Phytochemical Screening and Antimicrobial Activity of *Entandrophragma angolense*

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Abstract: *Entandrophragma angolense* (Welw.) C.DC. (Meliaceae) is a deciduous plant commonly found on in west Africa and it is used for treatment microbial infections, wounds, rheumatic and arthritic pains. The methanol stem bark extract was investigated for its antimicrobial activity using the agar well diffusion method and macro-dilution technique and time kill kinetics. The antimicrobial activity of the methanol extract and its fractions were evaluated against five bacteria species and a fungus including *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* NCTC 10073, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and clinical strains of *Candida albicans* and some of the test bacteria. Phytochemical screening of the crude extract revealed the presence of tannins, flavonoids, alkaloids, terpenoids, saponins and cardiac glycosides. The MICs of the methanol extract against the test organisms were: *S. aureus* (11.0 mg/mL), *E. faecalis* (9.0 mg/mL), *B. subtilis* (13.0 mg/mL) *E. coli* (17.0 mg/mL), *P. aeruginosa* (19 mg/mL) and *C. albicans* (15.0 mg/mL). The fractions of the methanol extract (pet ether, ethyl acetate and aqueous fractions) exhibited varying antimicrobial activities, with the highest activity exhibited by the aqueous fraction. The extract exhibited bacteriostatic and fungistatic activity against all test organisms. Time kill studies showed that the extract exhibited an inhibition of bacterial and fungal growth. The results indicate that the methanol stem bark of *E. angolense* has antibacterial and antifungal activities and may justify the medicinal uses of the plant in the management of bacterial and fungal infections.

Keywords: Phytochemical screening, antibacterial, antifungal, time kill kinetics.

1. INTRODUCTION

The widespread use of herbal remedies and healthcare preparations can be traced to the occurrence of medicinal properties in these herbs. For thousands of years people have used these plant products to flavour and preserve foods as well as in the treatment of diverse ailments. Popular observations on the use and efficacy of medicinal plants have significantly contributed to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not completely known [1]. Resistance to two or more classes of antimicrobial agents has been a common finding reported in human and veterinary medicine which has been limiting the available therapeutic options. Therefore the discovery and development of new compounds that would either block or circumvent these resistance mechanisms could improve the containment, treatment and eradication of these strains [2].

There is a an increasing need for the research and development of newer antibiotics from other sources such as plants and natural products as some microorganisms have developed resistant to existing antibiotics due to over prescription and misuse of these antibiotics [3]. The medicinal value of medicinal plants lies in the bioactive phytochemical compounds that produce normal physiological action on the human body [4]. Some of the most valuable bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds and many more compounds [5]. In recent years, there has been tremendous research interest in the possible role of phytochemicals derived from natural sources in the prevention and treatment of many diseases.

Entandrophragma angolense (Welwitsch) C.DC. belongs to a genus of eleven species of deciduous trees of the Meliaceae family. The aqueous decoctions of the root and stem bark is used in African folk medicine for the treatment of fevers, malaria, wounds and other gastrointestinal disorders including diarrhoea and peptic ulcers in humans [6, 7, 8]. It is also used in external applications as an anodyne against stomach and earaches, rheumatic or arthritic pains, a diuretic in

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the kidney problems as well as treatment of eye and ear infections and swellings [7, 8]. Antiulcer activities of the methanol stem bark extract of this plant [9] as well as its effects on the gastrointestinal smooth muscle and transit time in mice have recently been reported [10]. The stem bark extract has been found to possess antiplasmodial activity [11] and two limonoids 7α-acetoxydihydronomilin 1 and 7α- obacunyl acetate 2 isolated from the stem bark of *E. angolense* and two new semi-synthesized limonoids 7α-acetoxy-14, 15-deepoxynomilin 3 and 1, 2- dihydro-1, 4-dihydroxy-6-deoxoatalantin 4 derived by partial synthesis from 7α-acetoxydihydronomilin 1 have been found be to active against W2 strain of *Plasmodium falciparum* [12]. Relatively little information is available on the antimicrobial property of the stem bark of *E. angolense*. This study therefore investigates the antimicrobial activity of *E. angolense* stem bark and also performs phytochemical screening on the plant material and extract.

2. MATERIALS AND METHODS

2.1. Plant Material

E. angolense plant was identified and parts of the stem bark were collected from the forest at the bottom of mountain Ejuanoma, Nkawkaw in the Eastern region of Ghana. It was identified by Mr. G.H. Sam of the Department of Herbal Medicine, where a voucher specimen (P'cog*/*He*/*0036) has been deposited at Herbarium of Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

2.2. Extraction of Plant Material

The stem bark was cleaned, air dried, powdered and stored in air-tight container. The methanol extract was prepared by soaking 400 g of the dry powdered plant materials in 2.5 L of methanol 70% v/v (GPR, BDH Ltd. Poole, UK) using the cold maceration method for 3 days. The extract was filtered using Whatmann filter paper No.10. The filtrate was then concentrated using a rotary evaporator at 40°C and lyophilized. The percentage yield of extract was 10.7 % w/w related to the dried material.

2.3. Test Organisms

Typed Gram-positive bacterial strains including *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 10073, and *Enterococcus faecalis* ATCC 29212 and Gram-negative bacterial strains comprising *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and a clinical isolate of *Candida albicans* were used as test organisms. The clinical isolate was obtained from Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana.

2.3. Phytochemical Screening

Phytochemical screening of methanol stem bark extract of *E. angolense* was carried out using standard procedures [13-15].

2.4. HPLC Finger-Printing of Extract

The HPLC finger-printing of methanol stem bark extract of *E. angolense* was determined with Thermo Finnigan HPLC system using Shim Pack ODS column (150 x 4.6 mm). The concentration of the extract used was 10 mg/mL. HPLC optimum conditions: injection volume: 10 µL, detection wavelength: 254 nm, mobile phase: methanol/water/70:30 v/v (isocratic condition), temperature: 22°C, Pump pressure: 28 MPa, flow rate of 1 mL/min and running time: 14 min.

Table 1: Preliminary Phytochemical Screening of Methanol Stem Bark Extract of *E. angolense*

Chemical constituents	Observation	Inference
Flavonoids	Yellow colouration	+
Terpenoids	Red ppt. at the interface of test tube	+
Tannins	Darkish green colour/ red ppt. at the bottom of test tube	+
Alkaloids	Turbid buff- colour	+
Steroids	Absence of steroidal ring and no change in colour	-
Anthraquinones	Absence of a pink/violet colour	-
Cardiac glycosides	Violet ring formed at the interface of test tube	+
Saponins	Frothing and formation of emulsion	+

+ = present; - = absent; ppt. = precipitate.

2.5. Fractionation of the Methanol Extract of *E. angolense*

An amount of 10 g of the methanol extract was sequentially extracted into non-polar, intermediate and polar fractions with 100 mL of petroleum ether (Scharlau Chemicals, Ltd. UK), 100 mL of ethyl acetate (BDH Chemicals, UK) and 150 mL of methanol/water (7:3) respectively. The fractions obtained were concentrated under vacuum on a rotary evaporator at 40°C and lyophilized. The antimicrobial activity of the various fractions was determined using the agar diffusion method described above.

2.6. Determination of Antimicrobial Activity

2.6.1. Agar Well Diffusion Method

The activity of the methanol stem bark extract of *E. angolense* was determined using a modified agar diffusion method as described by Rauha *et al*. [16]. Several concentrations of the methanol extract (2, 4, 8, 16, 32, 64 mg/mL) were prepared in DMSO**.** Twenty millilitres Muller-Hinton agar (Oxoid, UK) was melted and stabilized at 45ºC for 15 min and inoculated with 100 µL of 18 h test organism containing 10^5 colony forming unit (cfu) per mL. A cork borer with a diameter of 10 mm was used to bore 4 wells per plate (equidistant from each other). Sabouraud dextrose agar (Oxoid, UK) was used as the culture medium for *C. albicans* and the above procedure repeated. The wells were labelled and filled with 100 µL of the above concentrations. Reference antibiotics, 10 µg/mL of ciprofloxacin (Zhejiang Xin Ltd.) and 1 mg/mL of ketoconazole (Sigma-Aldrich, Darmstadt, Germany) were used as positive controls. DMSO was used as negative control. The plates were allowed to stand for one hour to allow adequate diffusion of extract and reference drugs. The plates seeded with bacteria were then incubated at 37^{o}C for 24 h, while plates seeded with *C. albicans* were incubated at 30^{o}C for 48 h. The zones of inhibition were then measured and the results recorded. The procedure was performed in triplicate.

2.6.2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of methanol stem bark extract of *E. angolense* was determined according to the macro-dilution method described by Akinpelu and Kolawale [17]. Concentrations of 1 to 30 mg/mL of the extract were prepared in 2 mL nutrient broth. Sabouraud broth was used as the culture medium for the fungus and the above procedure repeated. One hundred microlitres of 18 h of test organism containing 10^5 cfu/mL and then inoculated into the different concentrations of the extract in 2 mL broth. Concentrations of the reference antibiotics ciprofloxacin (5 to 10 µg/mL) and ketoconazole (1.25 to 10.0 µg/mL) were also used. A control test tube containing DMSO was also prepared. The test tubes were incubated at 37°C for 24 h and at 30 °C for 48 h for bacteria and fungus respectively after which the plates were observed for growth. The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each micro organism. The procedures were performed in triplicate.

2.6.3. Determination of Minimum Bactericidal/ Fungicidal Concentration (MBC/MFC)

The MBC and MFC of the methanol stem bark extract of *E. angoelnse* were determined using the method described by Aibinu *et al*. [18]. Test tubes that showed no visible growth in the previous procedure for MIC determination (section 2.6) after 24 to 48 h incubation were streaked onto Muller-Hinton agar and incubated at 37^{o}C for 24 h for bacteria and sabouraud agar at 30^{o}C for 48 h for fungus. The MBC and MFC were determined as the lowest concentration that prevented bacterial and fungal growth respectively after incubation. Agar plates without extracts and any inoculated organisms were also incubated serving as positive and negative control plates respectively. All determinations were done in triplicates to ensure consistency.

2.7. Time Kill Kinetics

The killing rate of the methanol extract against the test organisms were performed according to the modified method of Adeniyi *et al*. [19]. Various concentrations of methanol extract (MIC, 2×MIC, 3×MIC and 4×MIC) were prepared in 5 mL nutrient broth. One hundred microlitres of 18 h test organism containing approximately 10^5 cfu/mL was added to each of the 5 mL nutrient broth and kept at 37°C. Aliquots of 100 µL were withdrawn at intervals of 0, 1, 2, 3, 4, 5, 6, 12 and 24 h and inoculated into 20 mL plate count agar (Oxoid, UK) stabilized at 40°C, thoroughly mixed and allowed to set. The plates were then incubated inverted at 37°C for 24 h for bacteria) and 30°C for 48 h for *C. albicans*. Control plates for positive and negative controls were also incubated. The cfu/mL of each plate determined after incubation. The number of surviving organisms per mL was calculated. The procedure was performed in triplicate to ensure consistency. A graph of Log10 surviving cells per mL against time (h) was plotted to determine the time-kill kinetics.

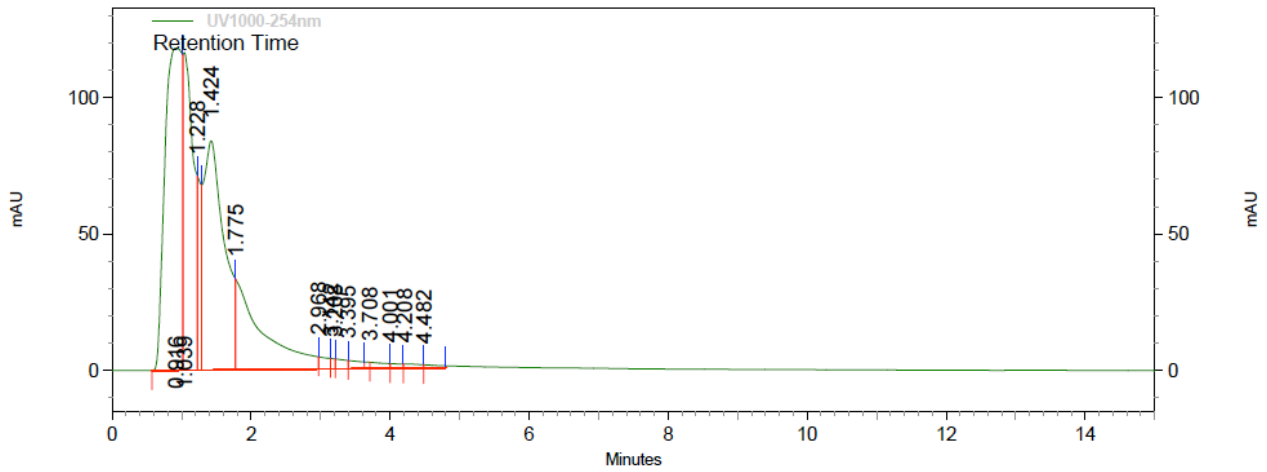

Figure 1: HPLC chromatogram (finger-printing) of methanol stem bark extract of *E. angolense* at λ 254 nm

3. RESULTS

3.1. Preliminary Phytochemical Screening

Methanol stem bark extract of *E. angolense* was found to contain tannins, flavonoids, alkaloids, terpenoids, cardiac glycosides and saponins (Table **1**).

3.2. HPLC Finger-Printing of Methanol Extract

The HPLC finger-printing of the extract was determined to identify the major constituents in the extract for the identification and quality control measures (Figure **1**).

3.3. Antimicrobial Activity

The methanol stem extract, fractions (pet ether, ethyl acetate and aqueous fractions) of *E. angolense* were active against the test organisms with different mean zones of inhibition (Tables **2** to **5**) and that of the reference antimicrobial agents recorded in Table **6**. The MIC of methanol stem extract of *E. angolense* against the Gram-positive bacteria was from 9.0 to 13 mg/mL and Gram-negative bacteria from 15 to 19 mg/mL (Table **7**). The time kill kinetics of the methanol extract of *E. angolense* against the test organisms were found to static (bacteriostatic and fungistatic) in nature (Figures **2** to **7**).

Table 2: Antimicrobial Activity (Agar Well Diffusion) of Methanol Extract *E. angolense* Against Test Organisms

Concentration (mg/mL)	Mean zones of inhibition (mm)					
	S. A	B. S	E. F	E. C	P. A	C. A
64	23.33± 0.33	19.00±0.33	24.33±0.33	18.67±0.33	18.00±0.58	20.33 ±0.33
32	21.00±0.58	16.33±0.33	21.67±0.88	16.00±0.33	16.00±0.58	18.00±0.58
16	18.00±0.58	15.33±0.33	18.67±0.33	14.67±0.33	14.25±0.25	15.75±0.25
8	15.33±0.33	13.33±0.33	16.67±0.33	13.33±0.33	12.50±0.50	14.50±0.50
4	13.33± 0.33	12.33±0.33	13.67±0.33	11.67±0.33	0.0	12.75±0.25
2	12.00± 0.0	11.33±0.33	11.67±0.33	0.0	0.0	0.0

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination. S.A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

Table 3: Antimicrobial Activity (Agar Well Diffusion) of Pet Ether Fraction of *E. angolense* Against Test Organisms

Concentration (mg/mL)	Mean zones of inhibition (mm)	
	S. A	C. A
100	17.67±0.33	16.00±0.33
25	15.00±0.58	14.33±0.33

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination. S.A: *Staphylococcus aureus*; C.A: *Candida albicans*.

Table 4: Antimicrobial Activity (Agar Well Diffusion) of Ethyl Acetate Fraction of *E. angolense* Against Test Organisms

Concentration (mg/mL)	Mean zones of inhibition(mm)					
	S. A	B. S	E. F	E. C	P. A	C. A
100	16.67±0.88	16.67±0.33	19.67±0.88	18.33±0.33	16.00±0.58	17.00±0.58
50	15.33±0.68	14.67±0.33	18.33±0.33	17.33±0.33	15.67±0.33	16.33±0.33
25	14.67±0.33	12.67±0.33	17.00±0.58	15.67±0.33	14.33±0.33	15.33±0.33
12.5	13.33±0.33	0.0	15.33±0.33	13.00±0.58	12.00±0.58	0.0
6.25	11.33±0.33	0.0	13.33±0.33	0.0	0.0	0.0
3.125	0.0	0.0	12.00±0.58	0.0	0.0	0.0

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.S.A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

Table 5: Antimicrobial Activity (Agar Well Diffusion) of Aqueous Fraction of *E. angolense* Against Test Organisms

Concentration (mg/mL)	Mean zones of inhibition(mm)					
	S. A	B. S	E. F	E. C	P. A	C. A
100	22.18±0.73	19.67±0.73	23.83±0.92	18.33±0.93	17.50±0.76	19.17±0.60
50	20.33±0.33	18.33±0.33	21.33±0.73	17.50±0.29	17.50±0.50	18.33±0.33
25	19.18±0.60	17.33±0.60	19.83±0.18	17.50±0.50	15.33±0.33	16.33±0.33
12.5	16.50±0.50	13.50±0.29	18.50±0.29	15.50±0.29	13.33±0.33	15.17±0.44
6.25	15.33±0.88	13.33±0.33	15.50±0.87	11.71±0.44	11.71±0.71	13.33±0.33
3.125	13.33±0.33	11.17±0.44	13.18±0.44	0.0	0.0	11.17±0.44

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination. S.: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

Table 6: Antimicrobial Activity (Agar Well Diffusion) of Reference Antibiotics Against Test Organisms

Concentration (mg/mL)	Mean zones of inhibition (mm)					
	S. A	B. S	E. F	E. C	P. A	C. A
Ciprofloxacin (0.01)	28.67±0.58	21.67±0.88	23.67±0.58	34.00±0.58	29.68±0.88	nd
Ketoconazole (1)	nd	nd	nd	nd	nd	28.33±0.33

Diameter of well = 10 mm; nd = not determined; values for the zone of inhibition are the mean of three independent determination. .A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

Table 7: Minimum Inhibitory Concentration (MIC) of the Methanol Stem Bark Extract of *E. angoense* and Reference Antibiotics Against Test Organisms

Antimicrobial agent	Minimum inhibitory concentration (mg/mL)					
	S. A	B. S	E. F	E. C	P. A	C. A
Methanol extract	11.0	13.0	9.0	17.0	19.0	15.0
Ciprofloxacin	0.04	0.02	0.03	0.0025	0.08	nd
Ketoconazole	nd	nd	nd	Nd	nd	0.0025

nd= not determined, .A: Staphylococcus aureus; B.S: Bacillus subtilis; E.F: Enterococcus faecalis; E.C: Escherichia coli; P.A: Pseudomonas aeruginosa; C.A: Candida albicans.

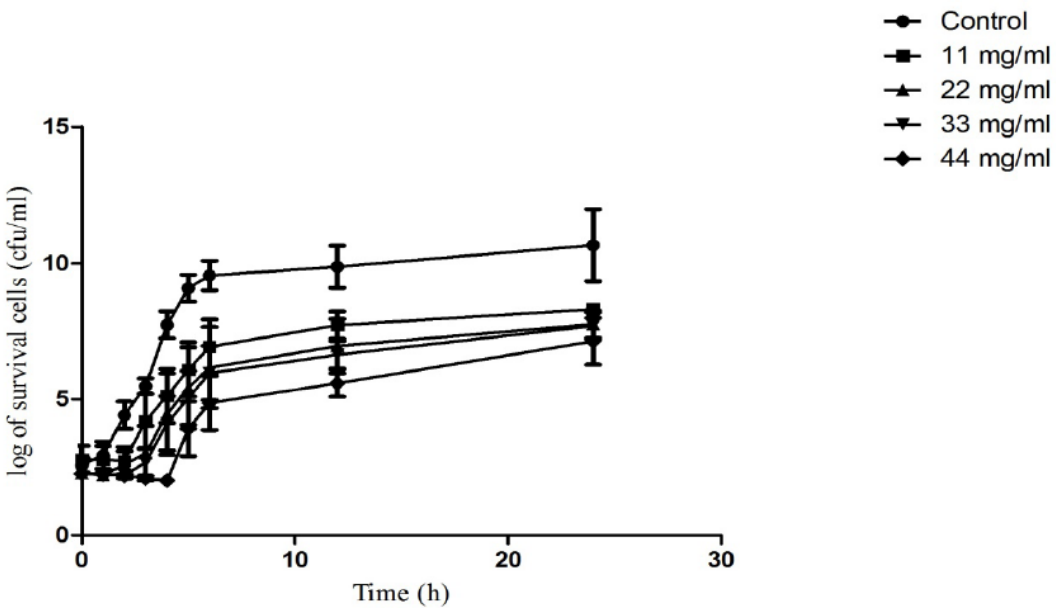

Figure 2: Survival of *S. aureus* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *S. aureus* without the methanol extract.

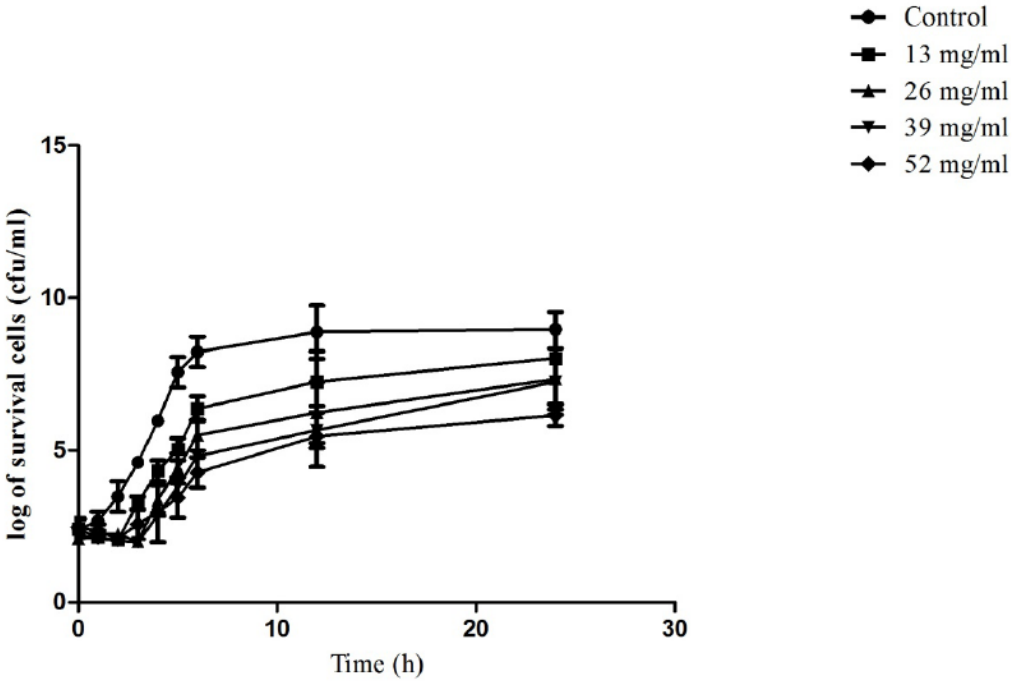

Figure 3: Survival of *B. subtilis* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *B. subtilis* without the methanol extract.

4. DISCUSSION

The phytochemical screening of the methanol extract revealed the presence of tannins, flavonoids, terpenoids, alkaloids, cardiac glycosides and saponins. Each of this group of compounds has been reported to possess antimicrobial activity [20] and exert their effects by affecting the cell membrane integrity of the bacteria [21, 22].

The methanol extract exhibited a broad spectrum of activity against the test bacteria. This antimicrobial of the extract and fractions may be due to the presence of the phytochemical constituents found in them. Gram-positive bacteria were found to be more susceptible to the crude extract than the Gram-negative bacteria. The difference in sensitivity patterns could be as a result of the morphological differences between these two types of bacteria [23]. The Gram-positive bacteria have only

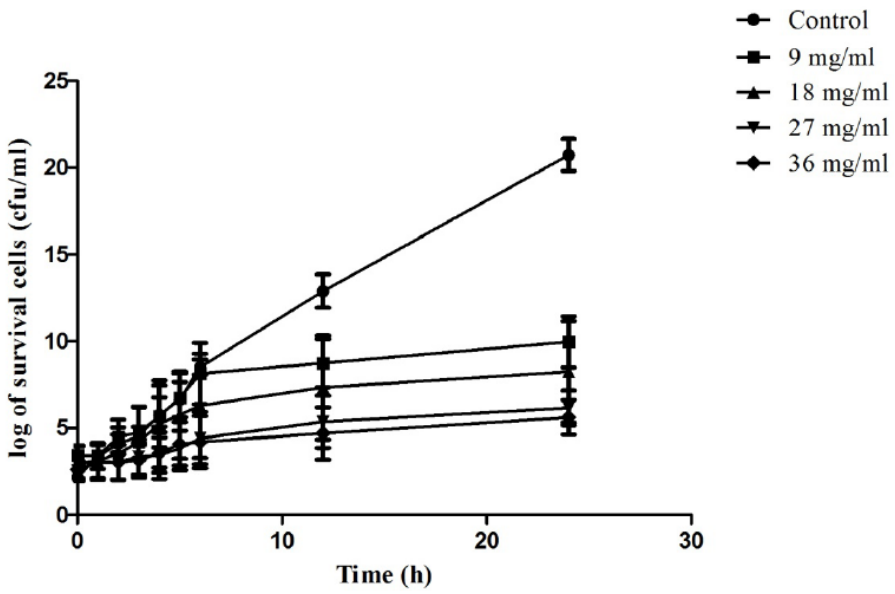

Figure 4: Survival of *E. faecalis* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *E. faecalis* without the methanol extract.

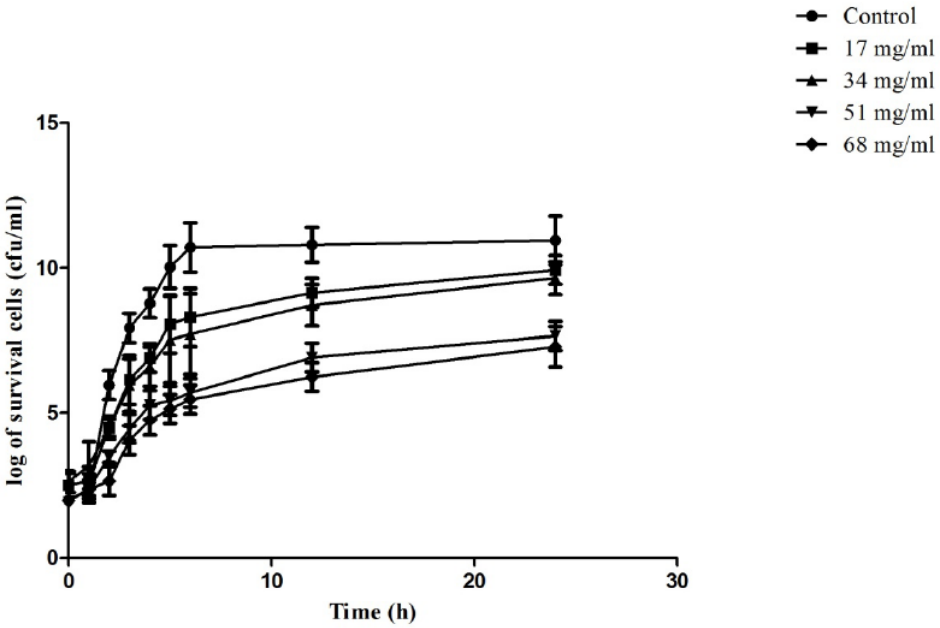

Figure 5: Survival of *E. coli* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *E. coli* without the methanol extract.

an outer peptidoglycan layer which is not an effective barrier against antimicrobial agents. Gram-negative bacteria on the other hand do possess an outer phospholipidic membrane which carries the structural lipopolysaccharide components, hence making the cell wall complex and impermeable to antimicrobial chemical substances [23].

The solvent fractions of the methanol extract exhibited varying antimicrobial activity with the aqueous fraction having the highest zone of inhibition (Table **5**) followed by the ethyl acetate fraction (Table **4**) whilst the pet ether fraction gave the least activity (Table **3**). In comparing the antimicrobial activities of the fractions to the crude extract (Table **3.2**), it was observed that the extract exhibited a higher activity against all test organisms as compared to the individual fractions. This may explain the synergistic effect of the bioactive compounds present in the crude extracts. The bioactive compounds or principles may be present in the polar fractions (aqueous and ethyl acetate fractions).

From the time kill kinetics, bactericidal or fungicidal activity could not be established even at concentrations

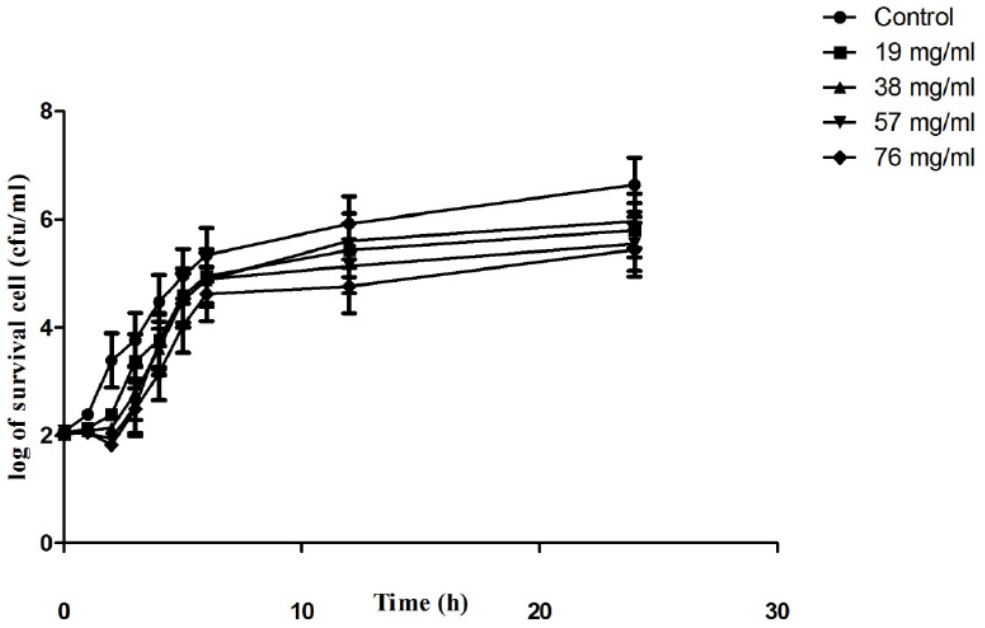

Figure 6: Survival of *P. aureginosa* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *P. aureginosa* without the methanol extract.

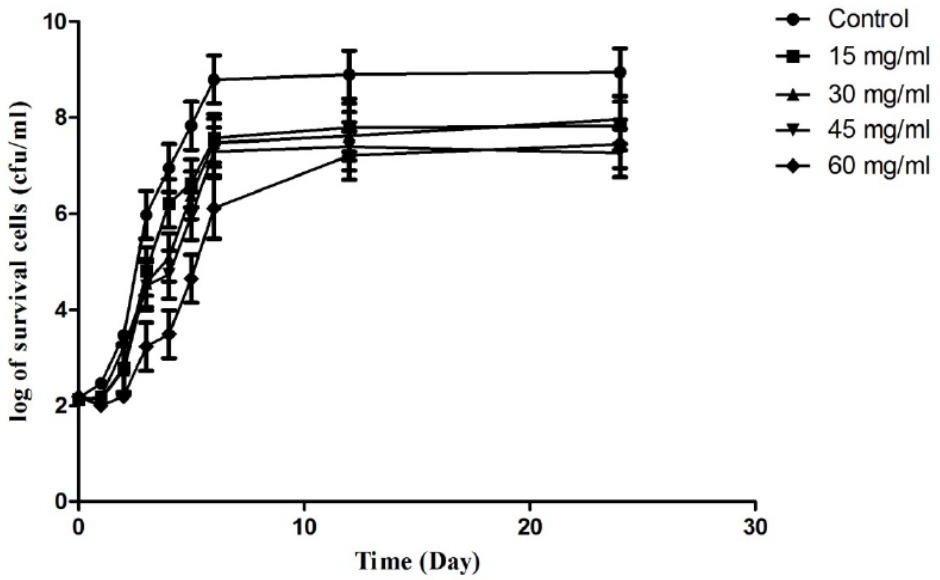

Figure 7: Survival of *C. albicans* in extract at different concentrations of methanol extract of *E. angolense* within 24 h. Control: Broth culture of *C. albicans* without the methanol extract.

as high as 150 mg/mL and this indicates that extract acts by static means.Research carried out by Fabry *et al*., [24] has shown that extracts having MIC values below 8 mg/mL possess some high antimicrobial activity. The MIC of the crude extract found in the range of 9.0 to 19.0 mg/mL and this may explain its bacteriostatic or fungistatic activity against the test organisms. Low antimicrobial activity could be associated with either the presence of either small amounts of potent active compounds or agents, or large amounts of less potent compounds/agent in the methanol extract of *E. angolense*.

From the time kill studies; although there were inhibitory effects exerted by the methanol stem bark extract against the test organisms as compared to the control, it was also observed that from the 3rd to the 24th h, there was a gradual increase in the level of surviving organisms (Figures **1** to **6**). The increase in surviving cells could have resulted from resistance built by the organisms due to their ability to adapt and metabolize the extract, hence diminishing its potency with time as result of its bacteriostatic nature. Further isolation and structural elucidation of the bioactive compounds would be performed.

CONCLUSION

The antimicrobial activity of *E. anglense* may justify the ethno-medicinal uses of plant for treatment of microbial infections. There is a need to isolate and characterize the active principle(s) responsible for the antimicrobial activity and possibly their mechanism of action.

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CONFLICT OF INTEREST

Authors declare that they have no competing interests.

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