Original Research Article

Antimicrobial and antioxidant properties of kaempferol-3-O-glucoside and 1-(4-Hydroxyphenyl)-3-phenylpropan-1-one isolated from the leaves of *Annona muricata* (Linn.)

ABSTRACT

Aim: To evaluate the antimicrobial and antioxidant activities of bioactive compounds isolated from *Annona muricata* (Linn.) leaf extract.

Study Design: *In vitro* antimicrobial assay of bioactive compounds isolated from solvent fractions of plant leaf extract against selected clinical bacterial and fungal isolates. Antioxidant assay of plant leaf extract.

Place and Duration of Study: All the work was carried out in the Departments of Chemistry and Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria between March, 2015 and January, 2016.

Methodology: Isolation of bioactive compounds was by column and thin layer chromatographic techniques. Isolated compounds were characterized by nuclear magnetic resonance spectroscopic analysis. Antimicrobial activities were evaluated by disc diffusion and broth microdilution methods while antioxidant activity was investigated using the 2,2-dipheny-1-picrylhydrazyl (DPPH) radical-scavenging assay.

Results: Two compounds kaempferol-3-O-glucoside **(1)** and 1-(4-Hydroxyphenyl)-3-Phenylpropan-1-one **(2)** were isolated from the ethyl acetate fraction of leaf extract of *A. muricata*. The two compounds showed broad spectrum antimicrobial activities with zones of inhibition ranging from 26.00 ± 1.73 to 31 ± 1.00 mm and 17.33 ± 1.15 to 31.33 ± 1.15 mm respectively, for compounds 1 and 2 for the test bacteria species and 15.33 ± 1.15 to 31.33 ± 1.15 mm and 17.67 ± 0.58 to 29.67 ±1.53 mm respectively, for compounds 1 and 2 for the test fungi. Minimum inhibitory concentrations ranged between 0.625-5.00 µg/mL and 1.25-5.00 µg/ml respectively, for compounds 1 and 2. Minimum bactericidal

concentrations ranged between 2.5-10.00 µg/mL for both compounds which compared favourably with the reference drugs used. DPPH radical-scavenging activities were IC_{50} = 13.41 ± 0.64 µg/mL and 7.42 ± 0.90 µg/mL for compounds 1 and 2 respectively, compared with IC_{50} = 51.99 ± 1.44 µg/ml obtained for the standard ascorbic acid. The results show that both isolated compounds from *A. muricata* leaf possess *in vitro* antimicrobial and antioxidant properties and they may be useful as active ingredients in antimicrobial drug formulations and as agents for the control of free radical-related pathological disorders.

Keywords: *Annona muricata*; Antimicrobial activity; Antioxidant activity; kaempferol-3-O-glucoside; 1-(4-Hydroxyphenyl)-3-phenylpropan-1-one; Phenolics.

1. INTRODUCTION

Medicinal plants have been used for many centuries by different human traditions to alleviate or treat diverse ailments [1,2]. Pathogenic strains of microrganisms such as bacteria, fungi and viruses have been implicated as the major causes of several infectious diseases of man [3,4]. Antibiotics, discovered along the course of human history, equipped man with the arsenal needed to stem the tide of ravaging human infectious diseases. However, the emergence of antibiotic-resistant bacterial strains is a current global problem resulting in antibiotic therapeutic failures especially in clinical settings [5,6]. This worrisome development has been attributable to a combination of microbial characteristics and selective pressures of antibiotic use [7]. Antibiotic resistance has necessitated the search for alternative sources of new and effective antimicrobial agents for the treatment of infections. Medicinal plants are continually investigated by scientists for their possession of antimicrobial and other health-promoting properties [8,9]. Plants contain several biologically active compounds such as alkaloids, flavonoids, tannins,

saponnins, steroids and other secondary metabolites which produce definitive physiological effects on living organisms which can be harnessed for medicinal purposes [10].

Annona muricata (Linn), variously known under the common names 'graviola', 'soursop' and 'guanadabana', belongs to the family Annonaceae [11]. It is an upright, evergreen tree that grows between 5 to 7 m in height, with large, smooth dark green leaves. It is indigenous to the warmest tropical climates of South and Central America but now has wide distribution throughout the tropical and subtropical parts of the world including Nigeria [12]. Several parts of the plant such as the leaves, bark, roots, fruits and seeds are traditionally used for medicinal purposes. The fruits and seeds are used for the treatment of worms and other parasitic infestations, and for their analgesic and antidiarrhoeal effects while the bark, roots and leaves are used for their anti-inflammatory, antiplasmodic, anticonvulsant, sedative and antimalarial effects [13-16]. In Nigeria, decoctions of the leaves and seeds are used to treat ailments such as gastric disorders, prostate cancer, diabetes and arthritic pains [17-19]. Previous reports have demonstrated that the leaf extracts of *A. muricata* possess antibacterial [20,21], antifungal [22], antiviral [23] and antioxidant]24] activities. A novel set of phytochemical compounds called acetogenins have been isolated from the leaf, stem, bark and fruits which have been demonstrated to have significant anticancerous properties and selective toxicity against various types of cancer cells, without harming healthy cells [25,26]. Despite its important medicinal uses, there is paucity of information on the constitutive phenolic compounds of *A. muricata* and their biological effects. This study was therefore designed to isolate and characterize chemical compounds from the ethyl acetate leaf extract of *A. muricata*. The antimicrobial and antioxidant potentials of the isolated compounds were then evaluated.

2. MATERIALS AND METHODS

2.1 Plant Material

Annona muricata leaves (Figure 1) were collected from several locations in the Obafemi Awolowo University, Ile--Ife, Nigeria campus in February, 2014 and authenticated taxonomically by Dr. A Folorunsho at the Ife herbarium in the University.

Fig. 1. Leaves of *Annona muricata* on its tree

2.1.1 Preparation of Plant Extract

The collected leaves were air-dried for three weeks and then blended into powder. Extractions were performed by maceration in which the powdered *Annona muricata* leaves (2000 g) were soaked in 50% aqueous-methanol (5 L) at room temperature for 72 h [27]. The extracts were then filtered using Whatman filter paper number 2 and concentrated *in vacuo* at 40 ^{o}C on a rotary evaporator (Heldolph, Germany) to about one-third of its original volume. Concentrated crude extract of the plant was in turn dissolved in distilled water and partitioned with n-hexane (3×1 L), dichloromethane (3×1 L), ethyl

acetate (3×1 L) and n-butanol (3×0.7 L). The partitioned fractions were concentrated to dryness *in vacuo* to obtain four different fractions with compounds of appropriate polarity.

2.2 Test microorganisms

The test bacteria species were *Staphylococcus aureus* (ATCC 6538*), Pseudomonas aeruginosa* (ATCC 19582), *Escherichia coli* (ATCC 8739), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), *B. stearothermophillus*, *Klebsiella pneumonia*, *Clostridium sporogenes*, *Salmonella typhi and Serratia marcescens* (ATCC 9986). *The test fungal species were Aspergillus niger* (ATCC 6275), *A. flavus, Penicillium camemberti, Fusarium oxysporium, Trichophyton mentagrophytes and Candida albicans* (ATCC 10231). The reference strains (ATCC) were obtained from American Type Culture Collection (Fockville, USA). The others were obtained from the culture collection of the Department of Microbiology, Obafemi Awolowo University, Ile- Ife, Nigeria. The bacterial species included reference strains (6), environmental strains (2) and clinical isolates (2). The fungal species included reference strains (2) and soil environmental strains (4).

2.3 Isolation of compounds

Ethyl acetate fraction (11.123 g) was fractionated by column chromatography on silica gel (60-200 mesh) on glass column (length 60 cm; diameter 4.5 cm) using n-hexane as the eluant. This was followed by an increasing gradient of ethyl acetate up to 100 % and in turn by an increasing gradient of methanol up to 100 %. One hundred and twenty-six test tubes fractions of 15 ml each were collected. Analysis of these test tube fractions on TLC plate using ethyl acetate/methanol (9:1) gave nine fractions (12A to 12I). Fraction 12F and 12G were combined having similar R_f values (1978 mg) was purified on Sephadex LH-20 using ethyl acetate/methanol (9.5:0.5) followed by an increasing gradient of methanol up to 100%. Five fractions 13A to 13E were obtained on analysis on TLC plate using ethyl acetate/methanol (9.5:0.5) as the solvent system. Purification of 13A and 13D (154 mg) on Sephadex

LH-20 using ethyl acetate/methanol (9.5:0.5) followed by an increasing gradient of methanol up to 100 % gave the isolated compounds, 82 mg of 3-((3R, 4S, 5S, 6S)-tetrahydro-3, 4, 5, 6-tetrahydroxy-2H-pyran-2-yloxy)-5,7-dihdroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one and 70 mg of 1-(4-hydroxyphenyl)-3-phenylpropan-1-one.

2.3.1 Characterization of isolated compounds

Structural elucidation of isolated compounds obtained from ethyl acetate fraction of *A. muricata* leaf extract was carried out using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopic analysis.

3-((3R, 4S, 5S, 6S)-tetrahydro-3, 4, 5, 6-tetrahydroxy-2H-pyran-2-yloxy)-5,7-dihdroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one: M.P = 176-178°C, R_f = 0.50 on TLC plate using ethyl acetate/methanol (4:1), ^{1}H NMR see Table 6, ^{13}C NMR see Table 6.

1-(4-hydroxyphenyl)-3-phenylpropan-1-one: 105-107 °C, R_f = 0.35 on TLC plate using ethyl acetate/methanol (4:1), ^{13}C NMR see Table 7.

2.4 Antimicrobial Assays

2.4.1 Preparation of inocula

The bacterial strains grown on nutrient agar at 37 ^{o}C for 18 h were suspended in sterile normal saline solution (0.85% w/v sodium chloride) and adjusted to a turbidity of 0.5 McFarland standard (10^8 CFU/ml). The suspensions were diluted 100 times with Mueller Hinton broth to give 10^6 CFU/ml [28]. Suspensions of fungal spores from fresh cultures were prepared in sterilized 0.85 % w/v sodium chloride solutions, which were compared with McFarland solution. These were then diluted to approximately 10^6 CFU/ml.

2.4.2 Disc diffusion assay

Antimicrobial activities were determined by the agar disc diffusion method [29], with modifications. Mueller Hinton agar and SDA were inoculated with microbial cell suspensions (200 µl in 20 ml

medium) and poured into sterile petri dishes. Stock solutions of isolated compounds were dissolved in 10% DMSO solution to give a final concentration of 1000 µg/ml. The solutions were then sterilized by filtration through 0.45 µm millipore filters. Sterile paper discs (6 mm) were impregnated with 10 µl of the sterile solutions of the isolated compounds and placed on the agar surface. Standard discs (6 mm) containing the broad spectrum antibiotic, streptomycin (10 µg/disc) (Oxoid, UK), and antifungal agent amphotericin B (10 µg/disc) (Abtek Biologicals Ltd, UK), were used as positive controls. Discs impregnated with 10% DMSO were used as negative control. The plates were incubated overnight at 37 ºC for 18-24 h. The plates of the fungal strains were incubated at 31 ºC for 72 h. The experiment was tested in triplicates and zones of growth inhibition were recorded in millimetres.

2.4.3 Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations of the isolated compounds from *Annona muricata* were determined based on the broth microdilution assay. The dried isolated compound was dissolved in 10% DMSO in water to give a concentration of 40 µg/ml. The broad spectrum antibiotic, streptomycin, which was used as positive control, was similarly treated. Serial two fold dilutions were then made in a concentration range 0.313 to 40.0 µg/ml. The 96-well microtiter plates were prepared by dispensing into each well 100 µl of Mueller Hinton broth. Inoculum (10 µl) of each bacteria and 50 µl (0.2 mg/ml) of ρ-iodonitrotetrazolium chloride (INT) were then added into each well. The plates were covered with parafilm, shaken to mix the contents and then incubated at 37ºC for 24 h. The MIC was defined as the lowest concentration at which no visible growth was observed. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms [30]. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT. Each experiment was done in triplicates.

2.4.4 Minimum bactericidal concentration (MBC)

Minimum bactericidal concentrations (MBC) of the isolated compounds were determined by removing 100 µl of bacterial suspension from subculture demonstrating no visible growth and inoculating this on Mueller Hinton agar plates. Plates were incubated at 37ºC for 24 h with experiment being carried out in triplicates.

2.4.5 Determination of rate of killing

Rate of killing studies on representative of each Gram-positive and Gram-negative bacterial isolates were carried out according to Akinpelu et al. [31]. *Staphylococcus aureus* was chosen for Gram positive while *Pseudomonas aeruginosa* represented the Gram-negative bacterial strains. Standardized inocula (10^6 CFU/ml) of test organisms (0.5 ml) were mixed with 4.5 ml of MBC of the isolated compounds. The preparations were allowed to stand at room temperature and the rate of killing was determined over 2 h. At each 15 min interval, 0.1 ml of mixture was taken and transferred to 4.5 ml of brain heart infusion broth recovery medium containing 3 % "Tween 80" to neutralize the effects of antimicrobial isolated compounds carry overs from the test organisms. The suspension was then serially diluted 10-fold with sterile normal saline and plated out on sterile Mueller Hinton agar in triplicates. The plates were incubated at 37 ºC for 24 h. Control plates containing organism suspension without solvent fraction were also set up. The numbers of surviving colonies were counted and recorded against time.

2.5 Antioxidant Activity

The free radical scavenging activity of the isolated compounds were evaluated by using 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) [32]. Different concentrations (0.625 to 10.0 µg/ml) of the isolated compounds were pipetted to the test tubes and volume adjusted to 3 mL with ethanol. One milliliter of 0.1 mM Alcoholic DPPH solution was added to the sample. The sample was vortexed, and incubated in dark at room temperature for 30 min. The absorbance was measured at 517 nm against blank samples.

Decreased absorbance of the sample indicates DPPH free radical scavenging capability. Ascorbic acid (Vitamin C) was used as reference positive control. The DPPH free radical scavenging activity was calculated using the expression:

% DPPH free radical scavenging activity = [1 – A517 nm of sample/A517 nm of control] X 100%.

2.6 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) for Window software version 12.0. The inhibition diameters of test substances were expressed as mean ± standard deviation. Group comparisons were done using one way analysis of variance (ANOVA) followed by Waller-Duncan Post Hoc test. A value of $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Structural elucidation of isolated compounds

Compound TEA 1 was obtained as white needles; mp 176-178 °C.

The ^{1}H-NMR spectrum showed a signal at δ 6.02 (1H, d, H-6), 6.4 (1H, d, J = 6.0 Hz, H-8), 6.9 (2H, d, J = 6.0 Hz, H-3', 5'), 8.06 (2H, d, H-2', 6'), 3.37 (1H, s, H-1), 3.34 (1H, s, H-2), 3.34 (1H, s, H-3), 3.33 (1H, s, H-4), 3.33 (1H, s, H-5), 3.32 (1H, s, H-6).

The ^{13}CNMR spectral (BB and DEPT) displayed resonances for 21 carbons. Resonances on 13C-NMR spectrum at δ 157.73(C-2), 123.10(C-3), 178.1 (C-4), 157.1(C-5), 98.52(C-6), 164.58(C-7), 93.39(C-8), 160.17(C-9), 104.36(C-10), 121.41(C-1'), 130.89(C-2'), 114.70(C-3'), 157.70(C-4'), 114.70(C-5'), 130.89(C-6'), 102.76(C-1''), 76.66(C-2''), 74.35(C-3''), 73.65(C-4''), 69.98(C-5''), 61.26(C-6'').

The isolated compound TEA 1 was identified as kaempferol-3-O-glycoside (Figure 2). The spectral data are in agreement with literature [33]. This is the first report of isolation of kaempferol-3-O-glycoside from the leaf of *Annona muricata*.

Compound TEA 2 was obtained as white needles; mp 105-107 °C.

The ^{1}H-NMR spectrum showed a signal at δ 7.90 (s, 1H), 7.90 (d, J=8.7 Hz, 2H, aromatic), 7.46 (d, J=6.6Hz, 2H), 7.21(t, 3H), 6.84 (d, J=8.7, 2H aromatic), 2.087 (s, 1H), 1.34 (d, J=14.7, 2H).

The ^{13}CNMR spectral (BB and DEPT) displayed resonances for 21 carbons. Resonances on 13C-NMR spectrum at δ 138.23 (C-1), 116.08 (C-2), 133.02 (C-3), 127.59(C-4), 133.02 (C-5), 116.08 (C-6), 177.76 (C-O), 34.99(Cα), 30.34(Cβ), 112.92(C-1'), 122.06 (C-2'), 122.79 (C-3'), 163.36 (C-4'), 123.61 (C-5'), 122.40 (C-6').

The isolated compound TEA 2 was identified as 1-(4-hydroxyphenyl)-3-phenylpropan-1-one (Figure 2). This is the first report of isolation of 1-(4-hydroxyphenyl)-3-phenylpropan-1-one from the leaf of *Annona muricata.*

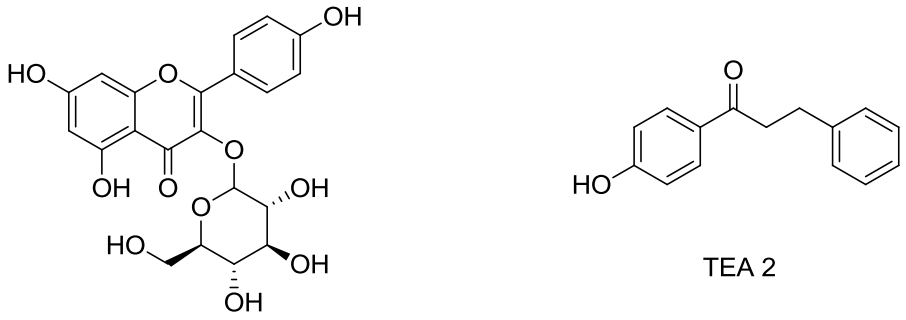

Fig. 2. Chemical structures of kaempferol-3-O-glycoside (TEA 1) and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one (TEA 2) isolated from *Annona muricata* leaves ethyl acetate fraction.

3.2 Antimicrobial activity

Results of our investigation showed that the isolated compounds from *Annona muricata* leaves exhibited varied antimicrobial activities against both the test bacterial and fungal strains (Tables 1 to 4).

The results of the study of the antibacterial activities of the isolated compounds, using the disc diffusion assay, are presented in Table 1. The zones of inhibition ranged from 26.00 ± 1.73 to 31.00 ± 1.00 mm and 17.33 ± 1.15 to 31.33 ± 1.15 mm, respectively for kaempferol-3-O-glycoside (compound 1) and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one (compound 2). The highest inhibitory effects were against *S. aureus* and the weakest activities demonstrated against *S. typhi*, in both cases. The isolated compounds were also effective in the inhibition of all the test fungal species with zones of inhibition ranging from 15.33 ± 1.15 to 30.67 ± 1.15 mm and 17.67 ± 0.58 to 29.67 ± 1.53 mm, respectively for compound 1 and compound 2 (Table 2). Greatest antifungal activity was against *A. niger* and the weakest activity against *C. albicans* by compound 1 while the greatest and weakest activities were against *A. flavus* and *C. albicans*, respectively, by compound 2 (Table 2). Activities of the two isolated compounds compared favourably with those of reference drugs streptomycin and amphotericin B.

In view of the results obtained in the disc diffusion assay, the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of isolated compounds were determined. The MIC values ranged between 0.625 and 5.00 µg/ml for compound 1 and between 1.25 and 5.00 µg/ml for compound 2. The MBC ranged between 2.50 and 10.00 µg ml^{-1} for both compounds 1 and 2. The compounds were most inhibitory against *S. aureus* and *E. faecalis* but least inhibitory against *S. typhi* and *P. aeruginosa* (Table 3). The results obtained in the disc diffusion assay were found to correlate with that obtained in the MIC assay.

Table 1. Antibacterial activities of compounds isolated from the leaf of *Annona muricata*, against some species of bacteria.

Test bacteria	Diameter of inhibition zones (in mm)		
	kaempferol-3-O-glucoside	**1-(4-hydroxyphenyl)-3-phenylpropan-1-one**	**Streptomycin**
S. aureaus	26.00±1.73	17.33±1.15	28.33±0.58
E. faecalis	27.67±1.53	26.67±1.15	28.6±0.58
B. subtilis	29.00±1.73	19.00±1.00	27.33±1.53
K. pneumonia	27.30±1.53	27.67±1.53	28.67±0.58
E. coli	28.0±2.00	30.33±1.53	27.33±1.15
B. stearothermophillus	30.30±0.58	27.67±0.58	29.0±1.00
C. sporogenes	29.67±2.33	30.00±1.00	27.33±0.58
S. marcescens	29.67±0.58	28.33±1.15	29.67±0.58
S. typhi	31.00±1.00	31.33±1.15	27.33±1.15
P. aeruginosa	26.33±1.53	28.67±0.58	26.67±0.58

Inhibition zones diameters were determined at 200 µg (for the isolated compounds) or 10 µg (for streptomycin). The results are mean ± standard deviation of triplicate tests.

Table 2. Antifungal activities of compounds isolated from the leaf of *Annona muricata*, against some species of fungi.

Test fungi	Diameter of inhibition zones (in mm)		
	kaempferol-3-O-glucoside	**1-(4-hydroxyphenyl)-3-phenylpropan-1-one**	**Amphotericin B**
A. niger	25.67±1.15	20.33±1.15	23.33±2.08
A. flavus	27.67±1.53	29.67±1.53	19.00±1.00
P. camemberti	30.67±1.15	29.0±1.73	21.33±1.15
F. oxysporium	18.67±1.15	19.67±1.53	24.67±1.15
T. mentagrophytes	30.67±0.58	26.0±1.53	20.66±1.53
C. albicans	15.33±1.15	17.67±0.58	14.67±1.53

Inhibition zones diameters were determined at 200 µg (for the isolated compounds) or 10 µg (for amphotericin). The results are mean ± standard deviation of triplicate tests.

Table 3. Minimum inhibitory concentrations (MIC) of compounds isolated from the leaf of *Annona muricata* against some species of bacteria.

Test bacteria	Minimum inhibitory concentrations (in µg/ml)		
	kaempferol-3-O-glucoside	1-(4-hydroxyphenyl)-3-phenylpropan-1-one	Streptomycin
S. aureus	0.625	1.25	0.625
E. faecalis	0.625	1.25	1.25
B. subtilis	1.25	1.25	0.625
K. pneumonia	1.25	2.50	2.50
E. coli	1.25	2.50	2.50
B. stearothermophillus	1.25	2.50	2.50
C. sporogenes	0.625	1.25	2.50
S. marcescens	2.50	2.50	5.00
S. typhi	5.00	5.00	5.00
P. aeruginosa	5.00	2.50	2.50

Values (in µg/ml) are the means of three trials which did not show any variation. Streptomycin is used as reference antibiotic.

Table 4. Minimum bactericidal concentrations (MIC) of compounds isolated from the leaf of *Annona muricata* against some species of bacteria.

Test bacteria	Minimum bactericidal concentrations (in µg/ml)		
	kaempferol-3-O-glucoside	1-(4-hydroxyphenyl)-3-phenylpropan-1-one	Streptomycin
S. aureaus	2.50	2.50	5.00
E. faecalis	2.50	5.00	5.00
B. subtilis	5.00	5.00	5.00
K. pneumonia	5.00	5.00	10.00
E. coli	10.00	10.00	10.00
B. stearothermophillus	5.00	5.00	10.00
C. sporogenes	5.00	5.00	10.00
S. marcescens	10.00	10.00	10.00
S. typhi	10.00	10.00	10.00
P. aeruginosa	5.00	10.00	10.00

Values (in µg/ml) are the means of three trials which did not show any variation. Streptomycin is used as reference antibiotic.

3.3 Killing rate

The killing rate test was carried out to determine the time-dependent bactericidal effects of the 1 x MIC and 2 x MIC of the isolated compounds of *A. muricata* leaf extract on *Staphylococcus aureus* and

Pseudomonas aeruginosa (Figures 3 and 4). At a concentration of 1 x MIC of compound 1, the percentage of *Staphylococcus aureus* cells killed after 15 min exposure was 34.8 ± 0.71 %. The percentage of cells killed rose to 57.2 ± 0.55 % after 60 min exposure time. When the contact time was increased to 105 min and 120 min, 84.5 ± 0.571 % and 100 % of the *Staphylococcus aureus* cells had been killed respectively (Figure 3). The concentration of compound 1 was increased to 2 x MIC and at 15 min exposure, 38.5 ± 0.71 % of the *Staphylococcus aureus* cells were killed. After 60 min exposure, 76.2±0.61 % of cells had been killed while there was complete eradication (100 %) by the end of 105 min exposure to this concentration of the compound (Figure 3). At a concentration of 1 x MIC of compound 2, the percentage of *Staphylococcus aureus* cells killed after 15 min exposure was 28.0 ± 0.20 %. The percentage of cells killed rose to 57.8 ± 1.25 % after 60 min exposure time. When the contact time increased to 90 min and 105 min, 87.2 ± 0.30 % and 100 % of the *Staphylococcus aureus* cells had been killed respectively (Figure 3). The concentration of compound 2 was increased to 2 x MIC and at 15 min exposure, 37.0 ± 1.60 % of the *Staphylococcus aureus* cells were killed. After 60 min exposure, 81.4 ± 1.29 % of cells had been killed while there was complete eradication (100 %) by the end of 90 min exposure to this concentration of the compound. A linear relationship was also observed between the percentages of *Pseudomonas aeruginosa* cells killed and increase in MIC concentrations and exposure time of the two isolated compounds (Figure 4).

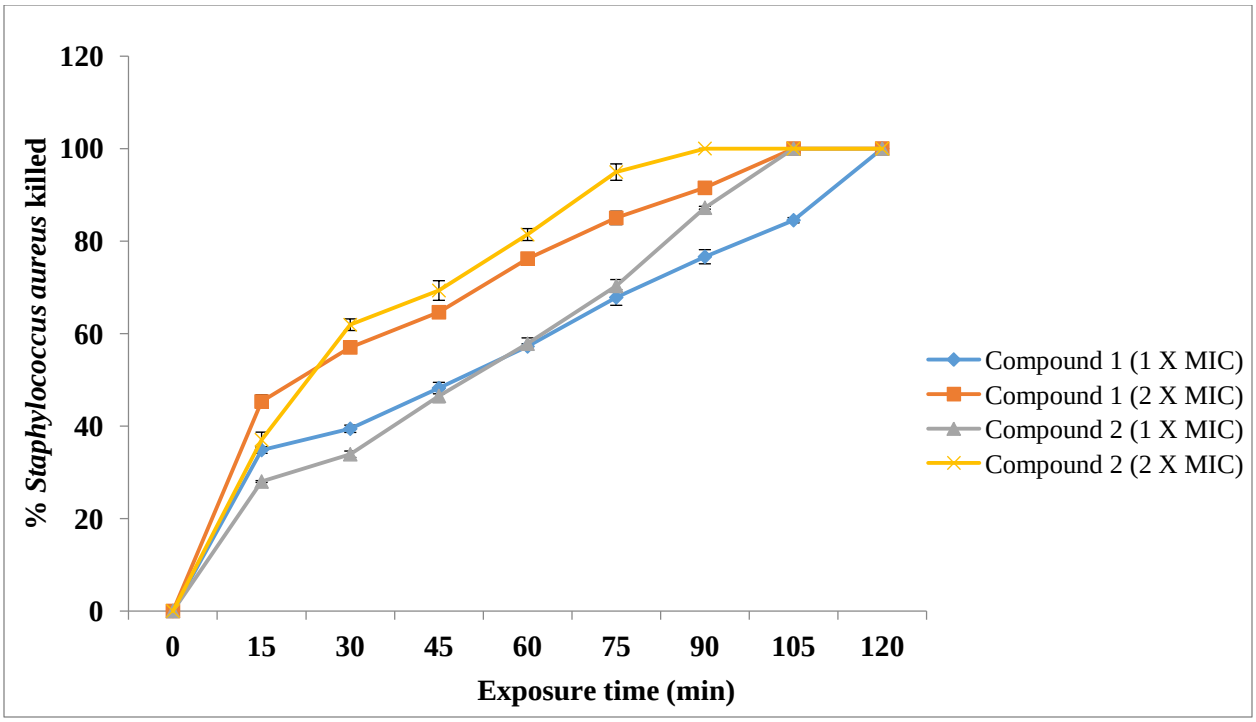

Fig. 3. Rate of killing of *S. aureus* by the minimum inhibitory concentrations of isolated compounds from *A. muricata* leaf.

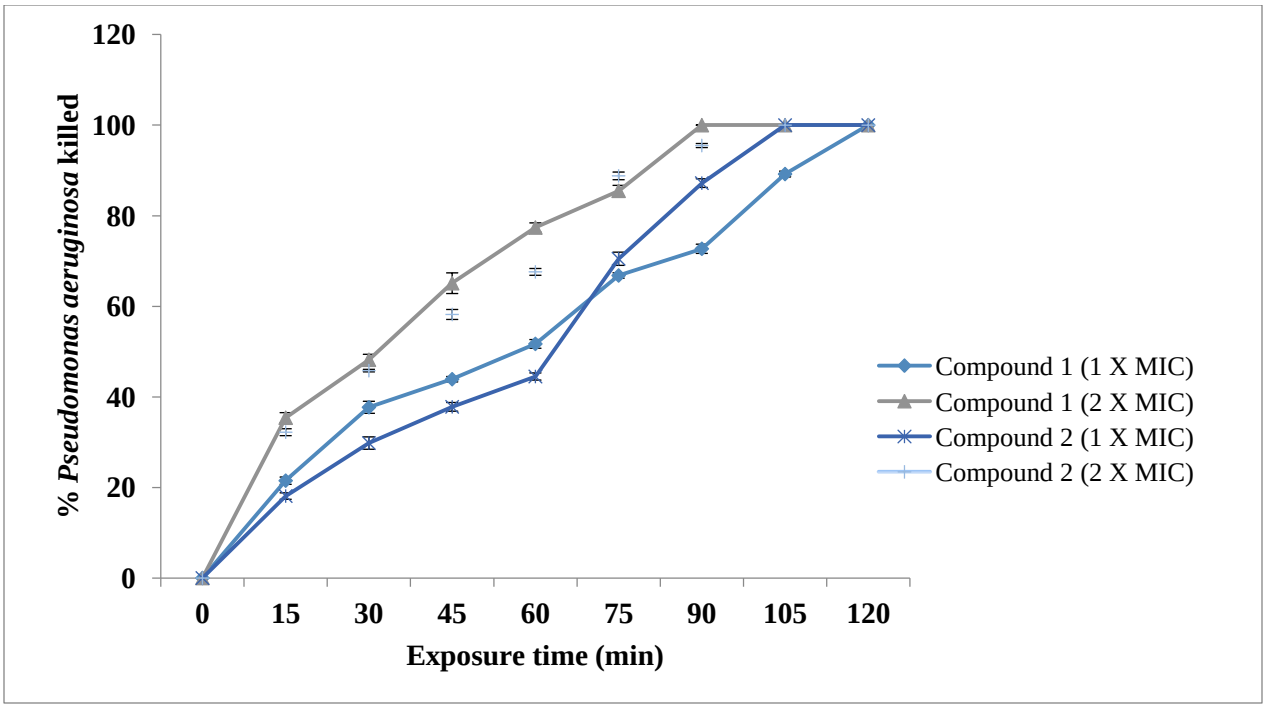

Fig. 4. Rate of killing of *P. aeruginosa* by the minimum inhibitory concentrations of isolated compounds from *A. muricata* leaf.

3.4 Antioxidant Activity

Antioxidant activities of the isolated compounds were determined using the DPPH-radical scavenging capacity. The DPPH radical scavenging capacity of the isolated compounds from *A. muricata* leaf extract relative to that of the reference standard ascorbic acid is presented in Table 5. The IC_{50} values were found to be 1.25 ± 0.09 and 1.87 ± 0.06 µg/ml respectively, for compound 1 and compound 2 and 3.08 ± 0.06 µg/ml for the standard ascorbic acid (Table 5).

Table 5. DPPH radical scavenging activity of isolated compounds from *A. muricata* leaf extract.

Test Sample	Mean IC_{50} (µg/ml)
Isolated compounds:	
kaempferol-3-O-glucoside	1.25 ± 0.09
1-(4-hydroxyphenyl)-3-phenylpropan-1-one	1.87 ± 0.06
Reference substance:	
Ascorbic acid	3.08 ± 0.06

Data represents mean ± S.D of triplicate determination

4. DISCUSSION

There is increasing trend in the development of resistance against the existing antimicrobial agents by infectious microorganisms [6]. This has resulted in interests in the exploration of plants used in traditional medicines as potential sources of novel antimicrobial compounds.

A. muricata is widely distributed throughout the tropical and subtropical areas of the world and is used as a natural remedy for variety of illnesses including infections caused by microorganisms (16). In our earlier study, phytochemical screening of the leaves of the plant had revealed the presence of tannins, anthraquinones, saponnins, flavonoids, alkaloids and caqrdiac glycosides (21). These metabolites were also detected in leaves of the plants by other researchers (34,35). Several researchers have demonstrated the antimicrobial properties of leaf extracts of the plant against Gram positive and Gram negative bacteria and fungi (21,34,35).

The phenolic compounds identified as kaempferol-3-O-glucoside and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one isolated from *Annona muricata* leaves ethyl acetate fraction showed antimicrobial effects which compared favorably with those of reference drugs - streptomycin and amphotericin B. They both exhibited broad spectrum inhibitory effects on both Gram positive and Gram negative bacteria, and all the tested fungal species. Also, the killing rate exhibited by the two compounds on test microbial cells indicated their ability to eliminate the test

338 microorganisms within a short period of time acting in a dose-dependent manner. These compounds
339 could therefore serve as antimicrobial agents or precursors of antimicrobial agents that could be used to
340 combat infections caused by these microorganisms and other pathogens. Kaempferol-3-O-glucoside,
341 isolated from ethyl acetate extracts of the capitula of *Helichrysum compactum*, was reported to exhibit
342 antibacterial and antifungal activities [36]. The antibacterial effect of this chemical compound isolated
343 from ethyl acetate fraction of the fresh flower extracts of *Propolis julifora* was also established [37].
344 Antioxidant evaluation by the DPPH assay revealed that the isolated compounds from *Annona muricata*
345 leaf extract enclosed antioxidant activity. Several studies have shown the relationship between the
346 antioxidant activity and total phenolic compounds [38,39]. Phenolic compounds like flavonoids, due to
347 their chemical structure, are ideal donors of hydrogen to the DPPH radical [40,41]. Our results
348 strongly suggest that phenolics are important components of *A. muricata*, and some of its
349 pharmacological effects could be attributed to these constituents. Kaempferol-3-O-glucoside isolated
350 from several other plant species was found to exhibit potent antioxidant activities [36,42]. The radical
351 scavenging abilities of these compounds are mainly due the presence of hydroxyl groups and their redox
352 properties [43]. These properties play important roles in the ability of phenolic compounds to absorb and
353 neutralize free radicals, quench active oxygen species and decompose peroxides [44-46]. The prevention
354 of risks of oxidative stress-associated diseases such as cardiovascular and
355 neurodegenerative diseases, cancer or osteoporosis has been partially ascribed to phenolic
356 compounds [47].
357 To our knowledge, this would be the first time the two isolated compounds have been isolated from the
358 plant. Kaempferol-3-O-glucoside and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one isolated from *Annona*
359 *muricata* leaves ethyl acetate fraction, in this study were found to demonstrate significant antimicrobial
360 and antioxidant activity. The compounds could be effective in the management of oxidative stress and

infectious-related diseases. This finding supports the traditional use of this plant in the treatment of infectious and other diseases. Kaempferol-3-O-glucoside is a flavonoid, a group of compounds which are reported to exhibit antimicrobial and pharmacological activities such as antioxidant, analgesic and anti-inflammatory effects [48-50]. Flavonoids have been reported to display strong antibacterial activity by mechanisms such as formation of complexes with extracellular proteins and bacterial cell wall, thereby inhibiting the microbial growth [51,52]. The compound 1-(4-hydroxyphenyl)-3-phenylpropan-1-one could be a precursor for the development of useful pharmaceuticals.

5. CONCLUSION

Our results indicate that kaempferol-3-O-glucoside and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one isolated from the ethyl acetate fraction of *Annona muricata* leaves presented broad spectrum antimicrobial activities which compared favorably with those of reference drugs used. These phenolic compounds also exhibited strong antioxidant activity based on DPPH radical scavenging capacity. These findings provided a rationale for the ethnomedicinal use of the plant in traditional medicine. The plant could therefore be a potential source of natural antimicrobial and antioxidant agents for the treatment of microbial infections and prevention of various oxidative stress-associated diseases.

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COMPETING INTEREST

The authors declare that no competing interest exist.

AUTHORS' CONTRIBUTION

FOT and OO designed the study and wrote sections of the article. FOT supervised the chemical part. MTK and MTO did the spectroscopic analysis. OO did the biological assays, helped in manuscript writing and editing. All authors read and approved the final manuscript.

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Table 6. Comparative NMR-chemical shift values for Kaempferol-3-O-glycoside (TEA 1)

C. No.	Observed data of kaempferol		Reported data of kaempferol [33]
	δC	***δH* (*J*, Hz)**	***δC***
2	157.73	-	157.60
3	134.10	-	134.90
4	178.1	-	177.90
5	157.1	-	161.30
6	98.52	6.2	99.20
7	164.58	-	164.90
8	93.39	6.4	94.30
9	160.17	-	157.00
10	104.36	-	104.30
1′	121.41	-	121.00
2′	130.89	8.06	131.00
3′	114.70	6.9	115.70
4′	157.70	-	160.10
5′	114.70	6.9	115.70
6′	130.89	8.06	131.00
1″	102.76	3.37	100.70
2″	76.66	3.34	74.20
3″	74.35	3.34	76.40
4″	73.65	3.33	69.90
5″	69.98	3.33	77.50
6″	61.26	3.32	60.80

Table 7. ^{13}C NMR chemical shift values for 1-(4-hydroxyphenyl)-3-phenylpropan-1-one (TEA 2)

C No	***δC***
1	138.23
2	116.08
3	133.02
4	127.59
5	133.02
6	116.08
C=O	177.76
C_{α}	34.99
C_{β}	30.34
1’	112.92
2’	122.06
3’	122.79
4’	163.36
5’	123.61
6’	122.40