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In vitro bactericidal activity of diterpenoids isolated from *Aframomum melegueta* K.Schum against strains of *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*

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ABSTRACT

Ethnopharmacological relevance: The ethnobotanical use of *Aframomum melegueta* in the treatment of urinary tract and soft tissue infection suggested that the plant has antimicrobial activity.
Materials and methods: To substantiate the folkloric claims, an acetone, 50:50 acetone:methanol and 2:1 chloroform:methanol extracts were tested against *Escherichia coli K12*; acetone extract and the fractions of acetone extracts were tested against *Listeria monocytogenes*. Bioassay-guided fractionation was performed on the extract using *L. monocytogenes* as the test organism to isolate the bioactive compounds which were then tested against all the other organisms.
Results: Four known labdane diterpenes (G3 and G5) were isolated for the first time from the rhizomes of *A. melegueta* and purified. These were tested against *E. coli*, *L. monocytogenes*, methicillin resistant *Staphylococus aureus* (MRSA) and *S. aureus* to determine antibacterial activity. The result showed that two compounds G3 and G5 exhibited more potent antibacterial activity compared to the current clinically used antibiotics ampicillin, gentamicin and vancomycin and can be potential antibacterial lead compounds. The structure of the labdane diterpenes were elucidated using nuclear magnetic resonance (NMR) spectroscopy and Mass spectrometry. A possible mode of action of the isolated compound G3 and its potential cytotoxicity towards mammalian cells were also discussed.
Conclusion: The results confirmed the presence of antibacterial compounds in the rhizomes of *A. melegueta* with a favourable toxicity profile which could be further optimized as antibacterial lead compounds.

1. Introduction

The continuous emergence of resistant genes in pathogenic bacteria has posed the greatest challenge to the efficacy of the current battery of antimicrobial agents and this has made the search for new antibiotics with different mechanisms of action an imperative. Natural product derived antibiotics, for example penicillins, cephalosporins and macrolides, have been extensively used but due to the development of reported cases of multiple resistance, new core structures are urgently required in order to permit the generation of new antibiotic candidates (Robards, 2003). Co-evolution has been given as one of the reasons why bacteria can easily acquire resistance against antibacterial molecules of natural origin. It is also the reason why it is more likely to obtain new antibacterial leads from natural sources rather than by chemical synthesis. This is because natural products are viewed as a group of structures which have evolved to interact with a wide variety of molecular targets for specific purposes (Katiyar et al., 2012; Koehn and Carter, 2005). Exploring the bio-discovery route from natural products has created new leads for pharmaceutical drug discovery and drug design (Katiyar et al., 2012). Herbal remedies have been used successfully in the treatment and prevention of various diseases (Nobili et al., 2009). They have been used as antispasmodic (Janbaz et al., 2013), antimalarial (Zhu et al., 2013), antihypertensive (Pang et al., 2008) anticancer (Ksouri et al., 2013; Liu et al., 2013) and antimicrobial agents (Frankova et al., 2014) among other uses. Plant roots, bark, stems, leaves and flowers have been administered as a whole and as extracts in folk medicine to treat these ailments (Nobili et al., 2009).

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Aframomum melegueta K.Schum (Zingiberaceae), commonly known as grains of paradise is a tropical herbaceous plant found in many African countries including Nigeria, Cameroon, Liberia, Ghana. The plant is used traditionally for various purposes in Africa ranging from wound healing to the treatment of infections such as urinary tract infection, skin infections and food poisoning (Doherty et al., 2010). Studies of the plant have indicated that different parts of the plants have different biological and medicinal properties. For instance it has been reported that oral ingestion of its seed extract increases whole-body energy expenditure (Sugita et al., 2013). Other properties reported include antioxidant and acetylcholinesterase inhibitory activity of the seed extract (Adefegha and Oboh, 2012), antihypertensive property (Gbolade, 2012), mycobacterial efflux inhibitory activity (Groblacher et al., 2012), hepatoprotective (Nwozo and Oyinloye, 2011) and reduction in gestational weight gain (Inegbenebor et al., 2009). Inhibition of cytochrome P450 3A' enzyme (Agbonon et al., 2010), anticancer properties (Gismondi et al., 2013) and increased the production of male hormones in rats (Mbongue et al., 2012) have also been reported.

Similar to other medicinal plants, all the therapeutic effects of this plants are due to the biological properties of a number of secondary metabolites contained within them (Alphonso and Saraf, 2012). Some of these have yet unknown functions to the plants while others are known to be synthesized for specific purposes. For instance, hydroxylated coumarins have been reported to accumulate in carrots in response to fungal invasion (Darvill and Albersheim, 1984). In addition, the accumulation of glucosinolates known for their antimicrobial properties (Al-Gendy et al., 2010) has been reported in *Brassica rapa* L. family, Brassicaceae in response to fungal infection (Abdel-Farid et al., 2009).

In the present study, an acetone extract of the rhizomes of *A. melegueta* was screened for antimicrobial activity against selected infectious agents of significant public health importance including *Escherichia coli K12* (surrogates of pathogenic *E. coli*), *Listeria monocytogenes*, a clinical isolate of *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA). Compounds which showed the highest levels of antimicrobial activity were isolated and purified from the extract followed by characterisation and structure elucidation.

2. Materials and method

2.1. General experimental procedure

Methanol, chloroform, hexane and HPLC grade acetonitrile were obtained from Sigma-Aldrich (Steinheim, Germany) while sodium chloride, disodium hydrogen phosphate & sodium dihydrogen phosphate used for the preparation of 50 mM PBS were sourced from VWR international limited (Poole, England). (*50 mM PBS formula: Na_2HPO_4 14.196 g, NaH_2PO_4, 5.999 g; NaCl, 8.766 g*). Acetone (TE Laboratories, Tullow, Ireland) was the major extracting solvent.

Mueller Hinton agar and broth, nutrient agar, nutrient broth, tryptone soya broth, tryptone soya agar and yeast extract were purchased from Oxoid ltd. (Basingstoke, Hampshire, England).

Stirring was carried out on an AGB1000 magnetic stirrer (Jenway Ltd., Felsted, Essex, England). A laboratory blender (Christison particles technologies, Gateshead, UK) was used for grinding and particle size reduction. Ninety-six well microtiter plates (Sarstedt AG and co.Nümbrecht Germany) were used for antimicrobial sensitivity testing. An Alliance 2695 HPLC machine equipped with 1525 binary HPLC pump and 2996 Photodiode Array (PDA) detector (Waters Milford Massachusetts, USA) was used for both analytical and semi-preparative separations. An Elga deionizer (Elga process water, Marlow Bucks, United Kingdom) was used for preparation of the purified water. Concentration of the extracts and fractions produced was carried out on a Stuart rotary evaporator (Stuart Scientific, Staffordshire, United Kingdom) and TurboVAP (Calipers Life Sciences, Hopkinton, Massachusetts, USA). HPLC columns – 150 mm × 4.6 mm I.D with 5 μm particle size and 250 mm × 10 mm ID, 5 um particle size were of supelcosil™ brand (Sigma-Aldrich, Steinheim, Germany). Bruker Avance DRX500 NMR (Bruker AXS Inc., Madison, WI, USA) was used for all nuclear magnetic resonance (NMR) experiment. Mass spectrometry was carried out on UPLC-MS–MS from Waters Division (Waters Milford Massachusetts, USA).

BD falcon 96-well tissue culture plates and BD falcon Tissue culture flasks (Becton Dickinson Labware, Franklin Lakes New Jersey, USA), Sapphire^2 ELISA Plate reader (Tecan Austria GmbH, Untersbergstrasse, Austria), Minimum essential medium Eagle, Sodium Pyruvate (Sigma-Aldrich company Ltd., Ayrshire, UK) and foetal bovine serum (GIBCO, Auckland, New Zealand) were used for cell culture experiments.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) *assay*: Dimethyl sulfoxide and MTT reagent m2128-1G were purchased from Sigma-Aldrich St. Louis MO, USA.

2.2. Biologicals

Plant materials: The rhizomes of *A. melegueta* was collected from Cameroon and were identified by Dr. Dene Bernadin Jiofack (a botanist) and a voucher specimen was deposited in the Millenium Ecologic Museum, Yaoundé, Cameroon.

E. coli K12 (NCTC 10538) and *L. monocytogenes* (NCTC 11994) were obtained from the National Collection of Typed Cultures (Colindale, London, United Kingdom). A Clinical isolate of *S. aureus* and methicillin resistant *S. aureus* (MRSA) (NCTC 12493) were obtained from the Agri-Food and Bioscience Institute, Newforge lane, Belfast, United Kingdom and the Health Protection Agency (Wiltshire, United Kingdom), respectively.

2.3. Extraction method

Protocol 1: The extraction procedure involved the use of protic and aprotic polar solvents in a stir-bar assisted maceration. In this procedure, 2 g of the plant specimen collected from Cameroon and sun-dried was ground to a coarse powder and extracted with 20 ml of acetone and 50:50 mixtures of acetone/methanol respectively. The extraction involved a 24 h-long maceration with at least 12 h of stirring. The solution was removed at 12 h and 24 h, pooled, centrifuged, filtered, and concentrated using a rotary evaporator.

Protocol 2: In a separate experiment, 150 g of the same material was extracted twice with 500 ml of acetone and subsequently 3 times with 2:1 chloroform–methanol solution.

2.4. Microbiological testing

The microbiological procedure was carried out as described by Friedman and co-workers with slight modifications (Friedman et al., 2003). The acetone, acetone:methanol (50:50) and 2:1 chloroform:methanol extracts were tested against *E. coli K12*; acetone extract and the fractions of acetone extracts were tested against *L. monocytogenes*, while the identified bioactive compounds were tested against all the test organisms.

Briefly, a serial dilution was prepared from the 1 mg/ml stock solution of extract to cover a range of concentrations (0.97 μg/mL–250 μg/mL). A stationary phase bacteria with optical density of 0.8 in 50 mM phosphate buffered saline was diluted so a 20 μl suspension contained between 100 and 200 colony forming units (CFU) of the bacteria (1:100 and then 1:2500). Using a 96-well

microtiter plate, a mixture of 100 μl of extract dilutions and 50 μl of bacteria suspension was incubated for 1 hour. This procedure was applied to all the test organisms. Twenty microlitres of the reaction mixture was then plated on agar and incubated for 13 h for *E. coli K12* and 18 h for *L. monocytogenes* and the other test organisms. All experiments were carried out in duplicate. A plate count was carried out and the 50% kill concentration (BA_{50}) was determined using the following equation:

$$\{(\text{No. of CFU in negative} - \text{No. of CFU in test})/\text{No. CFU in negative}\} \times 100$$

Also, each test performed on extracts and identified compounds had a positive control incorporated. The positive controls chosen were the first choice of clinical antibiotics used for the treatment of infections caused by each test organism. Ampicillin was used for *L. monocytogenes* and gentamicin was used for *E. coli* and *S. aureus* while vancomycin was used for MRSA. Microbiological tests were carried out on the fractions of the extract *en route* isolation as well as on the purified and identified bioactive compounds.

2.5. HPLC methods

The oily extract obtained from the plant was fractionated in a preparative reverse phase HPLC procedure. Using a Waters 2695 HPLC equipped with 2996 Photodiode Array (PDA) detector, a mobile phase made up of 0.05% aqueous trifluoroacetic acid (A) and acetonitrile (B) and *EMPOWER PRO* software, a gradient programme was set up for 60 min thus: 0 min (0% B); 10 min (60% B); 30 min (70% B); 45 min (70% B); 50 min (80% B); 55 min (0% B), 60 min (0% B). The column temperature was set at 25 °C throughout the experiment. The PDA was set between 200 nm and 500 nm in order to accommodate compounds with varying degrees of conjugation and those without chromophore. The purpose was to restrict fraction collection to observable peaks only. The injection volume was 100 μl of 350 mg/ml extract, giving a column load of 35 mg. Five major peaks were annotated (G1–G5) and collected as peak fractions. 'G' was derived from the first letter of the Cameroonian name of the herb 'Ghang kishog'.

Antimicrobial sensitivity testing carried out on the collected peak fractions as described above indicated that G3 and G5 had potent antimicrobial properties against the test organisms. Column chromatography in a glass column measuring 1 m × 5 cm was used to isolate pure G3 and G5 that would be sufficient for structural elucidation, toxicity testing and preliminary mode of action studies. Forty grams of silica was used as the column stationary phase which was suspended in hexane and poured as slurry into the column. The packing was washed and conditioned with hexane. Seven hundred milligrams of extract was dissolved in 80:20 mixtures of hexane:chloroform and loaded on top of the column.

The eluents used were hexane, chloroform and methanol. The polarity of the eluents was increased by increasing the percentage of chloroform in hexane. The extracts were separated by a serial elution with different mixtures of hexane, chloroform and methanol in 200 ml aliquots, starting with 100% hexane and then 50% chloroform in hexane. The percentage of chloroform in the hexane–chloroform mixture was increased by 10% for each subsequent elution to achieve a gradient. At 100% chloroform, methanol was introduced to increase polarity as up to 5% methanol in chloroform was required to completely elute all the extract from the column. Addition of methanol was from 0.5% (v/v) to 5% in chloroform with an increment of 0.5% at a time. The fractions were collected in 100 ml aliquots.

2.6. Structural elucidation

The isolated compounds were subjected to a range of NMR experiments for the purpose of structural elucidation. The experiments included ^{1}H-NMR (1D and 2D), ^{13}C-NMR (1D), heteronuclear multiple quantum coherence (HMQC), Heteronuclear multiple bond correlation (HMBC) and Distortionless enhancement by polarisation transfer (DEPT) 90 and 135.

Mass spectrometry was carried out on the isolated compounds using UPLC-MS-MS from Waters Division (Milford Massachusetts, USA). A total scan of each compound dissolved in acetone was carried out in positive electrospray mode in quadruple MS–MS. The other parameters set included; cone voltage (19 V), capillary voltage (350 kV), source temperature (100 °C), desolvation temperature (350 °C) and injection volume (10 μl).

2.7. MTT assay of G3 on CaCo-2-cell line

The cell line passage no. 24 was cultured in the culture media-minimum essential medium Eagle (MEME) supplemented with 1% v/v L-glutamine (2 mM), 1% v/v sodium pyruvate (1 mM), and 10% v/v foetal bovine serum. The cells were sub-cultured until passage no. 29 and were then passed into a 96-well plate at concentration of 8000 cells/well. The cells were incubated for 72 h before the introduction of different concentrations of G3 in a maintenance media and were further incubated for another 72 h at 37 °C under an atmosphere of 8% CO_2 in air. On the third day of incubation the media was removed.

Fifty microlitres of 40% MTT in maintenance media was added to each well and incubated for 4 h after which excess (unreacted) MTT was removed. Two hundred microlitres of dimethyl sulfoxide was added to each well and the plate was shaken for 15 min in a microplate shaker to dissolve the formazan crystals. The absorbance was measured with an ELISA plate reader at a wavelength of 570 nm with reference at 630 nm. The toxicity was calculated using the following formula:

$$\{(\text{Absorbance of Control} - \text{Absorbance of test})/\text{Absorbance of control}\} \times 100.$$

The LD_{50} (median lethal dose) of the compound was calculated using the GraphPad Prism 5 software.

2.8. Mode of action study

Peptidoglycan is a polymer made up of disaccharide subunits which are linked together by peptide bonds which are found in bacterial cell walls (Mott et al., 2008). It maintains the integrity of the bacterial cell wall. Inhibition of peptidoglycan synthesis is a known mechanism of antibacterial action.

Peptidoglycan extraction and HPLC analysis were carried out using a modified Pfizer peptidoglycan extraction protocol and HPLC assay procedure (Mott et al., 2008). *L. monocytogenes* (NCTC 11994) was grown in TSB at 30 °C overnight. The culture was diluted to OD_{600} of 0.1 and re-incubated in the same condition until it reached OD_{600} of 0.4. Then 20 μl each of the test compound and ampicillin (at a concentration of $8 \times BA_{50}$) were added to 980 μl of cell suspension.

The final concentration of G3 and ampicillin in the mixture (0.64 μg/mL for G3 and 4.8 ug/mL for ampicillin) was still 10 and 6 folds below their respective BA_{50}. The mixture was incubated for another 30 min at 30 °C. The cells were then centrifuged, re-suspended in 250 μl of ice-cold 1 M formic acid and incubated for 30 min on ice. After incubation, the cells were centrifuged again and the supernatants removed. Peptidoglycan precursors in the acid extract (supernatant) were analysed by HPLC. Chromatographic conditions: Column: Synergi 4 μm 150 mm × 4.6 mm i.d Polar RP, Flow rate: 0.5 ml/min, Injection: 100 μl; Buffer A: 10 mM

ammonium acetate pH 5.0; Buffer B: 2% acetonitrile in 10 mM ammonium acetate pH 5.0, Gradient: 0 min (0% B), 35 min (100% B), 37 min (100% B), 40 min (0%).

2.9. Reduction of G3 and G5

G3 and G5 were dissolved in methanol and treated with sodium borohydride at room temperature. The reaction was instantaneous and complete under 1 min. The mixture was filtered to remove any unreacted borohydride powder and dried and the purity of the product was determined using HPLC and NMR. The reduced forms of the compounds were subjected to NMR analysis and antibacterial senstivity testing against *L. monocytogenes* the model organism.

3. Results and discussion

Results of the present study showed that both the G3 and G5 (known compounds) isolated from *A. melegueta* have potent *in vitro* antimicrobial activity against Gram-positive pathogens such as *L. monocytogenes* and *S. aureus*. The results of this study also suggested that acetone may be the appropriate choice of solvent for the extraction of antimicrobial diterpenes from *A. melegueta* which has also been reported by Eloff (Eloff, 1998).

In Protocol 1, the extraction procedures were compared with regards to yield from acetone, an aprotic and moderately polar solvent, and methanol, a more polar and protic solvent. The potency of the extracts against *E. coli K12* and *L. monocytogenes* was also compared (Table 1).

The yields of the material when 2 g of the specimen was extracted with acetone and 50:50 acetone/methanol were 62 mg and 129 mg, respectively. The result of antibacterial test against *E. coli* showed no activity for 50:50 actetone extract while a BA_{50} of 20 μg/mL was obtained for acetone only extract.

Protocol 2, which involved sequential extraction with acetone and then 2:1 chloroform:methanol showed no significant difference ($p < 0.05$) between the yields from both procedures where approximately 10 g of extract from 150 g of starting material was recovered from each of the solvent systems. However, the HPLC chromatogram of each extract showed that the profile of the extracts differed by up to 95% reduction of the peak area of G5 peak from the 2:1 chloroform/methanol extract compared to the acetone extract with G5 being the major constituent (Abreu and Noronha, 1997) Fig. 1(i)–(ii).

G2 and G4 peak areas were also significantly reduced in the Chloroform/methanol extract.

Extraction using Protocol 1 resulted in a higher yield (2-fold) of extracts than using a mixture of acetone/methanol (50%, v/v) but no antimicrobial activity was observed against both *E. coli* (Gram-negative) and *L. monocytogenes* (Gram-positive) tested. It is possible that methanol can extract antimicrobial compounds from the plant but the process also extracts many other inactive compounds (Farnsworth, 1972) which reduces the concentration of the active principle per miligram of extract and thus reduces the observed antimicrobial activity. Another possibility could be that the addition of methanol to acetone may alter some structural features of the bioactive components resulting in the loss of their antimicrobial properties. Protic solvents such as methanol are known to be able to convert aldehydes to hemiacetals which in turn can react with methanol to form acetals (Li et al., 2010). Extraction of hemiacetal form of a dialdehydic compound with methanol instead of the original compound which was extracted with other solvents has been reported (Robards, 2003). If the pharmacophore resides on the aldehyde moiety, the compound will exhibit reduced or total loss of activity. Conversely, the hemiacetal could be active while the parent compound is inactive (Robards, 2003). In our case, both of the active compounds (G3 and G5) contain at least one aldehyde moiety. Many known plant derived antimicrobials such as cinnamaldehyde contain aldehyde moiety (Wong et al., 2008). Therefore it could be concluded that chemical inactivation was responsible for the loss of the antimicrobial properties in the methanol–acetone extract. The choice of solvent is therefore critical to the successful extraction of antimicrobially active compounds from plants.

Table 1
Table showing the 50% bactericidal concentrations (BA_{50}) of test samples against test organisms.

Test samples	50% Kill concentration (μg/mL)			
	E. coli	***S. aureus***	***Listeria monocytogenes***	**MRSA**
Gentamicin	**21.0**	**14.0**	–	–
Ampicillin	–	–	**29.5**	–
Vancomycin	–	–	–	**27.0**
Crude extract	**20.0**	–	**4.3**	–
G3	**15.6**	**7.8**	**6.4**	**10.0**
G3 (***reduced***)			**75.0**	
G4	–	**73.5**	–	–
G5	**44.0**	**40.0**	**22.7**	–
G5 (***oxidized***)	–	**5.2**	**1.8**	**1.9**
G5 (***reduced***)	**–**	**–**	**5.1**	**–**

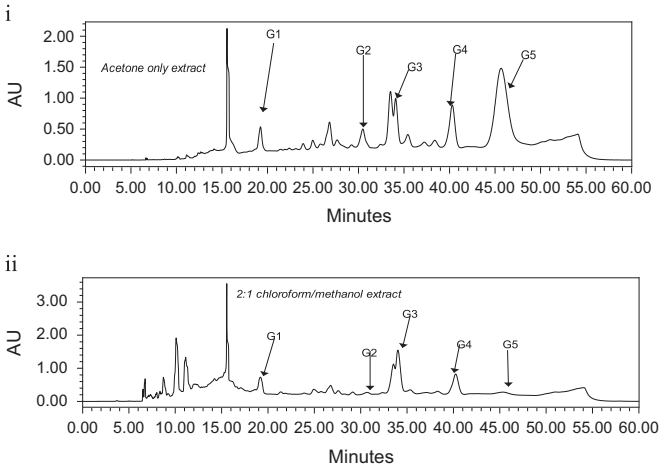

Fig. 1. Chromatogram showing the effectiveness of acetone as extractant (i) for G5 when compared to 2:1 mixture of chloroform and methanol (ii).

When the extracts from Protocol 1 were tested for antimicrobial property against *E. coli*, a $BA_{50} = 20$ μg/mL was observed in acetone extract while the methanol/acetone had no measureable activity (Fig. 2). The BA_{50} of gentamicin (the positive control) was 21.0 μg/ml while the BA_{50} of the extract against *L. monocytogenes* was 4.3 μg/mL which was 7 times more potent than the 29.5 μg/ml observed for ampicillin, (the positive control) against the same organism (Table 1).

The potency of the extract when juxtaposed with that of gentamicin, a drug used effectively in the treatment *E. coli* infection compares very well (Yoon et al., 2011). Though the effect of gentamicin seemed to be superior at higher concentrations, the extract was more effective at lower concentration (Fig. 2) which is a more desirable feature of a therapeutic agent. The BA_{50} of the extract was slightly better than that of gentamicin against *E. coli* but against *L. monocytogenes* NCTC 11994 the extract was shown to be several times more effective than ampicillin. Ampicillin is a drug of choice in the clinical treatment of *L. monocytogenes* infection (Korsak et al., 2005).

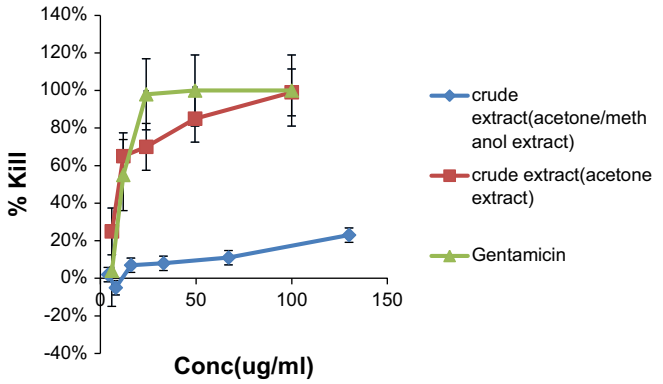

Fig. 2. Showing the comparison between antibacterial activities of gentamicin (positive control), crude acetone/methanol extract and acetone-only extract against *E. coli*.

The reason for greater antimicrobial activity against Gram-positive organisms could be because all the isolated bioactive compounds were found to be diterpenoids. Antibacterial diterpenoids are known to have potent antimicrobial activity against Gram-positive organisms (Chinou, 2005; Fonseca et al., 2013; Porto et al., 2012). Both G3 and G5 are potent antibacterial diterpenoids isolated from the rhizomes of *A. melegueta* as will be discussed later.

Two chromatographic methods were used for the isolation and purification of the compounds, i.e column chromatography and high performance liquid chromatography. Bioassay-guided fractionation was carried out using reverse phase HPLC and column chromatography. Five major peaks labelled G1–G5 eluting at 18 min, 29 min, 33 min, 40 min and 42 min respectively were obtained and isolated. To reduce the analysis time, column chromatography was applied for the isolation of G3 and G5 which had displayed good activity and HPLC was used to confirm the identity and the purity. The purity of the peaks were tested with empower software and the individul chromatograms of each isolated peak fractions confirmed the purity of the fractions. A total of 18 fractions were collected from column chromatography. Fraction G3 was eluted with 80% chloroform in hexane (fractions 8–12) while G5 eluted with 1% methanol in chloroform (fractions 14–17). As much as 90 mg and 30 mg of G5 and G3 respectively were recovered per gram of the acetone extract. G4 (15 mg) was collected from G4 peak fraction. G2 had no activity and 2 mg was isolated. About 35 mg of G1 was also isolated. A number of experiments were carried out in order to elucidate the structures of G1, G3, G4 and G5. G1 was found to be a plastcizer, di (2-ethylhexyl) phthalate (DEHP) as the NMR matched with published data (Uyeda et al., 1990). The source is not known since a procedure check did no indicate contamination from materials used. The quantity of G2 did not allow further studies and absence of observable activity against test organisms discouraged further effort to isolate more of the compound. Results of the NMR and mass spectrometry analysis confirmed G3, G4 and G5 were previously identified known diterpenoids. The masses of G3, G4 and G5 were searched and compared using SciFinder. Based on the findings of published data (Abreu and Noronha, 1997; Hong-Xi et al., 1996, 1995), the ^{1}H NMR and ^{13}C NMR of the G3, G4 and G5 compounds were identical to those corresponding to Zerumin A (G3) (Hong-Xi et al., 1996), Zerumin(G4) (Hong-Xi et al., 1995) and (E)-labda-8(17),12-diene-15,16-dial (G5) (Abreu and Noronha, 1997). The mass to charge ratios (m/z) for G3, G4 and G5 were found to be 318, 316 and 302 which also correspond to the published data (Abreu and Noronha, 1997; Hong-Xi et al., 1996, 1995). The structures of the identified compounds are shown in Fig. 3. All the compounds have the basic labdane diterpene back

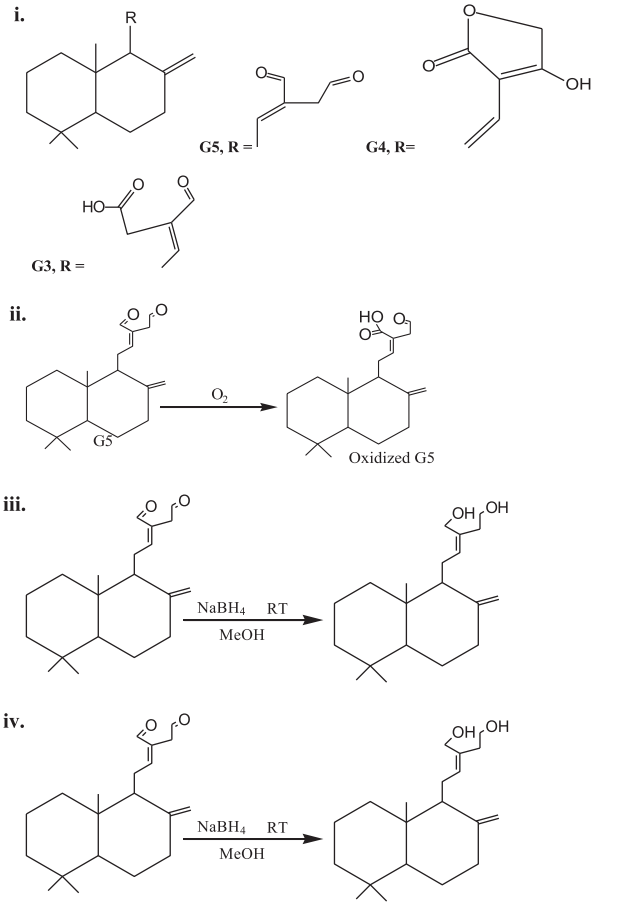

Fig. 3. (i) Structures of isolated compounds, (ii) the oxidation product of G5, (iii) reduction of G3 (iv) reduction of G5.

bone with modifications on the side chain Fig. 3. Although these compounds have been previously reported, they have not been isolated from *A. melegueta* as reported in the present study and more importantly no antibacterial activity relating to these compounds had been reported.

The ^{13}CNMR of G1, G3, G4 and G5 in comparison with published data is shown below Tables 2 and 3.

G3 was assigned the molecular formula of C_{20} H_{30} O_3 with $[M+H]^+$ m/z=319.2202, G4 was given a molecular formula C_{20} H_{28} O_3 $[M+H]^+$ m/z=317.2050, and G5 was assigned the formula C_{20} $H_{30}O_2$ and $[M+H]^+$ m/z=302. All have the signals for exo-methylene carbon at 108 ppm and a mass peak at m/z 137 which are characteristic of bicyclic labdane skeleton of diterpenes (Hong-Xi et al., 1996, 1995). The structural differences between the three compounds lie on the side chain which probably accounted for the variation in their potency against bacterial cells. G3 had one aldehyde group, G5 had two, and G4 had none. Furthermore, it was observed that when G5 was left on the bench at room temperature it changed from a yellowish oily liquid to a brownish liquid. The brownish oil called G5 brown (G5b) was subjected to NMR and antibacterial sensitivity studies. NMR showed a carboxylic acid carbon signal at 178.7 ppm resulting from the oxidation of the aldehyde group with a carbon signal at 197 ppm and the carboxylic acid proton was found at 9.2 ppm (data not shown). The proposed oxidation pathway is shown (Fig. 3ii).

The antimicrobial activity of the purified compounds against *L. monocytogenes*, *E. coli K-12*, *S. aureus* and *MRSA* were investigated. Their BA_{50} were determined against those organisms (Table 1). G1 and G2 have no measurable antibacterial properties against the

Table 2
^{13}C NMR signals of G1 and G3 compared with published data.

Test ^{13}C NMR (ppm) (G1)	Published ^{13}CNMR (ppm)	Test ^{13}CNMR (ppm) (G3)	Published ^{13}CNMR (ppm)
11	11	39.3	39.2
23	23	19.3	19.3
23.8	23.8	42.0	42.0
29	29	33.7	33.6
39	38.8	33.7	33.6
68.2	68.2	55.5	55.4
167.9	167.8	56.6	56.4
14.1	14.1	38.0	37.9
30.5	30.4	148.3	148
132.5	132.5	39.6	39.6
130.9	130.9	24.7	24.6
129	128.8	159.4	159.4
		136.1	135.7
		28.8	29.6
		174.8	175.3
		194.0	196.3
		108	107.9
		21.7	21.7
		14.4	14.4

Table 3
^{13}CNMR signal of G4 and G5 compared with published data.

Test ^{13}CNMR (ppm) (G4)	Published ^{13}CNMR (ppm)	Test ^{13}CNMR (ppm) (G5)	Published ^{13}CNMR (ppm)
41.3	40.9	14.5	14.4
19.4	19.1	19.4	19.2
42.5	42.2	21.8	21.7
33.9	33.5	24.1	24.2
55.0	54.6	24.8	24.6
23.6	23.3	33.7	33.5
37	36.7	33.7	33.5
149.7	149.3	38.0	37.8
62.3	61.9	38.0	37.8
39.7	39.4	39.3	39.2
135.6	135	39.4	39.3
121	120.9	39.7	41.9
128.3	128.2	42.1	42.0
136	136	55.5	55.3
68.2	68.2	56.6	56.4
171.6	171.6	108.0	107.8
1.08.5	108.3	135.0	134.9
33.9	33.5	148.2	148.1
15.5	15.1	160.1	159.9

test organisms. When tested against *E. coli* which is a gram negative organism, G3 was slightly more potent than gentamicin and the whole extract indicating that its presence may be largely responsible for the activity of the whole extract against *E. coli* (Table 1). On the other hand, G3 was less active against *L. monocytogenes* compared to the whole extract while the activities of G3 and G5 against wild *S. aureus* were similar. G4 had minor activity against *L. monocytogenes* but no activity against *E. coli* or *S. aureus*. The *in vitro* activity of G3 against *L. monocytogenes* was nine-fold better than the activity of ampicillin while that of G5b was16-fold better than ampicillin. G3 and G5 were also at least two-fold more active than gentamicin against *S. aureus*. This is in agreement with the findings reported by others that the diterpenoids was more active against Gram-positive organisms than the Gram-negative. The antimicrobial activities of these two compounds identified are highly noteworthy and could be regarded as new chemical entities worthy of further investigation. The antibacterial sensitivity testing carried out on G5b also indicated an increased activity with a BA_{50} of 1.8 ug/mL, 1.9 μg/mL and 5.2 μg/mL

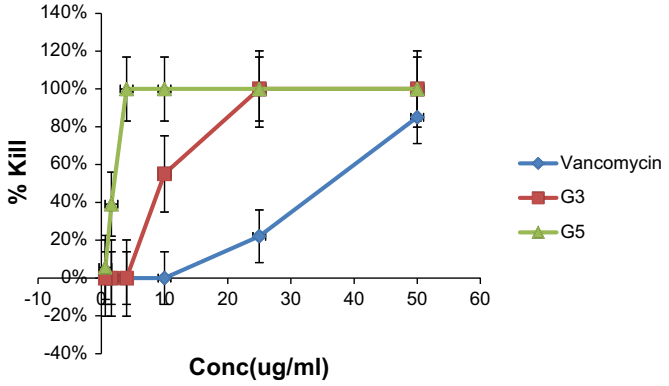

Fig. 4. Comparison of antimicrobial potency of G3 and oxidized G5(G5 brown) against MRSA.

against *L. monocytogenes*, *MRSA* and *S. aureus* respectively when compared to 22. 7 ug/mL and 40 ug/mL observed for G5 against *L. monocytogenes* and *S. aureus*. G5 had no measurable activity against MRSA.

MRSA is known to be resistant to most antibacterial agents, therefore, only the compounds with low BA_{50} against *Listeria* were tested on MRSA. The result showed that both G3 and G5b were more active than vancomycin which is the drug of last resort in the treatment of MRSA infections. Vancomycin had no activity at 25 μg/mL while G5b had a 50% bactericidal activity at 1.9 μg/mL (Fig. 4).

The BA_{50} of G3 (10 μg/ml) was also significantly ($p < 0.05$) lower than that of vancomycin (27 μg/ml) but higher than that of G5 brown. The increased activity of G5b could be attributed to its state of oxidation because G5 in non-oxidized form had a BA_{50} above 40 μg/mL against *S. aureus* which was 20 times less than the observed activity of the oxidized G5b.

On reduction, the aldehyde groups in the molecules (G3 and G5) were completely reduced as was confirmed by the disappearance of the aldedyde protons in the NMR spectrum (Supplementary material). In G5, the aldehyde signals at 9.3 and 9.6 ppm disappeared and the methylene signals appeared at 3.72 ppm and 3.99 ppm respectively. The reduction reaction is shown (Fig. 3iii–iv). An increase in the activity of reduced G5 (BA_{50}=5.1 μg/ml) was observed compared to G5 (BA_{50}=22.7 μg/ml) when tested against *L. monocytogenes* (Table 1) while reduced G3 showed decreased activity against MRSA (Table 1).

The cytotoxic and antifungal properties of G3 and G5 have been reported previously (Igwilo et al., 1991; Zheng et al., 2013). Antiplasmodial property of G5 has also been demonstrated (Duker-Eshun et al., 2002). The present study is the first to demonstrate the antibacterial properties of these compounds although the antibacterial property of the fruit extract (not rhizomes) of *A. melegueta* was reported in 1991 (Igwilo et al., 1991). G5 has a patent application filed for its antiplasmodial and antifungal activities (OKunji et al., 2000). The safety of G5 was established in the application. Therefore only G3 was investigated in this study for its toxicological properties against intestinal epithelial cells using CaCo2 cells as a model. The result showed that the LD_{50} of G3 against CaCo2 cells which were undifferentiated was 52.2 μg/ml which is about approximately 13 folds of the BA50 of the G3 against Gram-positive organisms and about 3 folds of its activity against *E. coli*. This value increased to 89.12 μg/ml when the cells were partially differentiated and further increased to 195 μg/ml. The LD_{50} against the fully differentiated cells is three times higher than the LD50 against the undifferentiated cells and about 48 times the BA_{50} against Gram-positive organisms. This suggests that fully differentiated CaCo2 cells which could be

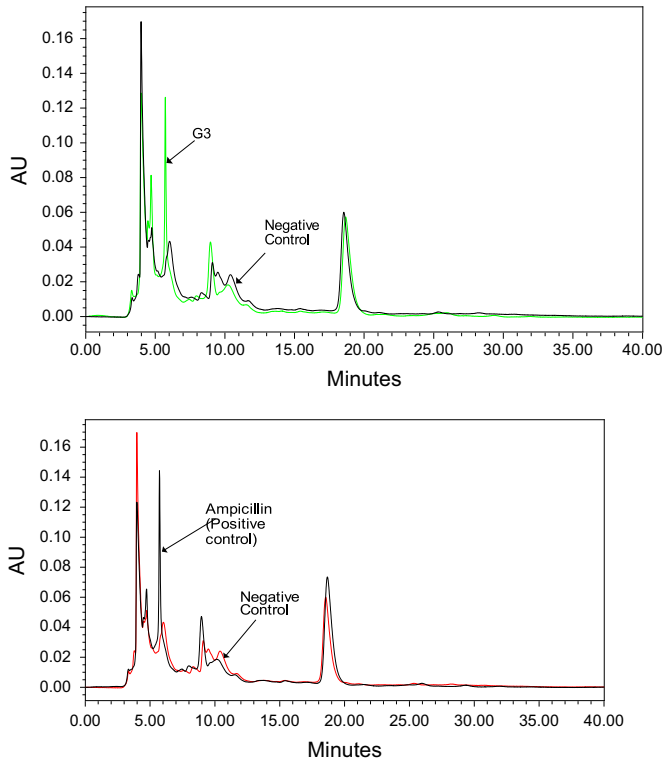

Fig. 5. Chromatograms of supernatant of NCTC 11994 treated with (i) G3 and (ii) ampicillin respectively.

considered to be normal cells are more tolerant to xenobiotics than cancer cells (undifferentiated cells). These findings was corroborated by a report of an investigation on Ergovaline toxicity on CaCo-2 cells. Ergovaline is an ergot alkaloid found in endophyte-infested fescue (Shappell, 2003).

While the reason for this is not yet known, it may be related to the continuously varying composition of gangliosides and the corresponding sialidase activity as the cells move from undifferentitated to differentiated cells. Gangliosides are glycosphyngolipids necessary for cell differentiation, proliferation, growth, signalling and apoptosis (Hakomori, 1990; Schnabl et al., 2009). Inhibition of ganglioside (a sphyngolipid) metabolism thus leading to accumulation, cell injury and cell death could be a mechanism of toxicity and may account for the differential toxicity of G3 on differentiated and undifferentiated CaCo2 cells (Soriano et al., 2005). This property can be exploited for cancer management as already their anti-tumor property has already been reported (Igwilo et al., 1991; Zheng et al., 2013).

A possible mechanism of antibacterial action of G3 was also investigated using *Listeria monocytogenes*, a gram positive bacterium as a model. G3 was chosen for this study because of its higher activity against *L. monocytogenes*. Ampicillin which was used as the positive control in this study is known to inhibit peptidoglycan (PG) synthesis (Matsumoto et al., 2012). This can result in the accumulation of PG precursors which could be extracted (Billot-Klein et al., 1992, Mott et al., 2008). HPLC analysis of the soluble extract in the supernatant showed a G3 profile which differed markedly from that of the negative control but identical to that of ampicillin (Fig. 5i–ii).

This suggests that G3 (as does ampicillin) inhibits peptidoglycan synthesis leading to the accumulation of the peptidoglycan precursors. One of the mass peaks of a preliminary UPLC-MS/MS electrospray analysis in the positive mode of the supernatant appeared at *m/z* at 597. This is the sodium adduct of *m/z* 574. The mass peak *m/z* 574 is a fragment of *m/z* 1148.7 which is the mass of UDP-MurNAcpentapeptide with alanine at the C terminus of the peptidoglycan precursors (David et al., 2004) suggesting the accumulation of peptidoglycan precurors.

It has been reported that aldehydes of plant derived antibacterials are highly reactive as they can form Schiff bases with membrane protein which may lead to the disruption of cell wall biosynthesis (Friedman, 1999; Uyeda et al., 1990). However, what membrane protein the aldehydes bind to is not known. Penicillin binding proteins are an example of bacterial membrane proteins and they play an in important role in mechanism of antibacterial action of penicillin. Whether G3 binds to penicillin binding protein has yet to be determined. It appears that whatever G3 binds to, it leads to the disruption of cell wall synthesis hence the similarity in the HPLC profile of the supernatants of ampicillin and G3. This suggests that G3 has a similar mode of action to the penicillins.

However, considering the potency of G3 which is far greater than that of ampicillin, it is possible that G3 may have additional mode of action. It has been suggested that aldehydes induce protein-DNA cross-linking resulting in replication failure in viruses (Permana and Snapka, 1994; Speit et al., 2010). This mechanism may also be possible in bacteria cells but this remains to be proven. G3 and G5 are highly promising compounds which require further investigation.

4. Conclusion

In conclusion, this study has validated the ethnobotanical use of *A. melegueta* in the treatment of urinary tract, skin infections and food poisoning. G3 and G5 are promising compounds and have potentials as antimicrobial leads in both human and veterinary medicine as *L. monocytogenes* is also known to cause listeriosis in farm animals. Further toxicological tests involving more cell lines should be carried out before proceeding to *in vivo* animal testing. Further attempt to synthesize these compounds in larger quantities beyond earlier efforts is necessary. This will enable studies on structure-activity relationship and also structure-toxicity relationship which was limited by the availability of starting material and pure compounds.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.12.035.

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