Original Research Article

Antibacterial activity of bioflavonoid from fruit pulps of *Acacia nilotica* Willd ex Delile.

Abstract

Emergence of multi-drug resistance in bacteria has led to call for research and development of new leads as antibiotics from medicinal plants. *Acacia nilotica* (Linn) is a plant of multipurpose medicinal uses, three bioactive flavonoids (methyl gallate, gallic acid and catechin) were isolated from its fruit pulps through a bioassay guided fractionation technique and characterized based on High Performance Liquid Chromatography, Liquid Chromatography-Mass Spectra and Nuclear Magnetic Resonance spectra. Antibacterial activity of these compounds was determined by microplate tetrazolium dye assay of broth microdilution technique against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and clinical isolates of *Salmonella typhi, Klebsiella pneumonia, Candida albicans* and *Bacillus subtilis*. Catechin, methyl gallate and gallic acid at 19.5, 39 and 39 µg/ml respectively caused a significant bio-reduction in cells of test organisms. Time kill kinetic study of the extract shows that there was percentage of growth reduction in test organisms at 2, 4, 6, 8 and 12 hrs of contact. The extent and rate of killing of the organism by the extract at 2 x MIC followed the same trend as rate of killing was time dependent. Antibacterial effects of these compounds are within the breakpoint of control drug chloramphenicol and could serve as leads in new drug development.

Keywords: *Acacia nilotica*, antibacterial, catechin, methyl gallate and gallic acid, bioactive compound, high through put techniques.

1.0 Introduction

Acacia nilotica Willd. Ex Delile is commonly known as Gum Arabic tree, Egyptian mimosa, Egyptian thorn, red thorn, Babool, babul in Indian [1]. It is recognized by the following names: Acacia, Acacia Arabica, Babhul – Hindi and Nepalese, Babool Baum – German etc [Steve, 2004]. It has naturalized in several countries where it has been introduced as a medicinal, forage and fuel wood plant [2; 1]. It is proverbial, medium sized tree and is broadly scattered in tropical and subtropical countries. It has an inspiring range of medicinal uses with potential antioxidant activity. It contains a profile of a variety of bioactive components such as gallic acid, (+)-catechin and methyl gallate. It also contains L-arabinose, catechol, galactan, galactoaraban, galactose, N-acetyl djenkolic acid, sulphoxides and pentosan. The seeds contain crude protein (18.6%), ether extract (4.4%), fiber (10.1%), nitrogen-free extract (61.2%), ash (5.7%) and silica (0.44%); phosphorus (0.29%) and calcium (0.90%) of dry matter (17%) [3]. Phytochemical analysis of stem bark of *A. nilotica* showed the presence of carbohydrates, saponins, tannins, and cardiac glycosides [4].

As a multipurpose medicinal plants, leaves, flowers, seeds, roots, fruits and gum act as anti-tuberculosis [5], anti-hypertensive and anti-spasmodic, , antibacterial and antifungal [6], antiplasmodial [7], and antioxidant [8, 9, 10]. Toxicologically, *A. nilotica*, at 2 and 8% levels, has a low toxicity potential [11]. In a survey of potentially allergenic plants in Pondicherry, it was reported likely to cause pollen allergy [12]. *A. nilotica* has a wealth of medicinal uses for stomach upset and pain, the bark is chewed to protect against scurvy, an infusion is taken as therapy for dysentery and diarrhea [13]. In Nigeria, it is one of the customary drugs for treating diarrhea and it has been authenticated to have an antidiarrheal property [3] and antimicrobial activity against neuro-pathogenic *E. coli* K1, MRSA and *Klebsiella pneumonia* [14].

This study describes the inhibitory effect of bioactive molecules of *A. nilotica* as leads for drug development against pathogenic organisms using tetrazolium salts reduction as indicators of cell inhibition.

2.0 Methodology

2.1 Chemicals/Reagents/equipment: Tetrazolium dye, Nutrient broth No 1, Muller Hinton Agar from Fluka Analytical, Sigma-Aldrich Co., USA; Nutrient Agar from HIMedia Laboratories Pvt Ltd., India; chloramphenicol disk (HIMedia Laboratories Pvt. Ltd, India), Uv/vis spectrophotometer (Jenway 6405, Britain), nuclear magnetic resonance (300 MHz, Varian machine), HLPC and LCMS.

2.2 Extract preparation and isolation: The pulp of *A. nilotica* fruit was collected from Suleja, Niger state, Nigeria, identified at Herbarium Unit of National Institute for Pharmaceutical Research and Development, Abuja, Nigeria and herbarium specimen was deposited. This part was air dried to constant weight at room temperature and pulverized. Extraction of both polar and non-polar components of the plants using 70% aqueous methanol was carried out. One hundred grams of dried plant part was soaked in 70% aqueous methanol (1000 ml) for 24 hrs. The extract was filtered, concentrated on rotary evaporator at 40°C and freeze dried. A portion of the crude extract was used to determine the preliminary antibacterial effect of the plant, while the other portion was subjected to bioassay guided isolation of the bioactive molecules (Figure 1).

Fractionation of the crude extract of *A. nilotica* (seeds) was carried out according to [15]. Activated silica gel (50 g) was packed by a wet method into a column. The extract (2.6g) which was absorbed on silica gel (60 – 120 mesh) and dried was loaded on the column. Gradient elusion was performed with 100 ml of each mobile phase mixture in a series. The elusion was

performed in a flash chromatography. The mobile phase consisted of hexane, ethyl acetate, methanol and water, starting from 100% hexane and 10% increment in the next polar component. The final elusion was performed with 70% methanol in water until the column appeared exhausted with a sign of colourless silica gel. The eluates were monitored by thin layer chromatography (TLC) using normal phase pre-coated silica gel K5 TLC plates. The TLC mobile phase consists of a mixture of ethyl acetate: hexane (7:3). The eluates were combined based on the similarity of TLC fingerprint to give six fractions labelled as FR1- FR6. The pooled fractions (0.8 g) were absorbed on Merck – Kiesegel and introduced into column packed with the same absorbent. It was eluted with petroleum either and an increasing gradient of ethyl acetate. A total of 6 fractions of 100 ml each were collected. Fractions with same similarity (2-4) were pooled together into 3 portions viz; P1, P2 and P3 based on their TLC profile (Figure 2). On staining with ferric chloride solution showed blue black colouration which is an indication of phenolic compounds. These fractions were purified further by HPLC technique by separating on a reverse phase column, eluting with a gradient of acetonitrile 5% - 98% in water for a period of 40 minutes. Some 100 mg of extract was dissolved in 1 ml of methanol, and water was added gradually to the point of precipitation before injecting on the HPLC reversed phase (C-18) column. Fractions were collected at intervals of one minute. The structural elucidation of the compounds was done using Nuclear Magnetic Resonance (300 MHZ, Varian machine) and LC-MS techniques. [2008]. The pure fractions from TLC analysis were dissolved in deuterated methanol (CD_3OD) and transferred to NMR tube for measurement of proton (^1H) and ^{13}C (carbon 13). Masses were acquired using liquid chromatography- Mass Spec (LC-MS).

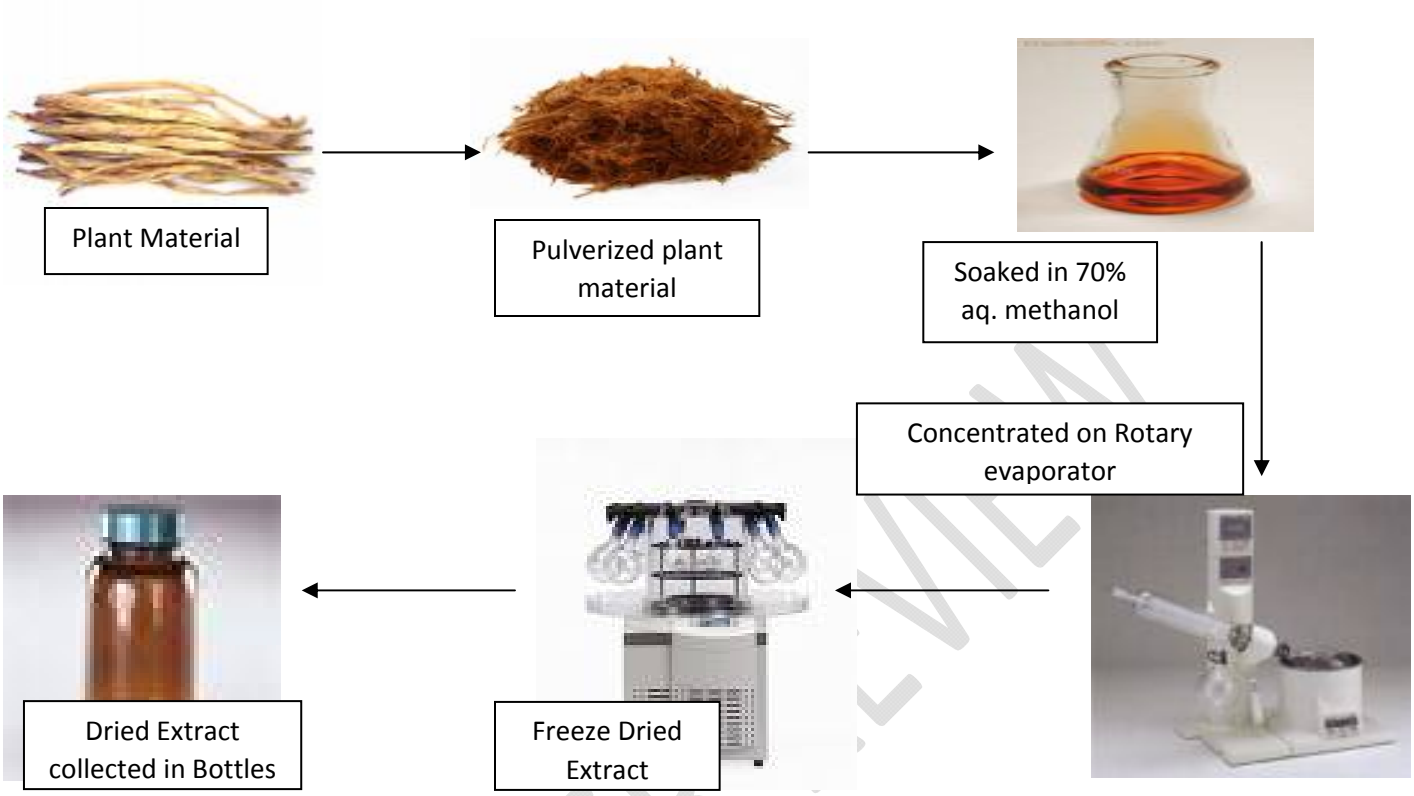


Figure.1. Extraction procedure of fruit pulps of *A. nilotica*

2.3 Phytochemical analysis: Phytochemical analysis of fruit pulp of *A. nilotica* was carried out according to Trease and Evans [16]. Metabolites tested for include alkaloid, saponins, tannins, anthraquinone, cardiac glycosides and flavonoids.

2.4 Antibacterial activity of the crude extract

2.4.1 Extract solution preparation: The extract was dissolved in dimethylsulphoxide (DMSO) and further diluted to 40 mg/ml concentration. The extract was sterile-filtered with 0.22 µm syringe filter (Fisher brand). From this, a 2 mg/ml concentration of extract in agar was prepared by dispensing 1ml of the extract solution (40 mg/ml) into 19 ml of molten Mueller Hinton Agar, maintained in water bath at 45°C. The molten agar/extract was poured into sterile Petri dish, allowed to gel and dried to remove moisture. Overnight broth cultures of the test organisms viz;

S. aureus (ATCC 28923), *P. aeruginosa* (ATCC 27853), *E. coli (ATCC* 25922*)* and clinical isolates of *S. typhi, K. pneumonia, C. albicans and B. subtilis* were standardized by diluting to 10^7 cfu/ml using a uv/vis-spectrophotometer as described by [17]. Two to three colonies of 20 hr growth on Mueller Hinton agar of the organisms to be studied were suspended on 50 ml pre-warmed (37°C) Mueller Hinton broth. The suspension was incubated overnight at 37° C, diluted 1/2500 in the same pre-warmed medium and incubated in water bath with agitation (50 rpm). The optical density of the culture was monitored at 450 nm until absorbance of 0.1 was reached (equivalent 2.5-3.0 x 10^7 cfu/ml for *E. coli* and *P. aeruginosa*, 1.8-2.0 x 10^7 for *S. aureus* and *B. subtilis)*. The agar containing extract was inoculated by dropping 50 µl of standardized organism on the surface of the agar, allowed to stand for 2 hrs. The plate was incubated for 24 hrs at 37^oC. The experiment was done in duplicate. Control plates containing 30 µg/ml concentration of chloramphenicol was used as drug control to confirm the susceptibility of the organisms. Agar containing solution of the extract served as extract sterility control. Post incubation, the presence or absence of growth of colonies of test organisms on agar plates indicated positive or negative results of the crude extract.

2.4.2 Minimum inhibitory concentration of molecules from *Acacia nilotica* (P1, P2 and P3)

The minimum inhibitory concentration of molecules of *A. nilotica* was determined by broth microdilution method according to British Society of Antimicrobial Susceptibility [18] recommendation with a slight modification using tetrazolium colorimetric assay as growth/inhibition indicator [19; 20]. Ten milligrams of each molecule was dissolved in Muller Hinton broth. 100 µl of each of the solutions was transferred to the first column of micro well plate, from where 50 µl was transferred to 2^{nd} well already containing 50 µl Muller Hinton broth,

mixed thoroughly and the procedure was repeated through to well 11 where 50 µl was discarded. All the wells were inoculated with 50 µl of overnight diluted cultures of each of *S. aureus, Pseudomonas aeruginosa, E. coli, S. typhi, K. pneumonia, C. albicans and B. subtilis* prepared as described above. The plates were incubated for 24 hrs at 37°C. Post incubation the plates were stained with tetrazolium dye; 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium (MTT). MICs were defined as lowest concentration of antimicrobial agents that red formazan of MTT was not observed.

2.4.3 Time kill Kinetic antibacterial study of fruit extract of *Acacia nilotica*

One hundred milligram per ml concentration of the crude extract was prepared in sterile water and diluted to 2 mg/ml concentration (being 2 X mic of the crude extract) filtered by centrifuging for 10 mins at 4500 rpm to remove woody sediments. The filtrate was inoculated with 10^5 cfu/ml of *S. aureus* (ATCC 28923) and *E. coli* (ATCC 25922) and incubated at 37°C, percentage growth decrease was measured at 2 hrs interval. Exactly 0.5 ml of each suspension was withdrawn at the appropriate time intervals and transferred to 4.5 mL nutrient broth recovery medium containing 3% Tween 80 to neutralize the carry-over effects of the antimicrobial compounds from the test suspensions. The suspension was shaken properly then serially diluted in sterile physiological saline. Exactly 0.5 mL of the final dilution of the test organism was transferred into pre-sterile Nutrient agar at 45ºC and plated out. The plates were allowed to set and incubated upside down at 37°C for 72 h. Optical density of growing culture in extract solution was measured using uv-spectrophotometer (Jenway 6405 uv/vis, UK) at 520 nm to determine decrease in cells growth. Control experiment which was set up without the inclusion of antimicrobial agent (i.e. excluding the crude extract). Viable counts were made in triplicates for each sample. Depression in the viable counts indicated killing by the antimicrobial agent.

3.0 Results and Discussion

Phytochemical analysis of fruit pulp of *A. nilotica* showed that the plant contains alkaloid, saponins, tannins (phlobatannins), anthraquinone and flavonoids. The presence of these phytochemicals could be responsible for the observed activity. For instance, tannins therapeutically have antiseptic properties and their precipitating quality is used in detecting gelatin, proteins and alkaloids [21]. Flavonoids and phenolic compounds are plants metabolites with at least one hydroxyl group [22] and are often found effective *in vitro* as antimicrobial substance against a wide array of microorganisms [23]. The presence of these phytochemicals could be responsible for the antibacterial activity recorded in this study.

Bioassay guided fractionation of the crude extract of fruit pulp of A. *nilotica* yielded 3 distinct spots on TLC (Fig. 2). The compounds were analyzed on Liquid Chromatography- Mass Spectrometer (LCMS) for purity and mass. Three compounds were isolated from the fractions by the preparative. The pure compounds were fractions 5, 9 and 12. Fractions in between were mixtures of these compounds as indicated by NMR spectra.

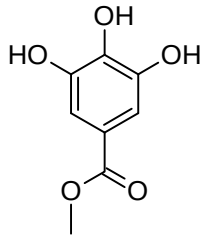
The ^{1}HMNR of fractions 5 revealed it to be methyl gallate, 9 to be gallic acid and 12 to be catechin through a library search [24, 15].

Figure 2. Thin Layer Chromatography plate of active fraction of *A. nilotica* (showing 3 distinct spots P1, P2 and P3).

3.1 Characterization of Isolated Compounds:

The first compound P1 (fraction 5) was a white solid with a melting point of 202-204 ℃ (Lit. 201-204 ℃). The ^{1}H NMR revealed the presence of aromatic protons at δ7.2, which integrated for two protons at positions 2 and 6 and that of methoxyl at δ3.3 which integrated for 3 protons at position 8. This indicated a simple aromatic system. The broad band was decoupled as; ^{1}H NMR: 7. 20 (2H, H-2 and H-6), 5.0 (OH), 3.3 (3H, CH_3), ^{13}C NMR: 167.9 (C-7), 146.6 (C-3), 146.6 (C-5), 139.4 (C-4), 122.2 (C-1), 110.3 (C-2), 110.3 (C-6), 52.5 (OMe).^{13}C NMR revealed the presence of 8 carbons at δ (167.9 for one C=O, 110.3 for two CH, 52.5 for OCH and four quaternary aromatic carbons at 122.2, 138.4, 146.6, 146.6) (Table 1). HRMS of this fraction gave 184.03717 amu, which was consistent with molecular formula $C_8H_8O_5$. A library search

revealed it to be methyl gallate (methyl 3, 4, 5-trihydroxybenzoate) [15] and [24]. The sample was also compared with an authentic sample by TLC and was confirmed to be methyl gallate: Methyl 3, 4, 5-trihydroxybenzoate (Fig 3). Molecular formula; $C_8H_8O_5$



Methyl gallate

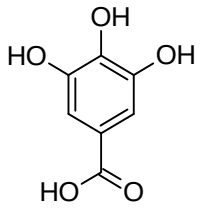
Figure 3. Chemical structure of methyl gallate with molecular formula $C_8H_8O_5$

Table 1. Carbon 13 and Proton NMR spectra of methyl gallate (P1) and gallic acid (P2)

		Compound P1		Compound P2	
Position	Group	^{13}C Chemical Shift (δ_C)	Proton Chemical Shift (δ_H)	^{13}C Chemical Shift (δ_C)	Proton Chemical Shift (δ_H)
1	C	122.2	-	122.2	-
2	CH	110.3	7.2	110.3	7.0
3	C-OH	146.6	6.8	146.6	4.789
4	C-OH	139.4	5.0	140.0	4.789
5	C-OH	146.6	6.8	146.6	4.789
6	CH	110.3	7.2	110.3	7.0
7	C=O	167.9	-		
8	OCH_3	52.5	3.30		

The second compound (P2) was a solid, light brownish in colour, m. p. = 237-239℃. ^{1}H NMR: 7.0 (2H, H-2 and H-6), 4.789 (OH), ^{13}C NMR: 170 (C-7), 146.6 (C-3), 146.6 (C-5), 139.4 (C-4), 122.2 (C-1), 110.3 (C-2), 110.3 (C-6).The ^{1}HNMR of this compound revealed the presence of aromatic protons at δ7.0, which integrated for two protons at positions 2 and 6 and the OH proton at position 7 at δ3.212. The broad band decoupled (Fig 4.) ^{13}C NMR revealed a total of 7 carbons at δ (170 for one C=O, 110.3 for two CH, and 4 quaternary aromatic carbons at 122.2, 138.4, 146.6, 146.6). These were almost identical with that of methyl gallate except for the

absence of the OCH_3 ^{13}C NMR peak. As a result this compound was subsequently identified as gallic acid [15]. Fig 4.



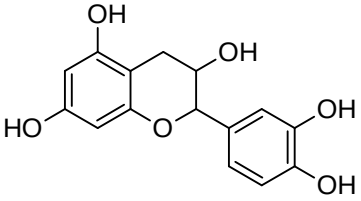
Gallic acid

Fig. 4 Chemical structure of gallic acid with molecular formula $C_7H_6O_5$

Table 2. Carbon 13 and Proton NMR spectra of Catechin (P3)

Position	Carbon 13 Chemical Shift (δ_C)	Proton Chemical Shift (δ_H)
1	-	
2	80.9	4.56
3	66.3	4.00
4	27.7	5.58
5	156.4	
6	93.9	
7		
8	95.1	
9		
1’		
2’	114	6.89
3’		
4’		
5’	115.1	6.79
6’	18.4	18.4

^{1}H-NMR spectra of the third compound (P3) showed peak at δ 4.56 (H-2, d), 4.01 (H-3, ddd), 2.54 (H-4, dd), 2.90 (H-4, dd), 5.87 (H-6, d), 6.01 (H-8, d) 6.89 (H-2’, d), 6.79 (H-5’, d) , 6.73 (H-6’, dd) and 8.00 (phenolic protons, m). ^{13}C-NMR, Carbon atoms showed peaks at δTMS 80.9 (C-2), 66.3 (C-3), 27.7 (C-4), 93.9 (C-6), 95.1 (C-8), 114.5 (C-2’), 115.1 (C-5’), 18.4 (C-6’) (Table 2). The NMR chemical shifts correlate well with those available in literature for catechin [25; 26]. The compound was identified as catechin (Fig 5).



Catechin

Fig 5. Chemical structure of catechin with molecular formula $C_{15}H_{14}O_6$

3.2 Antibacterial activity

Antibacterial activity of the crude fractions and compounds from fruit pulps of *A. nilotica* are as shown in table 3. The result shows that the crude extract was significantly active against all the test organisms but at varying degree of concentration. Minimum inhibitory concentration against *B. subtilis* and *S. aureus* were 500 and 750 ± 0.0 µg/ml respectively while the susceptibility of Gram negative organisms (*E. coli*, *P. aeruginosa, S. typhi, K. pneumonia*) and *C. albicans* were slightly higher at 1000 – 2000 µg/ml respectively). These concentrations are higher than the mic breakpoint for chloramphenicol (30 ± 0.1 µg/ml) the control drug. Hence the need for further purification of the extract to compound level for better activity. The fractions (F1-F6) produced by column chromatography with TLC finger printing yielded better minimum inhibitory concentrations (MICs) at 78 – 156 ± 0.0 µg/ml respectively. Fraction F1 had no antibacterial effect, fraction F5 had mic of 156 µg/ml against all the test organisms while F6 was only active against *S. aureus* and *B. subtilis* at 156 µg/ml. Fractions F2-F4 produced the best antibacterial effects at 78 µg/ml against Gram positive organisms and 156 µg/ml against Gram negative organisms. These fractions have a lot in common; TLC profile, positive test for phenolic and antibacterial effects. Microplate tetrazolium assay of isolated molecules against the

238 pathogenic organisms as shown in table 1, showed that methyl gallate, gallic acid at 39 µg/ml
239 and cathecin at 19.5 µg/ml concentration are significantly ($p<0.05$) more effective against *E.*
240 *coli, S aureus* and *B. subtilis* than against *P. aeruginosa, S. typhi* and *K. pneumonia* (78 µg/ml).
241 Catechin has shown to be the most effective of the molecules with MIC of 19±0.5 µg/ml against
242 *S. aureus* and *B subtilis* and *39* µg/ml against other Gram negative organisms.

243 This result shows that the purer the fractions the better the antibacterial activity. The use
244 of microplate tetrazolium assays to measure cell proliferation has increased exponentially since
245 their introduction [19]. Nevertheless, these assays do not actually measure the number of viable
246 cells in a culture or their growth but rather, an integrated set of enzyme activities that are related
247 in various ways to cell metabolism. They utilize the cofactor nicotinamide adenine dinucleotide
248 (NADH) and the dye3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium (MTT), and other
249 substrates like succinate and pyruvate which may also contribute to their reduction. Depending
250 on the particular dye chosen, reduction will be linked in various ways to cofactor/substrate
251 production, utilization and compartmentalization, and can be associated with the plasma
252 membrane, intracellular membranes, organelles and cytosol. Reduction can vary widely within
253 and between cell populations depending on the cell growth conditions, phase of cell growth and
254 stage of the cell cycle [20].

255 In the case of antibacterial activity of methyl gallate, the result is in agreement with a
256 similar study [27] that methyl gallate from the leaves of *Toona sureni* had antibacterial activity
257 against *E. coli, S. aureus* and *B. subtilis.* Other similar studies have reported that gallic acid by
258 mechanism of action in *P. aeruginosa, E. coli, S. aureus* and *Listeria monocytogens* led to
259 irreversible changes in membrane properties (charge, intra and extracellular permeability, and
260 physicochemical properties) through hydrophobicity changes, decrease of negative surface

charge, and occurrence of local rupture or pore formation in the cell membranes with consequent leakage of essential intracellular constituents [28]. Antibacterial activity of gallic acid of *A. nilotica* in this study corroborate the report that gallic acid inhibited the growth of *Campylobacter jejuni* and *E. coli* strains at15.63-250 µg/ml. [12].

Prolonged incubation of the microplates was used to determine the end point assay of the molecules as bacteriocidal. Wells with bacteriostatic effect at concentrations lower than 78 µg /ml showed tetrazolium reduction after 48 hours of incubation while bacteriocidal concentrations of 78 µg /ml upward remained colourless. This assay has been used to distinguish between dormant and metabolically active microbial cells (Tab. 3). The test organisms were able to reduce tetrazolium dyes in their electron transport chain, generating results within hours.

Molecules µg/ml	**MICROORGANISMS**						
	S. aureus	*P. aeruginosa*	*E. coli*	*B. subtilis*	*S. typhi*	*K. pneumonia*	*Ca*
Crude extract	750 ± 0.0	1000 ± 0.0	1000 ± 0.0	500 ±0.0	2000±0.0	2000±0.0	1000±0.0
F1	`NA	NA	NA	NA	NA	NA	NA
F2	78	156	156	78	156	156	156
F3	78	156	156	78	156	156	156
F4	78	156	156	78	156	156	156
F5	156	156	156	156	156	156	156
F6	156	NA	NA	156	NA	NA	156
Methyl gallate	39 ± 0.0	*78 ± 0.0	78 ± 0.0	39 ± 0.0	78	78	39
Gallic acid	39 ± 1.0	78 ± 0.0	78 ± 0.0	39 ± 0.0	78±0.0	78±0.0	39±0.0
Catechin	19.5 ± 0.0	39 ± 0.0	39 ± 0.0	19.5 ± 0.0	39±0	39±0.0	39±0.0

Chloramphenicol	30± 0.0	30 ± 0.00	30 ± 0.0	30±.0.0	30±0.0	30±0.0	

Table 3. Antibacterial activity of crude, fractions and compounds of fruit pulp of *A. nilotica*

Key: F= Fractions, NA = No activity, no growth inhibition

3.3 Time kill kinetic antibacterial of crude extract

The time kill kinetic antibacterial of crude extract against *S. aureus* and *E. coli* are as shown in Figs. 6 and 7. The percentage of growth reduction in *S. aureus* at 2, 4, 6, 8 and 12 hrs of contact were 52.2, 60.8, 70, 80 and 99.2% respectively. Percentages of *E.coli* growth reduction for each corresponding time were; 45.7, 51.7, 64.16 and 99.9% respectively. The extent and rate of killing of the organism by the extract at 2 x MIC followed the same trend as the concentrations of the extract increased with increase in contact time, the percentage of the organisms killed also increased.

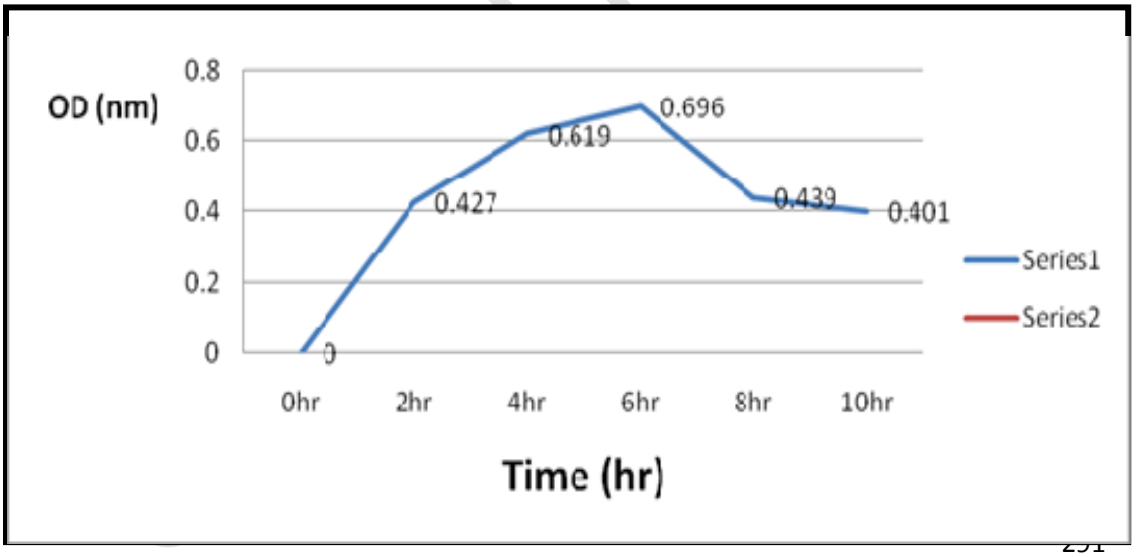


Fig. 6. Effect of extract of *A. nilotica* on the growth of *S. aureus* at different time

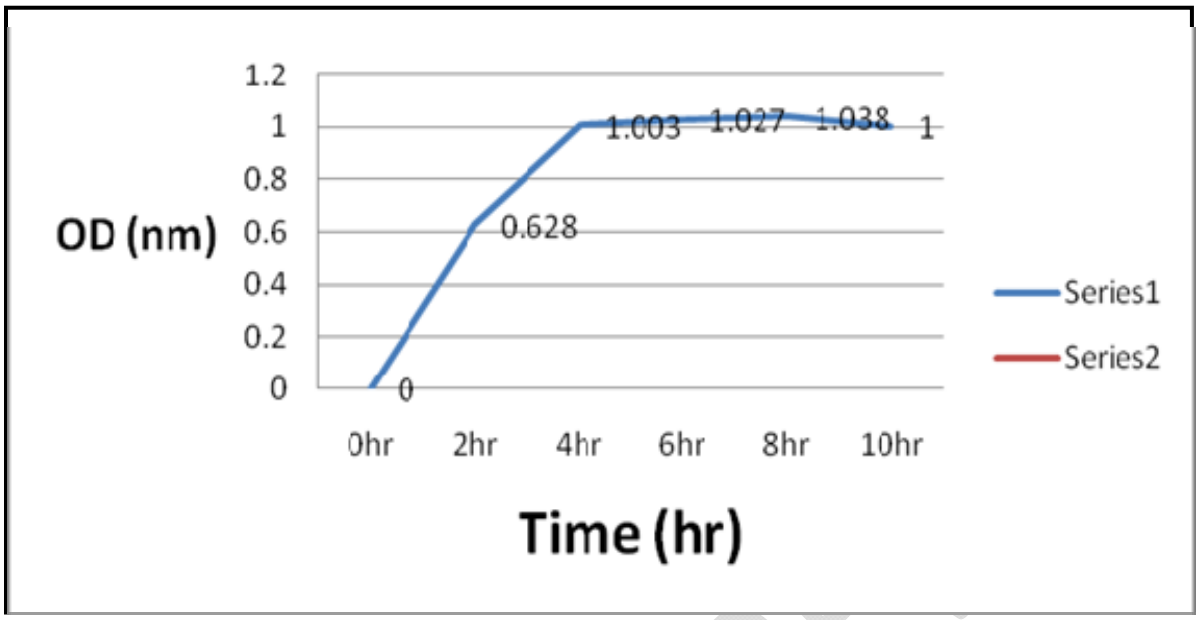


Fig 7. Effect of extract of *A. nilotica* on the growth of *E. coli* at different time

4.0 *Conclusion*

In conclusion, this study has demonstrated the antibacterial properties of bioflavonoids from fruit pulps of *A. nilotica*. The compounds isolated could serve as leads in the search for new and potent antibacterial compounds from indigenous medicinal plants.

Conflict of interest: Authors have declared that no competing interests exist

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