1

2 ***Original Research Article***

3

4 **Antibacterial activity of bioflavonoid from fruit pulps of *Acacia nilotica* Willd**

5 **Abstract**

6 The emergence of multi-drug resistance in bacteria has led to call for research and development

7 of new leads as antibiotics from medicinal plants. *Acacia nilotica* (Linn) is a plant of

8 multipurpose medicinal uses, three bioactive flavonoids (methyl gallate, gallic acid and catechin)

9 were isolated from its fruit pulps through a bioassay guided fractionation technique and

10 characterized based on High Performance Liquid Chromatography, Liquid Chromatography-

11 Mass Spectra and Nuclear Magnetic Resonance spectra. Antibacterial activity of these

12 compounds was determined by microplate tetrazolium dye assay of broth microdilution

13 technique against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and

14 clinical isolates of *Salmonella typhi, Klebsiella pneumonia, Candida albicans* and *Bacillus*

15 *subtilis*. Catechin, methyl gallate and gallic acid at 19.5, 39 and 39 µg/ml respectively caused a

16 significant bio-reduction in cells of test organisms. Time kill kinetic study of the extract shows

17 that there was percentage of growth reduction in test organisms at 2, 4, 6, 8 and 12 hrs of contact.

18 The extent and rate of killing of the organism by the extract at 2 x MIC followed the same trend

19 as rate of killing was time dependent. Antibacterial effects of these compounds are within the

20 breakpoint of control drug chloramphenicol and could serve as leads in new drug development.

21 Keywords: *Acacia nilotica*, antibacterial, catechin, methyl gallate and gallic acid, bioactive

22 compound, high through put techniques.

UNDER PEER REVIEW

23 **1.0 Introduction**

24 *Acacia nilotica* Willd. Ex Delile is commonly known as Gum Arabic tree, Egyptian
25 mimosa, Egyptian thorn, red thorn, Babool, babul in Indian [1]. It is recognized by the following
26 names: Acacia, Acacia Arabica, Babhul – Hindi and Nepalese, Babool Baum – German etc []. It
27 has naturalized in several countries where it has been introduced as a medicinal, forage and fuel
28 wood plant [2; 1]. It is proverbial, medium sized tree and is broadly scattered in tropical and
29 subtropical countries. It has an inspiring range of medicinal uses with potential antioxidant
30 activity. It contains a profile of a variety of bioactive components such as gallic acid, (+)-
31 catechin and methyl gallate. It also contains L-arabinose, catechol, galactan, galactoaraban,
32 galactose, N-acetyl djenkolic acid, sulphoxides and pentosan. The seeds contain crude protein
33 (18.6%), ether extract (4.4%), fiber (10.1%), nitrogen-free extract (61.2%), ash (5.7%) and silica
34 (0.44%); phosphorus (0.29%) and calcium (0.90%) of dry matter (17%) [3]. Phytochemical
35 analysis of stem bark of *A. nilotica* showed the presence of carbohydrates, saponins, tannins, and
36 cardiac glycosides [4].

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

UNDER PEER REVIEW

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

49 **2.0 Methodology**

50 **2.1 Chemicals/Reagents/equipment:** Tetrazolium dye, Nutrient broth No 1, Muller Hinton
51 Agar from Fluka Analytical, Sigma-Aldrich Co., USA; Nutrient Agar from HIMedia
52 Laboratories Pvt Ltd., India; chloramphenicol discs (REF OD 278R, HIMedia Laboratories Pvt.
53 Ltd, India), Uv/vis spectrophotometer (Jenway 6405, Britain), nuclear magnetic resonance (300
54 MHz, Varian machine), HLPC and LCMS.

55 **2.2 Extract preparation and isolation**: The pulp of *A. nilotica* fruit was collected from Suleja,
56 Niger state, Nigeria, identified at Herbarium Unit of National Institute for Pharmaceutical
57 Research and Development, Abuja, Nigeria and herbarium specimen was deposited. This part
58 was air dried to constant weight at room temperature and pulverized. Extraction of both polar
59 and non-polar components of the plants using 70% aqueous methanol was carried out. One
60 hundred grams of dried plant part was soaked in 70% aqueous methanol (1000 ml) for 24 hrs.
61 The extract was filtered, concentrated on rotary evaporator at 40°C and freeze dried. A portion of
62 the crude extract was used to determine the preliminary antibacterial effect of the plant, while the
63 other portion was subjected to bioassay guided isolation of the bioactive molecules (Figure 1).
64

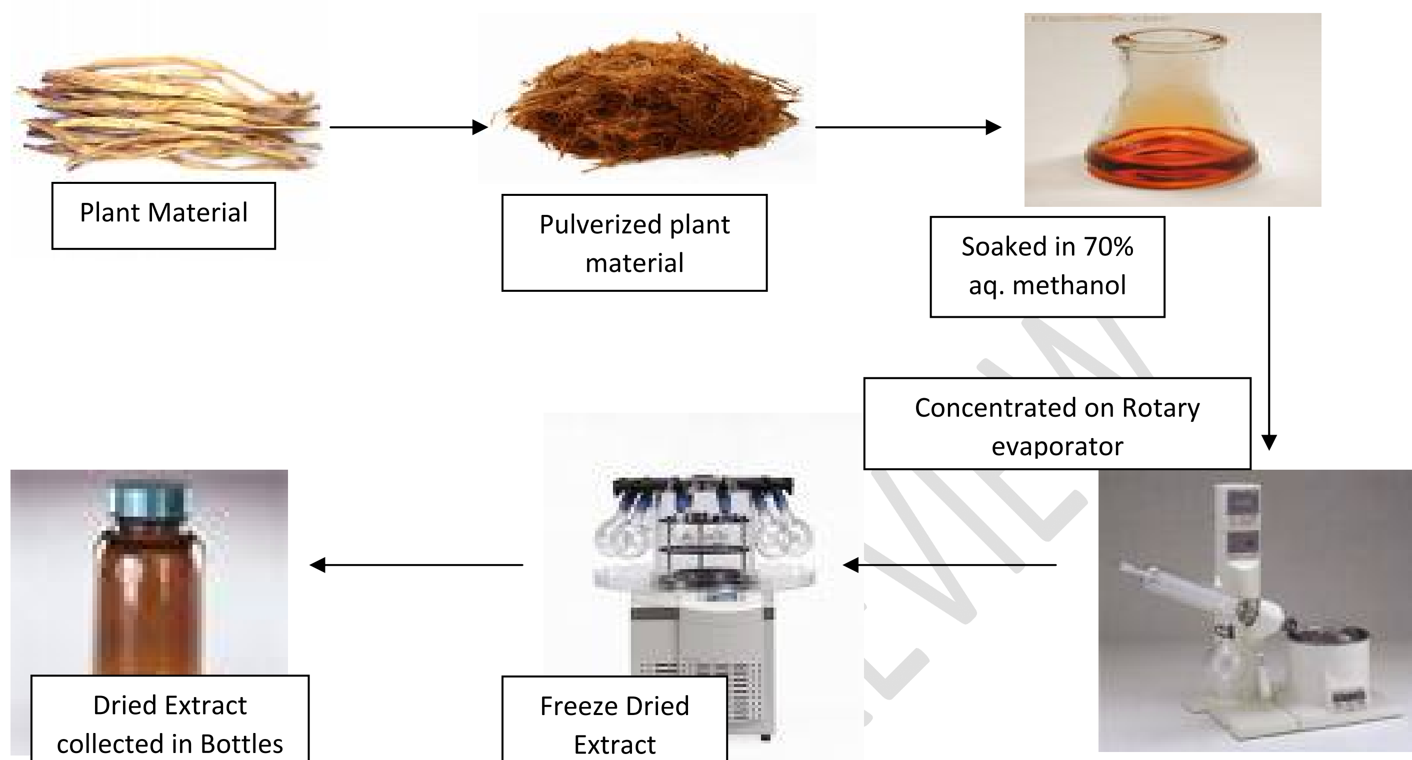
65 Fractionation of the crude extract of *A. nilotica* (seeds) was carried out according to [15].
66 Activated silica gel (50 g) was packed by a wet method into a column. The extract (2.6g) which
67 was absorbed on silica gel (60 – 120 mesh) and dried was loaded on the column. Gradient
68 elusion was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

UNDER PEER REVIEW

69 performed in a flash chromatography. The mobile phase consisted of hexane, ethyl acetate,
70 methanol and water, starting from 100% hexane and 10% increment in the next polar component.
71 The final elusion was performed with 70% methanol in water until the column appeared
72 exhausted with a sign of colourless silica gel. The eluates were monitored by thin layer
73 chromatography (TLC) using normal phase pre-coated silica gel K5 TLC plates. The TLC
74 mobile phase consists of a mixture of ethyl acetate: hexane (7:3). The eluates were combined
75 based on the similarity of TLC fingerprint to give six fractions labelled as FR1- FR6. The pooled
76 fractions (0.8 g) were absorbed on Merck – Kiesegel and introduced into column packed with the
77 same absorbent. It was eluted with petroleum either and an increasing gradient of ethyl acetate.
78 A total of 6 fractions of 100 ml each were collected. Fractions with same similarity (2-4) were
79 pooled together into 3 portions viz; P1, P2 and P3 based on their TLC profile (Figure 2). On
80 staining with ferric chloride solution showed blue black colouration which is an indication of
81 phenolic compounds. These fractions were purified further by HPLC technique by separating on
82 a reverse phase column, eluting with a gradient of acetonitrile 5% - 98% in water for a period of
83 40 minutes. Some 100 mg of extract was dissolved in 1 ml of methanol, and water was added
84 gradually to the point of precipitation before injecting on the HPLC reversed phase (C-18)
85 column. Fractions were collected at intervals of one minute. The structural elucidation of the
86 compounds was done using Nuclear Magnetic Resonance (300 MHZ, Varian machine) and LC-
87 MS techniques. [2008]. The pure fractions from TLC analysis were dissolved in deuterated
88 methanol (CD_3OD) and transferred to NMR tube for measurement of proton (^1H) and ^{13}C
89 (carbon 13). Masses were acquired using liquid chromatography- Mass Spec (LC-MS).
90

UNDER PEER REVIEW

91



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

93

94 **2.3 Phytochemical analysis**: Phytochemical analysis of fruit pulp of *A. nilotica* was carried out

95 according to Trease and Evans [16]. Metabolites tested for include alkaloid, saponins, tannins,

96 anthraquinone, cardiac glycosides and flavonoids.

97

98 **2.4 Antibacterial activity of the crude extract**

99 **2.4.1 Extract solution preparation**: The extract was dissolved in dimethylsulphoxide (DMSO)

100 and further diluted to 40 mg/ml concentration. The extract was sterile-filtered with 0.22 µm

101 syringe filter (Fisher brand). From this, a 2 mg/ml concentration of extract in agar was prepared

102 by dispensing 1ml of the extract solution (40 mg/ml) into 19 ml of molten Mueller Hinton Agar,

103 maintained in water bath at 45°C. The molten agar/extract was poured into sterile Petri dish,

104 allowed to gel and dried to remove moisture. Overnight broth cultures of the test organisms viz;

UNDER PEER REVIEW

105 *S. aureus* (ATCC 28923), *P. aeruginosa* (ATCC 27853), *E. coli (ATCC* 25922*)* and clinical
106 isolates of *S. typhi, K. pneumonia, C. albicans and B. subtilis* were standardized by diluting to
107 10^7 cfu/ml using a uv/vis-spectrophotometer as described by [17]. Two to three colonies of 20 hr
108 growth on Mueller Hinton agar of the organisms to be studied were suspended on 50 ml pre-
109 warmed (37°C) Mueller Hinton broth. The suspension was incubated overnight at 37° C, diluted
110 1/2500 in the same pre-warmed medium and incubated in water bath with agitation (50 rpm).
111 The optical density of the culture was monitored at 450 nm until absorbance of 0.1 was reached
112 (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.*
113 *subtilis)*. The agar containing extract was inoculated by dropping 50 µl of standardized organism
114 on the surface of the agar, allowed to stand for 2 hrs. The plate was incubated for 24 hrs at 37°C.
115 The experiment was done in duplicate. Control plates containing 30 µg/ml concentration of
116 chloramphenicol (prepared by dissolving 30 mg of chloramphenicol powder in 10 ml 10%
117 dimethyl sulfoxide (DMSO) and further diluted by taking 10 µl solution in 10 mL nutrient broth)
118 was used as drug control to confirm the susceptibility of the test organisms. Agar containing
119 solution of the extract served as extract sterility control. Post incubation, the presence or absence
120 of growth of colonies of test organisms on agar plates indicated positive or negative results of the
121 crude extract.
122
123 **2.4.2 Minimum inhibitory concentration of molecules from *Acacia nilotica* (P1, P2 and P3)**
124 The minimum inhibitory concentration of molecules of *A. nilotica* was determined by broth
125 microdilution method according to British Society of Antimicrobial Susceptibility [18]
126 recommendation with a slight modification using tetrazolium colorimetric assay as
127 growth/inhibition indicator [19; 20]. Ten milligrams of each molecule was dissolved in Muller

UNDER PEER REVIEW

128 Hinton broth. 100 µl of each of the solutions was transferred to the first column of micro well
129 plate, from where 50 µl was transferred to 2nd well already containing 50 µl Muller Hinton broth,
130 mixed thoroughly and the procedure was repeated through to well 11 where 50 µl was
131 discarded. All the wells were inoculated with 50 µl of overnight diluted cultures of each of *S.*
132 *aureus, Pseudomonas aeruginosa, E. coli, S. typhi, K. pneumonia, C. albicans and B. subtilis*
133 prepared as described above. The plates were incubated for 24 hrs at 37°C. Post incubation the
134 plates were stained with tetrazolium dye; 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium
135 (MTT). MICs were defined as lowest concentration of antimicrobial agents that red formazan of
136 MTT was not observed.

137 **2.4.3 Time kill Kinetic antibacterial study of fruit extract of *Acacia nilotica***

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

UNDER PEER REVIEW

151 of antimicrobial agent (i.e. excluding the crude extract). Viable counts were made in triplicates
152 for each sample. Depression in the viable counts indicated killing by the antimicrobial agent.
153 Statistical analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA)
154 software. The level of significance for all statistical tests was set at $p < 0.05$.
155
156

157 **3.0 Results and Discussion**

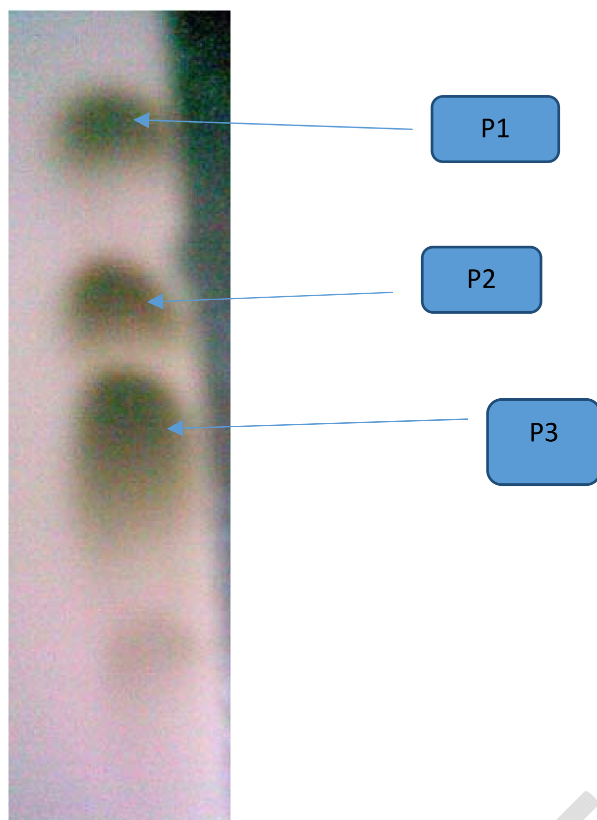
158 Phytochemical analysis of fruit pulp of *A. nilotica* showed that the plant contains
159 alkaloid, saponins, tannins (phlobatannins), anthraquinone and flavonoids. The presence of these
160 phytochemicals could be responsible for the observed activity. For instance, tannins
161 therapeutically have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

166 Bioassay guided fractionation of the crude extract of fruit pulp of A. *nilotica* yielded 3
167 distinct spots on TLC (Fig. 2). The compounds were analyzed on Liquid Chromatography- Mass
168 Spectrometer (LCMS) for purity and mass. Three compounds were isolated from the fractions by
169 the preparative. The pure compounds were fractions 5, 9 and 12. Fractions in between were
170 mixtures of these compounds as indicated by NMR spectra.
171

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

UNDER PEER REVIEW

174



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

177

178 **3.1 Characterization of Isolated Compounds:**

179 The first compound P1 (fraction 5) was a white solid with a melting point of 202-204 °C
180 (Lit. 201-204 °C). The ^{1}H NMR revealed the presence of aromatic protons at δ7.2, which
181 integrated for two protons at positions 2 and 6 and that of methoxyl at δ3.3 which integrated for
182 3 protons at position 8. This indicated a simple aromatic system. The broad band was decoupled
183 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
184 (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
185 revealed the presence of 8 carbons at δ (167.9 for one C=O, 110.3 for two CH, 52.5 for OCH and
186 four quaternary aromatic carbons at 122.2, 138.4, 146.6, 146.6) (Table 1). HRMS of this fraction
187 gave 184.03717 amu, which was consistent with molecular formula $C_8H_8O_5$. A library search

UNDER PEER REVIEW

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; $C_8H_8O_5$

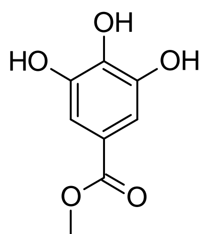


Figure 3. Chemical structure of methyl gallate with molecular formula $C_8H_8O_5$

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°C. ^{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

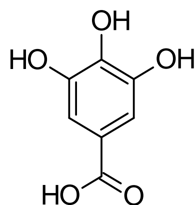
UNDER PEER REVIEW

205 absence of the OCH_3 ^{13}C NMR peak. As a result this compound was subsequently identified as

206 gallic acid [15]. Fig 4.

207

208



209 Gallic acid

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

211

212 **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

213

214

215 ^{1}H-NMR spectra of the third compound (P3) showed peak at δ 4.56 (H-2, d), 4.01 (H-3, ddd),

216 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

217 (H-6’, dd) and 8.00 (phenolic protons, m). ^{13}C-NMR, Carbon atoms showed peaks at δTMS 80.9

218 (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’)

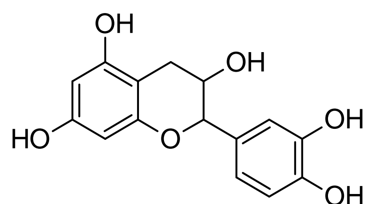
219 (Table 2). The NMR chemical shifts correlate well with those available in literature for catechin

220 [25; 26]. The compound was identified as catechin (Fig 5).

UNDER PEER REVIEW

221

222



223 Catechin

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

225

226

227 **3.2 Antibacterial activity**

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

UNDER PEER REVIEW

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

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

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

UNDER PEER REVIEW

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

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

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

Molecules µg/ml	**MICROORGANISMS**						
	S. aureus	*P. aeruginosa*	*E. coli*	*B. subtilis*	*S. typhi*	*K. pneumonia*	*C. albicans*
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

UNDER PEER REVIEW

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	

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.

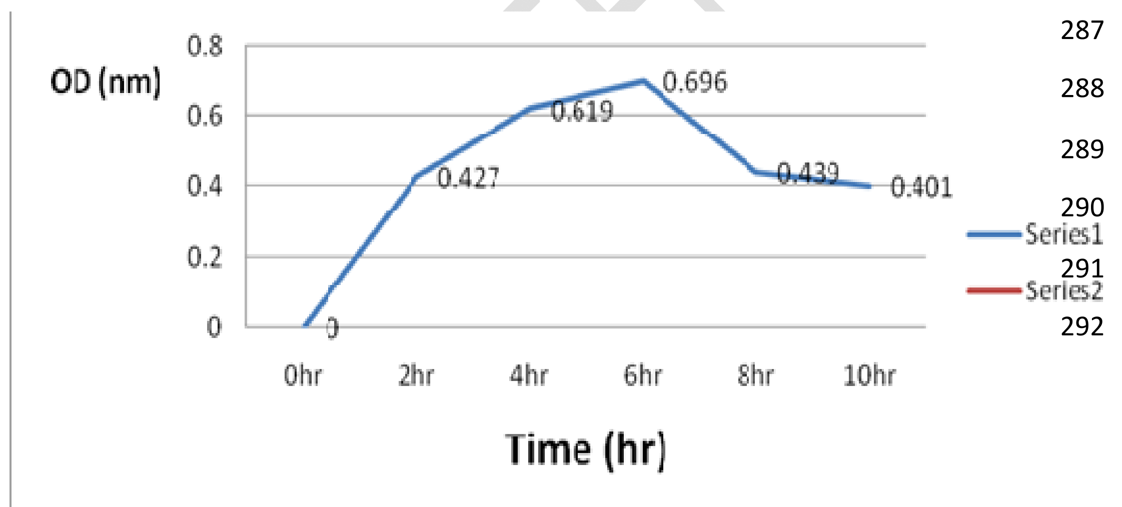
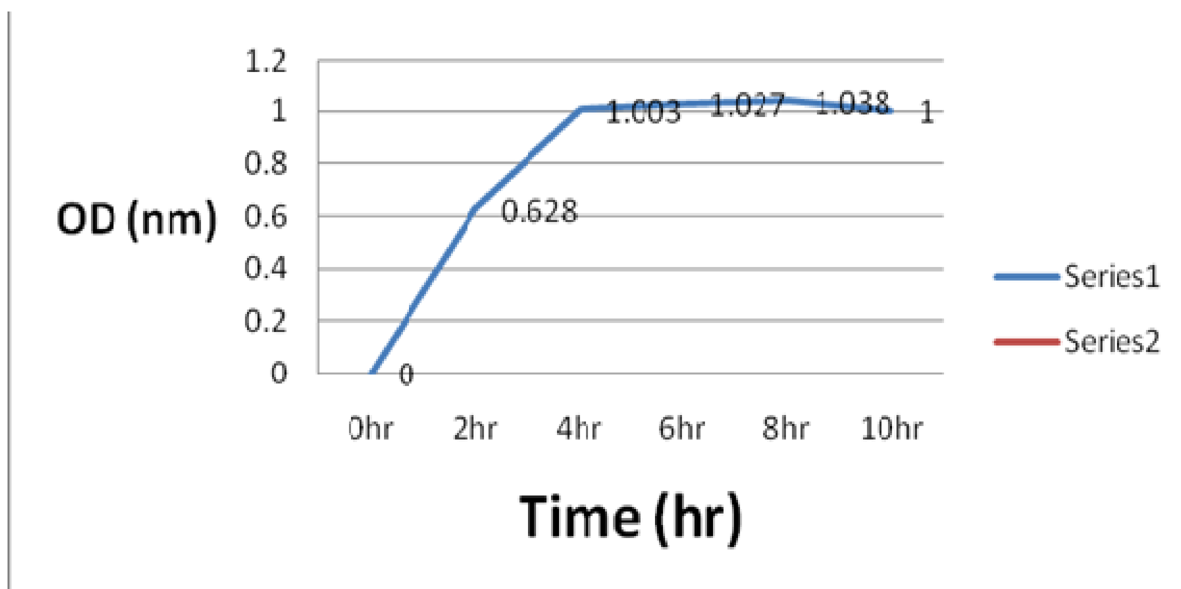


Fig. 6. Effect of extract of *A. nilotica* on the growth of *S. aureus* at different time

296



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

298

299

300

301 **4.0 *Conclusion***

302 In conclusion, this study has demonstrated the antibacterial properties of bioflavonoids

303 from fruit pulps of *A. nilotica*. Step by step bioassay guided isolation shows an improved

304 antibacterial effect from crude extract to pure compounds. The results of the minimum inhibitory

305 concentrations of the compounds showed that the compounds are active against all test

306 organisms, as seen the isolated compounds have antibacterial effect close to the MIC breakpoint

307 of the control drug, although methyl gallate and gallic acid have better activity than the crude

308 extract, catechin has shown to be the most active compound against all the test organisms. The

309 time kill antibacterial study was able to demonstrate time dependent effect of the crude extract

310 against the test organisms. Generally, the compounds isolated could serve as leads in the search

311 for new and potent antibacterial compounds from indigenous medicinal plants.

312

UNDER PEER REVIEW

313

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

315

316

317

318 References

319 *1.* Bennison JJ, Paterson RT (1994). The use of Trees by Livestock. *Acacia Production*
320 *Programme 1:160-164.*

321 2. Atif Ali, Akhtar Naveed, Barkat Ali Khan, Muhammad Shoaib Khan, Akhtar RasulShahiq-
322 UZ-Zaman, Nayab Khalid, Khalid Waseem, Tariq Mahmmod and Liaqat Ali (2012). Acacia
323 nilotica: A plant of multipurpose medicinal uses. Journal of Medicinal plants Research; Vol
324 6(9). Pp.1492-1496.

325 3. Ogbadoyi EO, Garba MH, Kabiru AY, Mann A, Okogun J (2011). Therapeutic evaluation of
326 Acacia nilotica (Linn) stem bark extract in experimental African trypanosomiasis.
327 *International Journal of Applied Research of Natural Products, 4(2):11-18.*

328 4. Sawant R. S., Godghate A. G., Sankpal S.A., S. A. Walaki1 and S. S. Kankanwadi (2014).
329 Phytochemical analysis of bark of Acacia nilotica. Asian Journal of Plant Science and
330 Research (Pelagia Research Library), 4(2):22-24.B.

331 5. Oladosu P, Isu NR, Ibrahim K, Okolo P and Oladepo DK (2013b). Time kill-kinetics
332 antibacterial study of *Acacia nilotica. African Journal of Microbiology Research, vol 7(46),*
333 *5248-5252.*

334

UNDER PEER REVIEW

335 6. Banso A (2009). Phyochemical and antibacterial investigation of bark extracts of Acacia
336 nilotica. Journal of Medicinal Plants Research., 3: 082-085
337 7. Jigam AA, Akanya HO, Dauda BEN, Okogun JO (2010). Polygalloytannin is effective
338 against Plasmodium berghei in mice. J. Med. Plants Res., 4(12): 1169-1175
339 *8.* Del WE (2009). In vitro evaluation of peroxyl radical scavenging capacity of water
340 extract/fractions of A. nilotica (L). African Journal of Biotechnology. 8(7) 1270-1272.
341 9. Amos S, Akah PA, Odukwe CJ, Gamaniel KS, Wambebe C (1999). The pharmacological
342 effect of an aqueous extract from Acacia nilotica seeds. Phytotherapy Research13: 683-685
343 *10.* Kalavaini T, Mathew L. (2010). Free radical scavenging activity from leaves of Acacia
344 nilotica (I) Wil. ex. Delile, an Indian medicinal tree. Food Chem. Toxicol., 48: 298-305.
345 11. Al-Mustafa ZH, Dafallah AA (2000). A study on the toxicology of *Acacia nilotica*. *Am. J.*
346 *China Med. 28(1): 123-129.*
347 12. Amrreta Sarjit, Yi Wang and Gary A Dykes (2014). Antimicrobial activity of gallic acid
348 against thermophilic *Campylobacter* is strain specific and associated with a loss of calcium
349 ions. Food Microbiology, Vol 46, 227-233
350 *13.* Misar A, Bhagat R, Mujumdar AM (2008). Antidiarrhoeal activity of Acacia nilotica Willd.
351 bark methanol extract. *Hindustan Antibiot Bull.; 49-50(1-4):14-20.*
352 14. Saba R, Muhammad F, Shahida H and Naveed Ak (2011). Antibacterial and Cytotoxicity of
353 Acacia nilotica Lam (Mimosaceae) Methanol extracts against extended spectrum Beta-
354 lactamase producing Escherichia coli and Klebsiella species. *Trop. J. Pharm. Res. 10*
355 *(6):785-791*
356 *15.* Orishadipe AT, Oladosu P, D'Ambroso M, Adesomoju AA, Ibrahim K, Barry CE 111 and
357 Okogun JI (2008). Isolation and characterization of 1, 2, 3, 4, 6- pentagalloyl glucose an

UNDER PEER REVIEW

358 antimycobacterial agent from the leaves of Entandrophragma angolense, *Journal of*
359 *Phytomedicine and Therapeutics 13; 16 – 22*.
360 *16.* Trease GE and Evans WC (2002). Pharmacognosy15th Edn., WB Sanders, London, ISBN:
361 8131200876, pp. 406
362 *17.* Dominguez, MC, Manuel de la Rosa, Victoria MB (2001). Application of spectrophotometric
363 method for the determination of post- antibiotic effect and comparism with viable counts in
364 agar. *Journal of Antimicrobial Chemotherapy, 47:391–398*
365 *18.* British Society of Antimicrobial Susceptibility (2005). Guidelines for the laboratory
366 diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA).
367 *Journal of antimicrobial chemotherapy; 56:1000-1018.*
368 *19.* Mozmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to
369 proliferation and cytotoxic assays. *Journal immunology Methods; 65: 55-63*
370 *20.* Marshall NJ, Goodwin CT and Holt SJ (1995). A critical assessment of micro culture
371 tetrazolium assays to measure cell growth and function. Growth regulation 5; 69-84
372
373 *21.* Adamczyk, J. Simon, V. Kitunen, S. Adamczyk, and A. Smolander (2017). Tannins and
374 Their Complex Interaction with Different Organic Nitrogen Compounds and Enzymes: Old
375 Paradigms versus Recent Advances. Chemistry Open (Reviews) 2017, 6, 610 – 614. DOI:
376 10.1002/open.201700113
377 22. *Duangjai Tungmunnithum, Areeya Thongboonyou, Apina Pholboon and Aujana Yangsabai*
378 *(2018). Flavonoids and other phenolic compoundsfrom Medicinal plants for Pharmaceutical*
379 *and Medical Aspects: An overview. Medicines (Basel), 5(3): 93. Doi: 10.3390/medicines*
380 *5030093*

UNDER PEER REVIEW

381 23. Bennett RN, Wallsgrove RM (1994). Secondary metabolites in plant defense mechanisms.
382 Phytotherapy Research, 127: 617-633
383 24. Pourchet, J Charles and Jacqlynn Benlinke (1993). *The Aldrich library of 1H and 13CNMR*
384 *(FT- Spectra). Vol. 111, Aldrich Chemical Co. Inc.*
385 25. Hye M A, M.A Taher, M.Y.Ali, M.U. Ali, and Shahed Zaman (2009). Isolation of (+)
386 catechin from Acacia catechu (Cutch tree) by Convenient Method*. J. Sci. Rese. 1(2): 300-*
387 *305.*
388 26. Kiehlmann E and A. S. Tracey (1986). Proton magnetic resonance spectra of catechin and
389 bromocatechin derivatives: C6' vs. C8-substitution. Can. J. Chem. 64: 1998-2005
390 27. Taufik M. Ekaprasada[#], Hazli Nurdin*, Sanusi Ibrahim*, Dachriyanus[1] (2005). Antibacterial
391 Activity of Methyl Gallate Isolated from the Leaves of Toona sureni. International Journal on
392 advanced Science Engineering Information Technology
393 28. Borges A, Ferreira C, Saavedra MJ, Simões M. (2013). Antibacterial activity and mode of
394 action of ferulic and gallic acids against pathogenic bacteria. Microbial Drug Resistant;
395 (4):256-65.

396

397

UNDER PEER REVIEW