	9	NZI	NZI	NZI	NZI	33
***A. baumannii* 12**	NZI	NZI	NZI	NZI	NZI	35
***A. baumannii* 13**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 14**	NZI	NZI	NZI	NZI	NZI	34
***A. baumannii* 15**	NZI	NZI	NZI	NZI	NZI	10
***A. baumannii* 16**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 17**	NZI	NZI	NZI	NZI	10	29
***A. baumannii* 18**	NZI	NZI	NZI	NZI	NZI	33
***A. baumannii* 19**	11	NZI	NZI	NZI	NZI	32
***A. baumannii* 20**	9	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 21**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 22**	10	10	NZI	NZI	9	27
***A. baumannii* NCTC 7363**	10	10	9	9	NZI	33

Key: "NZI" - NO ZONE OF INHIBITION; "A. baumannii" – Acinetobacter baumannii

Table 4: Antimicrobial Screening of *n*-hexane crude extract of *F. thonningii on Acinetobacter baumannii* isolates

Concentrations	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**6.125 mg/ml**	**3.125 mg/ml**	**Ciprofloxacin (5 µg/ml)**
Isolates	**Zones of Inhibition (mm)**					
***A. baumannii* 1**	11	11	10	NZI	NZI	NZI
***A. baumannii* 2**	11	10	NZI	NZI	NZI	NZI
***A. baumannii* 3**	11	10	NZI	NZI	NZI	NZI
***A. baumannii* 4**	11	11	10	10	NZI	30
***A. baumannii* 5**	12	12	10	NZI	NZI	40
***A. baumannii* 6**	12	10	10	NZI	NZI	12
***A. baumannii* 7**	12	10	10	NZI	NZI	NZI
***A. baumannii* 8**	11	11	10	10	8	37
***A. baumannii* 9**	11	10	NZI	NZI	NZI	NZI
***A. baumannii* 10**	NZI	12	10	11	NZI	35
***A. baumannii* 11**	NZI	NZI	NZI	NZI	NZI	33
***A. baumannii* 12**	NZI	NZI	NZI	NZI	NZI	44
***A. baumannii* 13**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 14**	NZI	NZI	NZI	NZI	NZI	35
***A. baumannii* 15**	NZI	NZI	NZI	NZI	NZI	10
***A. baumannii* 16**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 17**	NZI	NZI	NZI	NZI	NZI	39
***A. baumannii* 18**	NZI	NZI	NZI	NZI	NZI	33
***A. baumannii* 19**	NZI	NZI	NZI	NZI	NZI	38
***A. baumannii* 20**	NZI	NZI	NZI	NZI	NZI	NZI
***A. baumannii* 21**	12	10	8	NZI	NZI	NZI
***A. baumannii* 22**	12	11	10	NZI	NZI	33
***A. baumannii* NCTC 7363**	13	11	10	NZI	NZI	35

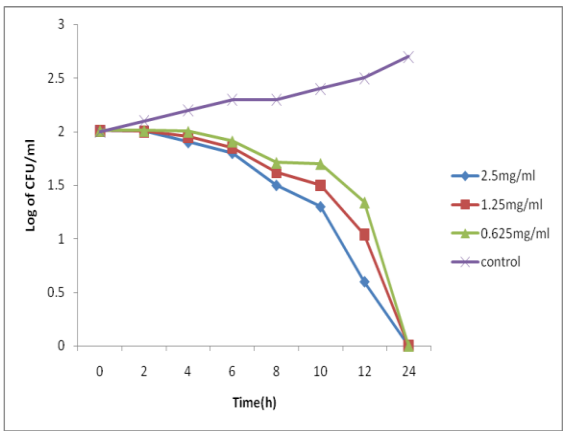

Fig 2. Time Kill kinetics of pooled fraction 1 on *Acinetobacter*

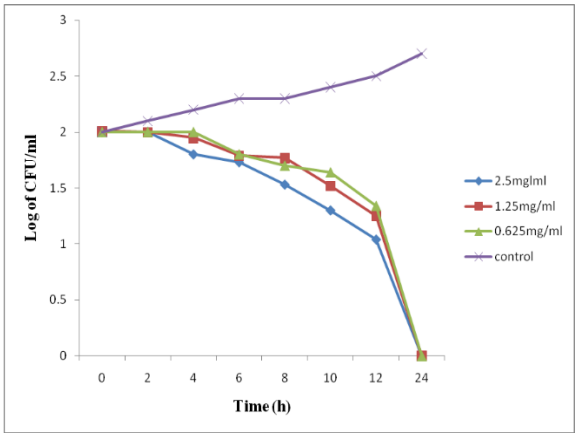

Fig 3. Time Kill kinetics of pooled fraction 2 on *Acinetobacter baumannii*

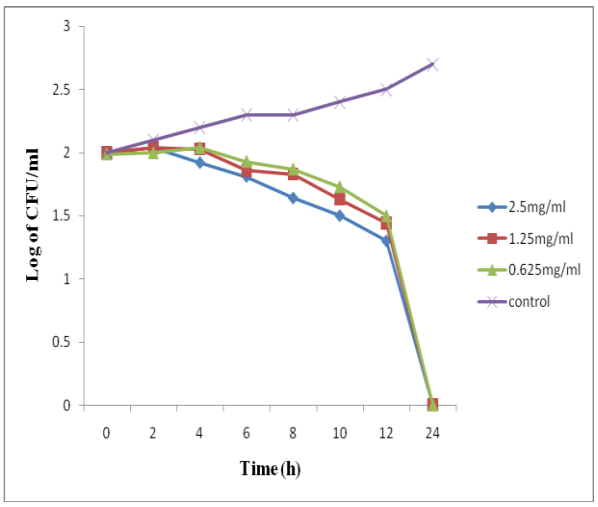

Fig 4. Time Kill kinetics of pooled fraction 3 on *Acinetobacter baumannii*

Out of the twenty-four isolates of *A. baumannii* and three typed strains which were assayed for pellicle formation; eighteen had pellicle-forming abilities of which twelve were *A. baumannii*. This confirmed the findings of Marti *et al*. (2011) on the pellicle-forming ability of this organism. Pellicles, like biofims have the potential of increasing the survival of *A. baumannii* in liquid environments. This is brought about by the exopolymeric substances released by organisms within a pellicle. There is also increased virulence within the pellicle as less pathogenic organisms become more pathogenic as a result of horizontal gene transfer within the pellicle. Pellicles formed by *A. baumannii* are classified into three groups (Giles et al., 2015) "small pellicles" having optical density (OD) of ≤0.19, "moderate pellicles" with OD of >0.20 and <0.50 and "well developed pellicles" having OD of >0.51. Based on this classification, none of the isolates produced "small pellicles", two strains of *A. baumannii* produced "moderate pellicles" while the rest had "well developed pellicles" in our study.

Cranberry extract has antiadherence properties against *Campylobacter jejuni* and *Campylobacter coli* strains (Ramirez-Hernandez *et al*., 2015). It also inhibits biofilm formation in *Enterococcus faecalis* (Wojnicz *et al*., 2016) which necessitated its use in the study. Pellicle formation is gaining wide attention because of its peculiarities. Aerobic bacteria survive stagnant environmental conditions because pellicles formed by these bacteria can have access to high levels of oxygen and nutrients (Yuan *et al*., 2013). In *Mycobacterium tuberculosis*, it contributes to the organism's resistance to antibiotics (Sambandan *et al*., 2013). It was observed that a mutant strain of *M. tuberculosis* which lacked pellicle was sensitive to rifampin. However, when the mutant strain was incorporated into a wild-type pellicle-biofilm, it acquired resistance to rifampin. A correlation has also been established between pellicle formation and acetic acid resistance ability in *Acetobacter pasteurianus* (Kanchanarach *et al*., 2010). These observations gave credence to the relevance of pellicle formation. In this study, though there was an increase in pellicle formation at 3.125 mg/mL and 12.5 mg/mL concentrations with *n*-hexane and methanol extracts, there was a reduction in the pellicles formed at both concentrations with ethyl acetate extracts as shown in Table 7. Therefore, ethyl acetate extract of *F. thonningii* in addition to inhibiting the growth of *A. baumannii* isolates, *also* has the ability to inhibit pellicle formation of *A. baumannii* at the air-liquid interface which will reduce its survival and adherence in such interfaces. Fig 5 showed the pictorial view of some of the pellicle assay result.

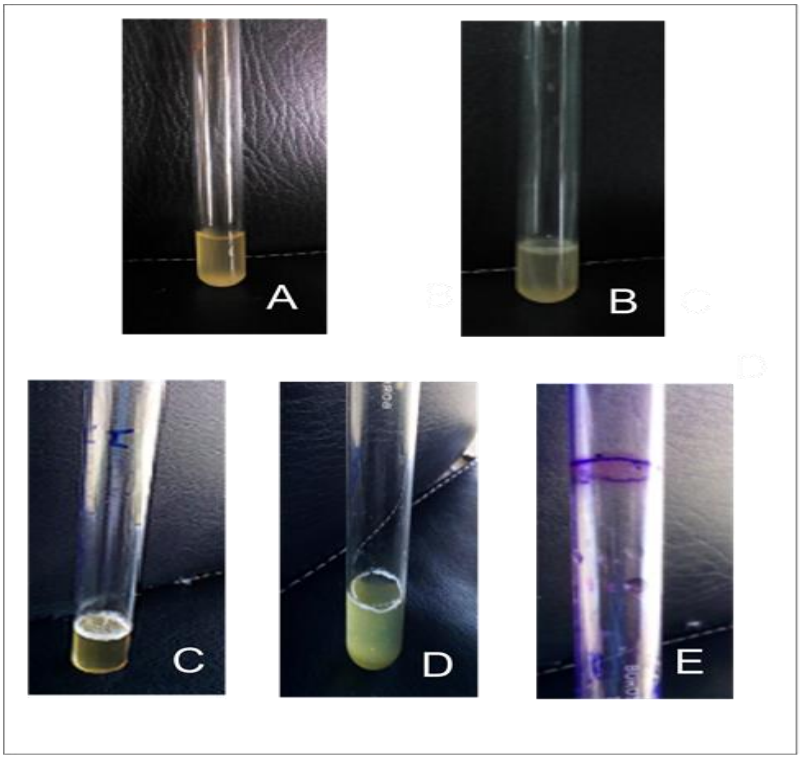

Figure 5: Pellicle formation. A = Absence of pellicle in one of the isolates, B = Pellicle formation in one of the isolates, C = Pellicle formation at 3.125 mg/mL of extract, D = Pellicle formation at 12.5 mg/mL of extract, E = Stained pellicle ready for elution

Table 5: MIC and MBC of Ethyl Acetate, Methanol and n-Hexane Extracts of *F. thonningii* on *Acinetobacter baumannii* clinical isolates

Isolate	Ethyl acetate extract		Methanol extract		*n*-Hexane extract	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
***A. baumannii* 1**	1.25	5	≤0.039	1.25	≥20	≥20
***A. baumannii* 2**	0.625	20	≤0.039	1.25	≥20	≥20
***A. baumannii* 3**	0.625	20	≤0.039	1.25	≥20	≥20
***A. baumannii* 4**	0.3125	5	≤0.039	20	2.5	20
***A. baumannii* 5**	0.3125	≥20	≤0.039	20	≥20	≥20
***A. baumannii* 6**	1.25	20	≤0.039	1.25	≥20	≥20
***A. baumannii* 7**	1.25	20	≤0.039	20	≥20	≥20
***A. baumannii* 8**	≤0.039	20	20	≥20	2.5	20
***A. baumannii* 9**	0.156	5	≤0.039	1.25	20	≥20
***A. baumannii* 10**	0.625	20	≤0.039	≥20	20	≥20
***A. baumannii* 11**	1.25	20	≤0.039	≥20	≥20	≥20
***A. baumannii* 12**	≤0.039	20	≤0.039	≥20	10	20
***A. baumannii* 13**	0.625	20	20	≥20	≥20	≥20
***A. baumannii* 14**	1.25	20	20	≥20	≥20	≥20
***A. baumannii* 15**	1.25	20	20	≥20	≥20	≥20
***A. baumannii* 16**	1.25	20	20	≥20	20	≥20
***A. baumannii* 17**	≤0.039	20	20	≥20	1.25	20
***A. baumannii* 18**	≤0.039	20	20	≥20	2.5	20
***A. baumannii* 19**	≤0.039	20	20	≥20	≥20	≥20
***A. baumannii* 20**	1.25	20	≤0.039	1.25	≥20	≥20
***A. baumannii* 21**	≤0.039	20	≤0.039	≥20	≥20	≥20
***A. baumannii* 22**	≤0.039	20	≤0.039	1.25	≥20	≥20
***A. baumannii* NCTC 7363**	0.625	20	≤0.039	1.25	≥20	≥20

Key: A. baumannii – Acinetobacter baumannii, MIC – Minimum inhibitory concentration, MBC – minimum bactericidal concentration

Table 6. Antimicrobial Screening of VLC pooled fractions of *F. thonningii*

Pool fraction 1							
Concentrations	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**6.25 mg/mL**	**3.125 mg/mL**	**Ciprofloxacin 5µg/mL**	**Methanol**
Isolates	**Inhibition Zones(mm)**						
***A. baumannii* 2**	15	12	12	12	12	10	NZI
***A. baumannii* 4**	13	13	12	12	10	25	NZI
***A. baumannii* 9**	15	15	11	10	10	10	NZI
***A. baumannii* 15**	15	15	12	10	10	12	NZI
Pooled fraction 2							
***A. baumannii* 2**	13	10	10	10	10	10	NZI
***A. baumannii* 4**	12	11	10	10	10	30	NZI
***A. baumannii* 9**	14	12	12	10	10	11	NZI
***A. baumannii* 15**	15	14	12	12	NZI	13	NZI
Pooled fraction 3							
***A. baumannii* 2**	15	11	10	10	10	NZI	NZI
***A. baumannii* 4**	13	12	10	10	10	27	NZI
***A. baumannii* 9**	12	10	10	10	10	11	NZI
***A. baumannii* 15**	14	15	13	13	13	12	NZI

KEY: A. baumannii - Acinetobacter baumannii; NZI - No zone of inhibition; VLC- Vacuum Layer Chromatography

TABLE 7: One-way ANOVA comparison of the Optical density of pellicles at 3.125 mg/mL and 12.5 mg/mL of crude extracts

Extract	**Mean ± Standard Deviation (at 3.125 mg/mL)**	**Mean ± Standard Deviation (at 12.5 mg/mL)**
Without extract	0.44 ± 0.167^{a}	0.44 ± 0.166^{a}
Cranberry extract	0.99 ± 0.813^{b}	0.79 ± 0.709^{a}
Hexane extract	1.41 ± 0.846^{b}	1.01 ± 0.373^{b}
Methanol extract	1.04 ± 0.451^{b}	1.02 ± 0.378^{b}
Ethylacetate extract	0.45 ± 0.261^{a}	0.49 ± 0.243^{a}

mean±standard deviations with same superscripts are not significantly different p-value at 0.05

CONCLUSION

The antimicrobial activity of *Ficus thonningii* observed in our study has shown that the plant can be used in the treatment and management of infections caused by *A. baumannii*. Methanol and ethyl acetate extracts of *Ficus thonningii* had more activity against *A. baumannii* than the hexane extract.

Pellicle formation is an emerging field in bacteria especially in Gram-negative bacteria. Reports on the inhibition of pellicle are scarce in literature. In this study, at the lowest concentration of extracts, there was an increase in the pellicle formation. However, gradual reduction in pellicle formation was observed in few isolates at increased concentrations of ethyl acetate extract.

Determination of MIC seems to be a reliable method of predicting the efficacy of plant extracts. Future studies should be focused more on it. Also, attention should be given to the use of plants in antimicrobial studies as it may also trigger some other physiological functions in organisms such as pellicle formation which aids in the expression of some virulence factors in the organism.

Furthermore, studies should be directed towards evaluating the mode of action of extracts and fractions of *Ficus thonningii* against *A. baumannii*. Attempts towards isolation and characterization of pure antimicrobial compounds should be made.

More investigations should be directed towards the pellicle formation in Gram-negative organisms especially on ways of dismantling the formed pellicles.

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