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ScienceDirect
Phytochemistry 68 (2007) 1436–1441
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Antibacterial and antioxidant cassane diterpenoids from *Caesalpinia benthamiana*

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Received 12 October 2006; received in revised form 6 March 2007
Available online 5 April 2007

Abstract

Bioactivity-guided fractionation of the light petroleum extract of *Caesalpinia benthamiana* (=*Mezoneuron benthamianum*) root bark has led to the isolation of two cassane diterpenoids, designated as benthaminin 1 and 2. A third compound, a deoxy form of caesaldekarin C (also referred to as methyl vouacapenate) which has previously been isolated from *Caesalpinia major*, *C. bonducella*, *Vouacapoua americana* and *V. macropetala*, was also isolated, together with β-sitosterol and stigmastenone. The antibacterial and antioxidant activities of these cassane diterpenoids have been assessed using the microdilution assay method and DPPH spectrophotometric and TBA lipid peroxidation assays. Benthaminin 1 was the more active antibacterial compound with MIC values of 47.8 μM for both *Staphylococcus aureus* and *Micrococcus flavus*. Benthaminin 2 was the more active antioxidant compound and showed IC_{50} values of 42.7 μM and 74.2 μM for the DPPH and TBA assays, respectively. Deoxycaesaldekarin C possessed both antibacterial and antioxidant activities. The presence of methyl ester and methyl functional groups as well as an unsaturated furan ring appears to confer antibacterial activity. On the other hand, the relatively stronger antioxidant activity of benthaminin 2 may be associated with the presence of an exocyclic methylene function.

Keywords: *Caesalpinia benthamiana* (Baill.) Herend. and Zarucchi; *Mezoneuron benthamianum* Baill.; Caesalpinaceae; Cassane diterpenoids; Antibacterial and antioxidant activities

1. Introduction

Caesalpinia benthamiana (Baill.) Herend. and Zarucchi (=*Mezoneuron benthamianum* Baill.) (Caesalpinaceae) (Herendeen and Zarucchi, 1990) is a shrub found mostly in secondary forest in Ghana and finds use in the treatment of topical infections and wounds (Irvine, 1961; Abbiw, 1990). Traditionally, a paste made from powdered root bark mixed with shea butter, palm oil or palm kennel oil may be used topically (Attah, 2003). Microbial infections and the presence of oxygen free radicals are known impediments to wound healing (Houghton et al., 2005). Notable among the micro-organisms delaying or inhibiting wound healing are *Staphylococcus*, *Streptococcus* and *Pseudomonas* species (Thomas, 1990). Any agent capable of eliminating or reducing the number of micro-organisms present in a wound as well as reducing the levels of reactive oxygen species may facilitate the wound healing process (Houghton et al., 2005). A number of *in vitro* models have been employed to assess relevant antimicrobial and antioxidant properties. Motivated by the severe health hazards caused by the ever increasing resistance of human pathogens to currently administered antimicrobial agents the roots of *C. benthamiana* collected from Ghana were investigated. Previous studies on the leaves of this plant resulted in the isolation of gallic acid and gallic acid derivatives possessing antibacterial activity (Cordell and Binutu, 2000), but no studies have been carried out on the root bark. However, piceatannol, *trans*-resveratrol, apigenin and scirpusin A have been isolated from a related species, *Mezoneuron cucullatum* (Lee et al., 1998). A variety of cassane diterpe-

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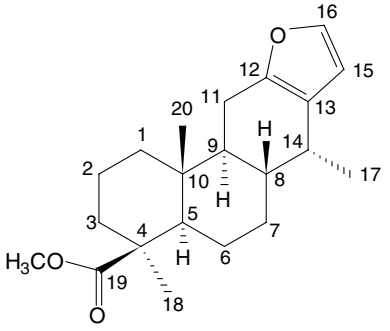
doi:10.1016/j.phytochem.2007.03.008

noids have been isolated from *Caesalpinia* species (Kitagawa et al., 1996; Peter et al., 1998).

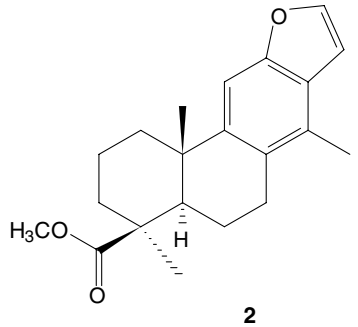
2. Results and discussion

From bioactivity-guided analysis carried out using agar overlay bioautography of five main fractions (MPF1-MPF5), obtained from the light petroleum extract of *C. benthamiana*, MPF1 and MPF2 were found to be active against *Bacillus subtilis*. MPF1 was subjected to column chromatography and aliquots were monitored by TLC to obtain five active fractions (MPF11–MPF15). Further repeated column chromatography of MPF11, MPF13 and MPF15 on silica produced solid materials which were then recrystallized from CH_2Cl_2 to afford compounds **1** (deoxycaesaldekarin C), **2** (designated benthaminin 1) and **3** (designated benthaminin 2).

The structures of these compounds were elucidated using a combination of 1D and 2D NMR spectroscopy, mass spectrometry (LREIMS, HREIMS and ESI) and reference to the literature (Peter et al., 1998; Kitagawa et al., 1996).



1



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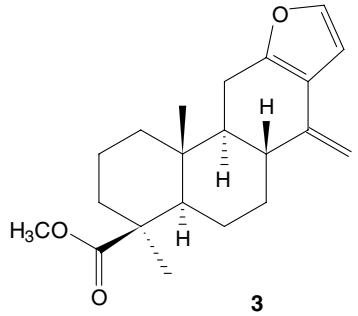

3

Accurate mass spectral measurements indicated that **1**, **2** and **3** had 20 C atoms and, in view of the cassane diterpenes isolated from the related *Caesalpinia* species, it seemed logical to assume that these compounds had an identical skeletal type.

Compound **1** was established to be deoxycaesaldekarin C by the identity of its spectral data with those of the literature (Peter et al., 1998; Kitagawa et al., 1996).

Benthaminin 1 (**2**) was isolated as white crystals from CH_2Cl_2 and had molecular formula $C_{21}H_{26}O_3$ based on HREIMS and ESI spectral analysis, *i.e.*, four hydrogens less than in **1**. The presence of ester and furan functional groups was evident from IR absorptions at 1725 and 725 cm^{-1}, respectively. The UV spectrum had absorption maxima at 220, 255, 280 and 295 nm, which was consistent with the presence of a benzofuran moiety in the structure. The ^1H NMR spectrum (Table 1) is broadly similar to that of **1**, but showed resonances for the 2,3-disubstituted furan at δ7.26 (*d*, $J = 1.6$ Hz) and δ6.71 (*dd*, $J = 2.0$, 0.7 Hz), downfield from the corresponding positions in the spectrum of **1**. The presence of an additional aromatic singlet at δ6.71 and an aromatic methyl singlet at δ1.30 confirmed the presence of a 2,3-disubstituted benzofuran moiety, the aromatic ring of which being ring C in a caesaldekarin-type nucleus. A 3H singlet also appeared at δ3.67, indicating the presence of a methyl ester group. These data are broadly consistent with the structure being an aromatized form of deoxycaesaldekarin C, and is so assigned structure **2** and is named benthaminin 1. Analysis of the HMQC spectrum revealed that the aromatic hydrogen at δ6.71 was directly attached to a carbon giving a signal at δ104.3 (assigned to C-11). This hydrogen also showed HMBC correlations to aromatic carbons at δ153.5 (C-12), δ127.5 (C-13), δ125.4 (C-8) and a quaternary carbon at δ37.8 (C-10). These findings confirm that ring C is aromatic. The methyl group signal at δ1.26 also had HMBC correlations to the ester carbonyl signal at δ179.2, the quaternary carbon at δ47.7 (C-4) and a methylene carbon at δ38.9 (assigned to C-3). These observations allowed assignment of the NMR data as shown in Tables 1 and 2.

Benthaminin 2 (**3**) was also obtained as white crystals from CH_2Cl_2 and had a molecular formula of $C_{21}H_{28}O_3$ as determined by HREIMS. The UV spectrum showed maxima at 210 and 235 nm, similar to the maxima in the spectrum of **1**, suggesting the presence of a furan ring, confirmed by its absorption at 729 cm^{-1}. The absorption at 1725 cm^{-1} indicated the presence of an ester carbonyl. The ^1H NMR spectrum showed resonances due to two quaternary methyls at δ1.23 (*s*) and δ0.97 (*s*) attributable to a methyl ester at δ3.67 and a 2,3-disubstituted furan ring δ6.43 (*dd*, $J = 1.9$, 1.8 Hz) and δ7.23 (*d*, $J = 1.6$ Hz) (see Tables 3 and 4).

The presence of a 2,3-disubstituted furan was evident from signals at δ7.23 (*s*) and 6.43 (1 H, *dd*, $J = 1.9$, 1.8 Hz), similar to the data for **1**, for two adjacent olefinic hydrogens in the furan, confirmed by the ^{13}C NMR signals for four olefinic carbons, (δ152.1, 141.4, 118.8 and 106.3). The ^{13}C NMR spectrum of this compound also showed 21

Table 1
^{1}H NMR spectral data (δ, Hz) of compounds **1**–**3** (500 MHz, $CDCl_3$)

H	1	2	3
1α	1.45 (*m*)	1.53 (*m*)	1.50 (*m*)
1β	1.75 (*m*)	1.77 (*m*)	1.75 (*m*)
2α	1.48 (*m*)	1.55 (*m*)	1.50 (*m*)
2β	1.71 (*m*)	1.67 (*m*)	1.72 (*m*)
3α	1.12 (*m*)	1.25 (*m*)	1.25 (*m*)
3β	1.70	1.74 (*m*)	1.79 (*m*)
5	1.77 (*m*)	1.80 (*m*)	1.81 (*m*)
6α	1.56 (*m*)	1.58 (*m*)	1.57 (*m*)
6β	1.73 (*m*)	1.88 (*m*)	1.78 (*m*)
7α	1.5 (*m*)	2.77 (*m*)	2.22 (*m*)
7β	–	2.88 (*dd, J*=6.7, 16.9)	2.26 (*m*)
8	1.75 (*m*)	–	1.79 (*m*)
9	1.57 (*m*)	–	1.56 (*m*)
11α	2.57 (*dd*, $J = 7.0$, 16.8)	6.72 (*s*)	2.69 (*dd*, $J = 5.4$, 16.7)
11β	2.37 (*dd*, $J = 10.2$, 16.8)	–	2.47 (*dd*, $J = 11.2$, 16.7)
14	2.6 (*m*)	–	–
15	6.18 (*d*, $J = 1.6$)	6.71 (*dd*, $J = 2.0$, 0.7)	6.43 (*dd*, $J = 1.9$,1.8)
16	7.22 (*d*, $J = 1.6$)	7.26 (*d*, $J = 1.6$)	7.23 (s)
17a	0.98 (*d*, $J = 7.1$)	–	5.07 (s)
17b			4.84 (*s*)
18	1.22 (*s*)	1.30 (*s*)	1.23 (*s*)
20	0.93 (*s*)	1.26 (*s*)	0.97 (*s*)
OMe	3.68 (*s*)	3.68 (*s*)	3.67 (*s*)

Table 2
^{13}C NMR spectral data (δ) of compounds (500 MHz, $CDCl_3$)

C	**1**	**2**	**3**
1	36.9	36.6	36.5
2	17.9	18.7	17.9
3	38.6	38.9	38.3
4	47.5	47.7	47.4
5	45.7	44.3	49.0
6	30.8	27.5	29.9
7	24.1	21.7	22.4
8	31.5	125.4	52.1
9	35.7	147.2	36.2
10	36.8	37.8	36.8
11	22.0	104.3	23.8
12	149.4	153.5	152.1
13	122.5	127.5	118.8
14	49.6	128.3	142.6
15	109.6	105.0	106.3
16	140.4	144.2	141.4
17	17.6	25.6	103.9
18	17.1	16.6	16.9
19	179.5	179.2	179.3
20	14.7	15.9	14.6
OMe	51.9	52.0	52.0

carbon signals (Table 2), two of which were accounted for by the presence of a methyl ester functionality, plus two methyl at δ16.9 and 14.6, seven methylene (δ36.5,17.9, 38.3, 29.9, 22.4, 23.8 and 142.6) and three methine (δ49.0, 52.1 and 36.2) carbons. The ^{1}H NMR spectrum of **3** is similar to that of **1**, but the signal for the secondary methyl in **1** was replaced by exocyclic methylene hydrogen signals at δ5.07 in the spectrum of **3**. The 2D HMQC spectrum of **3** showed that the exomethylene hydrogens were directly attached to a carbon which resonated at δ103.9. The HMBC spectrum also indicated correlations from a quaternary methyl at δ1.23 to a methyl ester at δ179.3 (C-19), a quaternary at carbon δ 47.4 (C-4) and a quaternary carbon at δ 36.8 (C-10). The rather high chemical shift down field observed for C-17 (δ103.9) appears to confirm the presence of an exocyclic methylene group. The conformations of the Hs at C-8 and C-9 appeared to be the same as for **1**, since no nOe correlation was seen between the ^{1}H NMR signals for these in the NOESY spectrum, which did however show a correlation between 9-H and 5-H. These results are summarized in Tables 1 and 2 and led to the structural proposal for **3** (which is named benthaminin 2).

Bioactivity studies of the compounds revealed that compounds **1-3** possess antibacterial activity, with **2** being the most active (MIC of 47.0 μM against *S. aureus* and *M. flavus*) followed by **3** (48.7 μM) for both bacteria. Although of interest in explaining the traditional use of the plant, these values are too high to attract commercial interest.

Compounds **1** and **3** showed free radical scavenging and antioxidant activities with **3** being the more active compound, having IC_{50} values for DPPH and TBA assays of 42.7 and 74.2 μM, respectively. The relatively stronger antioxidant activity of **3** appears to be associated with the presence of the exocyclic methylene functional group, C-17. The antioxidant property of this plant has not been previously reported but the antioxidant activity in a related taxon *Mezoneuron cucullatum*, has been documented (Lee et al., 1998). This study is the first report of the presence of cassane-type diterpenoids in this plant. β-Sitosterol and stigmastenone are plant sterols that are widely distributed in nature – however this is the first report of their isolation from this genus. The folkloric use of this plant for the treatment of infections and wounds may be partly due to its antibacterial and antioxidant properties.

Table 3
Minimum inhibitory concentration (MIC; μM) of compounds on test organisms

Test organism	Strain	1	2	3
Staphylococcus aureus (wild)	NCTC 4263	189	47	48
Bacillus subtilis	NCTC 10073	47	95	190
Streptococcus faecalis	NCTC 775	>1000	383	>1000
Pseudomonas aeruginosa	NCIMB 1042	757	383	>1000
Micrococcus flavus	NCTC 7743	94	47	48
Methicillin-resistant *S. aureus*	SA 1199B	194	98	96
Tetracycline-resistant *S. aureus*	XU 212	388	98	97
Erythromycin-resistant *S. aureus*	RN 4220	>1000	>1000	>1000

All tests were performed in triplicate; tetracycline (200 μ/ml) served as positive control and all organisms tested against it showed no growth.

Table 4
IC_{50} values for free radical scavenging activity and inhibition of lipid peroxidation, respectively, of bovine brain extract liposomes by **1** and **3**

Compound	IC_{50} DPPH (μM)	IC_{50} TBA (μM)
1	97.4	134.8
3	42.7	74.2
Catechin	45.2	NA
Propyl gallate	NA	2.7

Catechin and propyl gallate served as positive controls for DPPH and TBA assays, respectively; NA, not Applicable.

3. General experimental procedures

3.1. General

Mps were determined using a Gallenkamp instrument and are uncorrected. The UV was obtained on a Perkin–Elmer Lambda 2 UV/VIS spectrophotometer in MeOH. IR spectra were recorded on a 1720X Perkin–Elmer FT-IR spectrometer. LREIMS spectra were run on the JEOL ASW505, the high resolution spectra on the Bruker Apex III system. Low resolution ESI mass spectra were run on a QToF (Waters) instrument. The NMR spectra were obtained on a Bruker 500 spectrometer with chemical shifts reported in δ (ppm) using TMS as an internal standard.

3.2. Plant material

The root bark of *C. benthamiana* (Baill.) Herend. and Zarucchi was collected from Akwapim Mampong in the Eastern region of Ghana in August, 2003. It was authenticated by Mr. H R Blagogee, a taxonomist at the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong Akwapim, Ghana, where voucher specimen #RADMB11 is deposited.

3.3. Extraction and isolation

2.7 kg (dry weight) of coarsely powdered root bark of *C. benthamiana* were extracted with light petroleum (5 L) using a Soxhlet apparatus for 24 h. The extract was concentrated under reduced pressure at 40 °C to dryness to obtain yellowish-brown syrup [40.9 g (1.5%) yield]. This extract was subjected to VLC on silica gel 1500 g, eluted initially with light petroleum (5 L) and subsequently with 5% EtOAc in light petroleum (12 L), 10% (15 L), 15% (6 L), 25% (5 L), 50% (2 L) and finally with EtOAc (4 L). The TLC profiles of aliquots collected enabled five major fractions to be bulked: MPF1 (12.8 g), MPF2 (10.6 g), MPF3 (2.6 g), MPF4 (3.2 g) and MPF5 (10.2 g). MPF1 and MPF2 were active. MPF1 was subjected to repeated column chromatography on silica gel (450 g), eluting with light petroleum and gradually increasing the polarity of the solvent by 1% increments of EtOAc. Aliquots were monitored on TLC to obtain five active fractions (MPF11– MPF15). Impure crystals were obtained from fractions MPF11, MPF13 and MPF15 after subjecting the three fractions individually to further chromatography on a smaller column. Recrystallization from CH_2Cl_2 of the crystals obtained afforded compounds **1**, **2** and **3** (30 mg, 5.5 mg, and 12 mg, respectively). The TLC profiles of MPF12 and MPF14 revealed that MPF12 was a mixture of **1** and **2**, and MPF14 was a mixture of **2** and **3**, with other impurities. MPF12 was repeatedly CC on silica gel eluting with light petroleum with gradual increments (0.5%) of polarity using EtOAc to yield two major solids. These were recrystallized in CH_2Cl_2 to afford further quantities of **1** (180 mg) and **2** (35.5 mg). MPF15 was further purified using PTLC to yield **3** (58 mg). The final yields of compounds obtained from MPF1 were 210 mg (**1**), 41 mg (**2**) and 70 mg (**3**). MPF2 was also CC to yield fractions MPF21–MPF22. Further CC of MPF21 led to fractions MPF211 and MPF222. Further CC of MPF222 and MPF22 in smaller columns produced β-sitosterol and stigmastenone, respectively.

3.3.1. Compound **1** (deoxycaesaldekarin C)

Colourless crystals; m.p., 103.9–104.1°, uncorrected; UV (MeOH) λ_{max} 220 nm; IR (KBr) ν_{max} 726, 1725 cm^{-1}; LREIMS *m/z* 330; ESI observed: MH^+ at *m/z* 331.22676; calculated for $C_{21}H_{31}O_3$, 331.22712; corresponding with molecular formula for compound **1** of $C_{21}H_{30}O_3$. See Tables 1 and 2 for ^1H and ^{13}C NMR data.

*3.3.2. Compound **2** (benthaminin 1) (systematic name: 4,7,11b-trimethyl-1,2,3,4,4a,5,6,11b-octahydro-10-oxa-cyclopen[b]phenanthrene-4-carboxylic acid methyl ester)*

Colourless crystals; m.p. 196.8–197.0°, uncorrected; UV (MeOH) λ_{max} 220, 255, 280, 295 nm; IR (KBr) ν_{max}, 725, 1725 cm^{-1}; LREIMS m/z 326; ESI observed: MH^+ at *m/z* 327.19568; calculated for $C_{21}H_{27}O_3$, 327.18470; corresponding with molecular formula for compound **2** of $C_{21}H_{26}O_3$. See Tables 1 and 2 for ^1H and ^{13}C NMR data.

*3.3.3. Compound **3** (benthaminin 2) (systematic name: 4,11b-dimethyl-7-methylene-1,2,3,4,4a,5,6,6a,7,11,11a,11b-dodecahydro-10-oxa-cyclopental[b]phenanthrene-4-carboxylic acid methyl ester)*

Colourless crystals; m.p. 107.2–107.3°, uncorrected; UV (MeOH) λ_{max} 210, 235nm; IR (KBr) ν_{max}, 729, 1725 cm^{-1}; LREIMS *m/z* 328; ESI observed: MH^+ at *m/z*; 329.21220; calculated for $C_{21}H_{29}O_3$, 329.20490; corresponding with molecular formula for compound **3** of $C_{21}H_{28}O_3$. See Tables 1 and 2 for ^1H and ^{13}C NMR data.

3.4. Determination of minimum inhibitory concentration (MIC)

Stock solutions of all compounds were prepared by dissolving 4 mg of the compounds in 80 μl of DMSO. Sterile water was added with slight heating to aid dissolution and the volume made up to 2 ml in a sterile bottle. This was then sonicated to ensure complete dissolution. The stock mixture was passed through a 0.2 μm pyrogenic filter to sterilize the solution and serially diluted to arrive at concentrations between 1000 μg/ml and 7.8 μg/ml. The inocula of micro-organisms were prepared from broth cultures, and serial dilutions were made to achieve a suspension of approximately 10^5CFU/ml. For every experiment, a sterility check (2% DMSO and medium), negative control (2% DMSO, medium and inoculum), and positive control (2% DMSO, medium, inoculum and water-soluble antibiotic) were included. The 96-well plates were prepared by dispensing into each well 100 μL each of double strength nutrient broth, 100 μL of test solutions and 20 μl of the inoculum. Contents of each well were thoroughly mixed with a sterile-tipped multi-channel pipette and the plates incubated at 37 °C for 24 h. Any growth of the micro-organisms was determined by adding 20 μl of a 5% solution of tetrazolium salt (MTT) and incubating for a further 30 min. Dark coloured wells indicated growth whilst colourless wells indicated inhibition of growth of organisms. Tetracycline (200 μg/ml) served as a positive control. All experiments were performed in triplicate.

3.4.1. Micro-organisms used

The following bacterial strains obtained from the UK National Culture Collection and School of Pharmacy, University of London, were used in the bioassay: *M. flavus* (NCTC 7743), *B. subtilis* (NCTC 10073), *Staphylococcus aureus* (NCTC4163), multidrug-resistant *S. aureus* SA-1199B, tetracycline-resistant *S. aureus* XU 212, erythromycin-resistant *S. aureus* RN 4220, *Streptococcus faecalis* (NCTC 775) and *Pseudomonas aeruginosa* (NCIMB 10421).

3.5. Scavenging activity on DPPH radical

The free radical scavenging activity of the compounds were measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Brand-Williams et al. (1995) with slight modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 0.5 ml of the samples dissolved in methanol and using a range of 12.5–200 μg/ml. After 20 min, the absorbance was measured at 525 nm. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH}^{\cdot}\ \text{scavenging activity}(\%) = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the test substance. A graph of % inhibition against concentration was plotted and IC_{50} determined using the GraphPad Prism programme (Brand-Williams et al., 1995). DPPH solution alone served as control (A_0).

3.6. Antioxidant activity against the formation of lipid peroxide

The lipid peroxidation assay was performed to determine the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation (LPO) on bovine brain liposomes (Galvez et al., 2005). Bovine brain liposomes obtained from Sigma were suspended in phosphate-buffered saline (PBS) (5 mg/mL) and sonicated in an ice-water bath until the suspension appeared to be homogenous. Glass balls (borosilicate solid-glass beads) were included to aid the process. The suspension was not allowed to become hot during the sonication process, as this might have caused lipid degradation. Lipid peroxidation was assayed using the formation of malondialdehyde (MDA) as indicator. For each compound a range of concentrations (12.5–200 μg/ml) were tested. The solution test reaction mixture (ST) consisted of 0.2 mL of liposomes, 0.1 mL of aqueous $FeCl_3$ (1 mM), 0.1 mL of aqueous ascorbic acid (1 mM), 0.5 mL of PBS, and 0.1 mL of the solution of compound to be assessed. Different controls were used, the first being the full reaction mixture (FRM), where the test compound solution was omitted and the solvent (methanol) was added instead. In the second control, a suspension of liposomes alone (B) was employed to test if liposomes underwent a self-peroxidation process during the incubation period, and for the third control the absorbance was measured for each solution of compound with PBS (SA), to take into consideration the background. Propylgallate (10^{-4} M) in FRM was used as a positive control. Four replicates were carried out for each mixture (test, FRM and all controls) and each mixture was incubated at 37 °C for 20 min. After this time

the TBA test was performed by adding 0.1 mL of 2% butylated hydroxytoluene (BHT) in EtOH followed by 0.5 mL of 1% w/v thiobarbituric acid (TBA) in 50 mM NaOH and 0.5 mL of 25% HCl. The system was heated to 90 °C for 30 min. After cooling, 2.5 mL of butanol was added to each tube. The mixture was vortexed and centrifuged at 3500 rpm for 20 min at room temperature. The absorbance of the MDA–TBA complex in the upper layer was determined at 532 nm. The percentage of lipid peroxidation inhibition was assessed by comparing the absorbance of the full reaction mixture with that of the test solution reaction mixtures where the substance to be assessed was included. Inhibition was calculated as follows:

$$\%\text{Inhibition} = [(\text{FRM} - B) - (\text{ST} - \text{SA} - B)]/(\text{FRM} - B) \times 100$$

where FRM is full reaction mixture, B is blank, ST is test reaction mixture, and SA is a test solution alone. A graph of % inhibition against concentration was again plotted and IC_{50} determined from the Sigma programme (Galvez et al., 2005).

Acknowledgements

We gratefully acknowledge Dr. Simon Gibbons for his generous gift of resistant strains of bacteria, and the assistance rendered by Messrs A. Ampomah Appiah and H.R. Blagogee of the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong Akwapim, Ghana, in providing and identifying plant materials. Mrs. J. Hawkes (University of London Intercollegiate NMR Service), A. Cakebread and R. Tye (ULIRS Mass Spectrometry from King's College London) are appreciated for NMR and MS measurements, respectively. R.A.D. is supported by the Commonwealth Scholarship Commission, UK.

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