(–)-Brunneusine, a new phenolic compound with antibacterial properties in aqueous medium from the leaves of *Agelanthus brunneus* (Engl.) Tiegh (LORANTHACEAE)

Moifo Kuete Thomas Wieland[1], Ambassa Pantaleon[1], Moungang Luciane Marlyse[2], Ngameni Bathelemy[3,4,*], Thomas E. Storr[4], Ngadjui Tchaleu Bonaventure[1] and G. Richard Stephenson[4,*]

[1] *Department of Organic Chemistry, University of Yaoundé I, Yaoundé, P.O. Box 812, Cameroon*
[2]*Department of Biology and Animal Physiology, Faculty of Science, University of Yaoundé I, Cameroon*
[3]*Department of Pharmacognosy and Pharmaceutical Chemistry, University of Yaoundé I, Yaoundé, P.O. Box 1364, Cameroon*
[4]*School of Chemistry, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK*

Corresponding authors':

Ngameni Bathelemy:bath_ngameni@yahoo.fr

G. Richard Stephenson:g.r.stephenson@uea.ac.uk

ABSTRACT

Agelanthus brunneus (Loranthaceae) is a hemiparasitic plant growing on *Senna siamea* (Fabaceae). The chemical investigation of its leaves and flowers led to the isolation of one new phenolic compound namely (–)-brunneusine (**1**), together with thirteen known compounds. The crude leaves and flowers extracts (CLE and CFLE) with their ethyl acetate fractions (EAFL and EAFFL) and some isolated compounds (**1-3**; **8-9**; **11-14**) have been tested on four bacterial species of sanitary importance isolated in an aquatic environment. All the samples except compound **3** showed antibacterial activity with MICs ranging from 0.43 to $8.88.10^3$ µg/mL and MBCs from 0.43 to $3.55.10^3$ µg/mL. Compounds **9** and **14** showed better activity on all bacterial species tested with MICs ranging from 0.43 to 27.77 µg/mL. Only CLE, EAFL, compounds **14**, **2**, **8**, and **9** showed bactericidal effects on all bacterial species tested.

Keywords: Brunneusine, *Agelanthus brunneus*, Loranthaceae, antibacterial activity

1. Introduction

Microorganisms are widely distributed in the environment and the majority inhabits terrestrial or aquatic environments [1]. Indeed, water, apart from its importance in the daily life of man for nutritional and practical needs (body hygiene, household tasks, etc.), is also a medium for waterborne human pathogen dissemination. Access to drinking water is an obstacle to improving the health of populations, despite the water supply and sanitation programs that have been put in

place, since one in three people in the world, i.e. 2.4 billion people, still live without adequate sanitation facilities, with sub-Saharan Africa being the greatest [2, 3].

Studies conducted by Ako *et al.* (2009) [4] on boreholes and rivers in the city of Douala and by Nnanga *et al*. (2014) [5] on wells and boreholes in the Mvog-Betsi district of Yaoundé, revealed a link between the contamination of different water sources and the waterborne diseases recorded in hospitals in these areas, particularly gastroenteritis. The situation is becoming increasingly alarming because of the emergence of strains of microorganisms that are multi-resistant to antibiotics as a result of the misuse and uncontrolled use of antibiotics [6]. To remedy this situation, in recent years various studies have focused on the search for new therapies from various natural resources, in particular medicinal plants [7]. The richness of plants in secondary metabolites and their accessibility justify their use in traditional medicine by almost 80% of the African population, who sometimes use conventional medicines in combination [8]. As part of our continuing search for bioactive compounds from Cameroonian medicinal plants displaying antibacterial activities, *Agelanthus Brunneus* (Eng.) Tiegh was selected and investigated.

Agelanthus brunneus (Engl.) Tiegh is a hemiparasitic plant belonging to the Loranthaceae family (mistletoes) which comprises more than 70 genera and about 900 species growing in Africa, Asia, America and Europe [9]. In Cameroon, the Loranthaceae family is represented by 26 species distributed in 7 genera [10, 11]. Some species of the *Agelanthus* genus are frequently used in Cameroonian traditional medicine to treat several diseases such as diabetes, abdominal pains, parasitic and bacterial diseases, urinary tract infections, malaria and cancer [12, 13]. Crude extracts from some species have exhibited vasorelaxant, anti-inflammatory, antiplasmodial and antibacterial activities [14-16]. Previous phytochemical studies on plants from this genus have led to the isolation of secondary metabolites belonging to tannins, anthracenosides, flavonoids, phenols anthraquinones, alkaloids, saponins, sterols and triterpenes classes [14, 17].

The present study reports the first phytochemical investigation on the species *A. brunneus* (Eng.) Tiegh and the structural elucidation of an unreported antibacterial phenolic compound.

2. Results and discussion

Leaves and flowers of *A. brunneus* (Eng.) Tiegh were collected from the host plant *Senna siamea*. The air-dried leaves and flowers of this plant were crushed and extracted at room temperature with a mixture of CH_2Cl_2/MeOH (v/v: 1:1). The residue obtained after evaporation of the solvent was fractionated using liquid-liquid extraction for the leaves and solid-liquid extraction for the flowers. The fractions obtained were purified by column chromatography on silica gel, eluted with *n*-hexane, and with mixtures of *n*-hexane/EtOAc and EtOAc/MeOH of increasing polarity. This process led to the isolation of a previously undescribed compound, brunneusine (**1**), together with the known compounds dodoneine (**2**) [18, 19], methyl gallate (**3**)

[20], lupeol (**4**), taraxerol (**5**) [21], globimetulin C (**6**) [22], pentacosanoic acid (**7**) [23], rel-(1*R*,5*S*,7*S*)-7-[2-(4-hydroxyphenyl)ethyl]-2,6-dioxabicyclo [3.3.1]nonan-3-one (**8**) [19, 20, 24, 25], gallic acid (**9**) [21], 3-*O*-β-D-glucopyranoside of β-sitosterol (**10**) [26], quercetin (**11**) [19, 26, 27], and quercetin 3-*O*-α-L-arabinofuranoside (**12**) [28, 29], quercetin-3-*O*-α-L-rhamnopyranoside (**13**) [19], and quercetin 3-*O*-α-arabinopyranoside (**14**) [30] (Figure 3). All the fourteen compounds are isolated for the first time from *Agelanthus brunneus* (Eng.) Tiegh. The known compounds were identified by their ^{1}H, ^{13}C NMR, 2D NMR and mass spectral data and confirmed by comparison of these data with those published in the literature.

Compound **1** was obtained as a white amorphous solid, $[\alpha]^{22.1}_{D} = -37.1$ (c 0.014, CH_2Cl_2) from the mixture *n*-hexane/EtOAc 55%. It gave positive $FeCl_3$ test, indicating the presence of phenol moiety. Its molecular formula was deduced as $C_{15}H_{20}O_5$ from the analysis of its HR-ESI-MS showing the fragment ion at *m/z* 263.1308 ($[M+H-H_2O]^+$, $C_{15}H_{19}O_4^+$; calc. 263.1283) corresponding to six degrees of unsaturation (see Supplementary Material, Fig.S1). The IR spectrum displayed bands at 3338 cm^{-1} (O-H), 2920 cm^{-1} (C-H), 1699 cm^{-1} and 1615 cm^{-1} (C=O), 1516 cm^{-1} (C=C), and 1081.5 cm^{-1} (C-O-C). All 15 carbons were resolved in the ^{13}C-NMR, DEPT, and HSQC spectra as signals corresponding to five sp^3 methylene groups appearing at δ_C 29.5, 30.4, 36.2, 36.8 and 37.7, seven methine groups (among them three oxygenated (O-CH) at δ_C 64.8, 65.8 and 73.6, as well as two pairs of magnetically equivalent aromatic CH positions (δ_C 129.2 (2C) and 154.8 (2C) on the 4- substituted phenolic ring) and three quaternary C-atoms (among them a carbon of a lactone group (δ_C 171.1). The ^{1}H NMR spectrum of compound **1** (see Supplementary Material, Fig. S3, S4) exhibited signals for a *p*-substituted benzene ring δ_H 6.59 (d, *J* = 8.4 Hz, 2H) and 6.84 (d, *J* = 8.4 Hz, 2H) (Table 1). The same spectra also showed the presence of three oxymethine (O-CH) groups at δ_H 4.76 (dd, *J* = 3.6, 1.8 Hz, 1H), 4.24 (brs, 1H) and 3.56 (ddd, *J* = 14.7, 7.3, 3.8 Hz, 1H). The ten remaining protons between δ_H 1.47 and 2.67 ppm were assigned to five methylene proton groups using correlations observed in the COSY spectrum in combination with those of the HMBC spectrum (Figure 1). In fact, COSY spectrum of **1** exhibited coupling between CH_2 H-atoms at 2.53 (m, H-4'b) and those at 1.54 (m, H-3'a) suggesting their vicinal position. This was confirmed by the HMBC spectrum of compound **1** in which cross-peaks were observed between the diastereotopic protons H-1' at 1.47 (ddd, 13.9, 11.7, 2.1,1H) and 1.84 (m, 1H) with C-atoms C-2' (δ_C 64.8), C-5 (δ_C 29.5), C-6 (δ_C 73.6). Similarly, there were cross-peaks between H-5 at δ_H 1.80 (m, 1H) and 1.86 (m, 1H) with C-4 (δ_C 65.8), C-6 (δ_C 73.6) and C-1' (δ_C 36.8) ; between H-3' at δ_H 1.54 (m, 1H) and δ_H 1.66 (m, 1H) and C-4' (δ_C 30.4), C-2' (δ_C 64.8) and C-1" (δ_C 132.4). The same spectrum also showed correlation between H-6 at δ_H 4.76 (dd, *J* = 3.6, 1.8 Hz) and C-1', C-5 and the ester at C-2 (δ_C 171.1) suggesting the tetrahydro-*2H*-pyran-2-one substituted at the 4 and 6 positions and a 4-phenyl(2-hydroxy)butyl moiety. At this junction, the NMR data of compound

1 were closely similar to those of dodoneine [18] **2** also isolated in this study. However, the signal of the ethylenic protons (δ_H 5.95 and 6.98) and carbons $\Delta^{3,4}$ (δ_C 134.8 and 121.8) were absent in the ^1H and ^{13}C RMR spectra of **1.** They were rather replaced by a methylene at C-3 ($\delta_{H/C}$ 2.67/36.2) and an oxymethine at C-4 ($\delta_{H/C}$ 4.24/65.8). This clearly shows that **1** derives from **2** by the hydration of the double bond $\Delta^{3,4}$.

The relative configurations of the stereogenic centres at C-4, C-6 and C-2' of compound **1** were deduced from the NOESY spectrum compared to those of dodoneine **2** in which H-2' and H-6 are both *Cis* as shown in figure 3. On this basis, the NOE interactions observed between H-2' and both H-6 and H-4 lead us to assign the relative stereochemistry as shown for **1b**, with both the alkyl and hydroxyl groups equatorial. The interconversion of the two chair structures [*cis* isomer **1a** (chair 1) and *cis* isomer **1b** (chair 2)] is thermodynamically possible but it is reasonable to expect the NOESY spectrum to be most strongly influenced by the $^1H\cdots{}^1H$ distances present in the more stable conformation. This type of equilibrium has been documented previously in the literature describing both natural and hemi-synthesized products [31-34]. Recently, similar hemi-synthesized cyclohexanic compounds using their chair forms have been reported [31]. All those Considerations allowed us to confirm that the relative stereochemistry of the chemical structure of the isolated new compound **1** is that of 1b where both the alkyl and hydroxyl substituents occupy the equatorial position.

Considering the cyclic lactone of **1** as an approximately chair conformation, slightly flattened by the exocyclic C=O bond of the lactone, structure **1b** is expected to be the more stable form because it avoids the unfavorable axial positioning of the alkyl substituent at C-6 in **1a** (Figure 2).

Thus, based on the known absolute configuration of its presumed precursor dodoneine (**2**) [(+)-(4*R*,6*S*)-(5,6-dihydro-[2'-hydroxy-4'-(4-hydroxyphenyl)butyl]-2H-pyran-2-one) proved by an X-ray crystallographic determination of the relative stereochemistry of the 10-camphorsulfonyl phenolic ester of a cyclisation product formed by Michael addition of the 2' hydroxyl group on the dihydropyran ring] [19], compound **1** was provisionally determined as (–)-(4*R*,6*S*,2'*S*)-4-hydroxy-6-[2'-hydroxy-4'-(4''-hydroxyphenyl)butyl]tetrahydro-2H-pyran-2-one or (–)-brunneusine, a new secondary metabolite isolated for the first time and not yet synthesized.

Agelanthus brunneus (Eng.) Tiegh leaf and flower dichloromethylene-methanol (1:1) crude extracts (CLE and CFLE) with their ethyl acetate fractions (EAFL and EAFFL) and some of the isolated compounds (**1-3**; **8-9**; **11-14**) were all tested on four multiresistant bacterial strains isolated in an aquatic environment and known to have damaging effect on human health: *Escherichia coli*, *Streptoccoccus faecalis*, *Salmonella typhi*, *Vibrio cholerea*. The results are summarized in Tables 2 to 4. All the tested products except 3 showed antibacterial activity with

MICs ranging from 0.43 to $8.88.10^3$ µg/mL. Compounds **14** and **9**, showed better activity on all the tested bacterial strains with MICs ranging from 0.43 to 27.77µg/mL.

The Minimal Bactericidal Concentrations (MBC) of the different extracts, fractions and isolated compounds was determined and the MBC/MIC ratios calculated. These MBC/MIC ratios varied between 1 and 10 for the bacterial species tested. The obtained MBC results and the calculated MBC/MIC ratios are presented in Tables 3 and 4 respectively. Considering the MBC/MIC ratio, the products tested exert either a bactericidal or a bacteriostatic effect on the bacterial species tested. Only the samples of CLE, EAFL, **14**, **2**, **8**, and **9** have a bactericidal effect on all bacterial species tested (Table 4).

3. Experimental section

3.1. General experimental procedures

The chemical constituents of *A. brunneus* (Engl.) Tiegh, were purified using open column chromatography on SiO_2 (70-230 mesh; Merck) and the column were monitored using thin layer chromatography (silica gel 60 F254 (SiO_2; Merck)) and elution with a Hex/EtOAc gradient. Specific optical rotation was measured using a Bellingham and Stanley ADP220 polarimeter. IR spectra were recorded on KBr discs using a Perkin Elmer Spectrum 100 FT-IR-410 spectrometer (reported $\bar{\upsilon}$ in cm^{-1}). ^{1}H-, ^{13}C-NMR, DEPT, COSY, HSQC, NOESY and HMBC spectra: Bruker AMX 500 instrument (at 500 and 125 MHz, resp.); in d4-methanol and $CDCl_3$; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS APEXIII (Bruker Daltonik) 7 Tesla (ESI-FT-ICR-MS); in m/z.

3.2. Plant Material.

The leaves and flowers of *A. brunneus* (Engl.) Tiegh, which is a synonym of *A. Brunneus* (Engl.) Balle & Hallé were collected on *Senna siamea* tree in the campus of the University of Yaoundé 1 in Cameroon in August 2018. The plant was identified by Mr. Nana Victor, botanist at the National Herbarium, Yaoundé, Cameroon, where a voucher specimen (N°26086SRFCAM) was deposited.

3.3. Extraction and Isolation.

The air dried and powdered of the leaves and flowers of *A. brunneus* (Engl.) Tiegh (1.60 kg and 0.50 kg, respectively) were extracted at room temperature by maceration with the mixture CH_2Cl_2/ MeOH (1/1 v/v) for 72 h. After filtration, the solvent was evaporated under reduced pressure to yield crude extracts of leaves (206.5 g) and flowers (118.1 g).

The crude extract of leaves was fractionated using a liquid-liquid extraction method with *n*-hexane, ethyl acetate and *n*-butanol to yield three fractions: F1 (*n*-hexane fraction, 40.15 g); F2 (EtOAc fraction, 20.68 g) and F3 (*n*-butanol fraction, 13.45 g).

The crude extract of flowers also yielded three fractions through solid-liquid extraction: F4 (*n*-hexane fraction, 20.15 g); F5 (EtOAc fraction, 14.25 g) and F6 (residual, 82.13 g).

Fraction F1(35.10 g) was subjected to column chromatography over silica gel Merck 60 F254 (0.1-0.5 mm) eluting with *n*-hexane, *n*-hexane/EtOAc and EtOAc of increasing polarity to yield 325 sub-fractions of 100 mL each. Those sub-fractions were collected as follows: [(1-35), *n*-hexane 100%], [(36-55), *n*-hexane/EtOAc 2.5%], [(56-75), *n*-hexane/EtOAc 5%], [(76-110), *n*-hexane/EtOAc 7.5%], [(111-150), *n*-hexane/EtOAc 10%], [(151-165), *n*-hexane/EtOAc 12.5%], [(166-180), *n*-hexane/EtOAc 15%], [(181-200), *n*-hexane/EtOAc 17.5%], [(201-220), *n*-hexane/EtOAc 20%], [(220-235), *n*-hexane/EtOAc 25%], [(221-235), *n*-hexane/EtOAc 30%], [(236-250), *n*-hexane/EtOAc 40%], [(251-265), *n*-hexane/EtOAc 50%], [(266-276), *n*-hexane/EtOAc 60%], [(277-290), *n*-hexane/EtOAc 70%], [(291-310), *n*-hexane/EtOAc 80%], [(311-325), EtOAc 100%]. These sub-fractions were combined on the basis of their TLC profiles to give seven main fractions (A1-A7) as follows: A1 (1-60, 4.05g), A2 (61-150, 5.18g), A3 (151-215, 2.50 g), A4 (216-253, 1.14 g), A5 (254-270, 3.24 g), A6 (271-290, 2.15 g) and A7 (292-325, 2.38 g). Taraxerol (**5**; 48.18 mg) and pentacosanoic acid (**7**; 35.02 mg) were isolated as white needles and white powder respectively through purification of main-fraction A1 over a silica gel column Merck 60 F254 (0.1-0.5 mm) eluting with *n*-hexane/EtOAc (95/5). From A2, lupeol (**4**;70.56 mg) and globimetulin C (**6**; 12.05 mg) were obtained respectively as white needles and white powder eluting with *n*-hexane/EtOAc (92.5/7.5) over a silica gel column Merck 60 F254 (0.1-0.5 mm). 3-*O*-β-D-glucopyranoside of β-sitosterol (**10**; 8.31 mg) was isolated over a silica gel column Merck 60 F254 (0.1-0.5 mm) as white needles eluting with *n*-hexane/EtOAc (75 / 25) from A7.

A 18.23 g portion of fraction F2 was also chromatographed [silica gel column Merck 60 F254 (0.1-0.5 mm)] eluting with *n*-hexane, *n*-hexane/EtOAc, EtOAc and EtOAc/MeOH of increasing polarity to yield 515 sub-fractions of 100 mL each. These sub-fractions were collected as follows: [(1-72), *n*-hexane/EtOAc 5%], [(73-120), *n*-hexane/EtOAc 10%], [(121-150), *n*-hexane/EtOAc 15%], [(151-201), *n*-hexane/EtOAc 20%], [(202-236), *n*-hexane/EtOAc 25%], [(237-260), *n*-hexane/EtOAc 30%], [(261-280), *n*-hexane/EtOAc 35%], [(281-310), *n*-hexane/EtOAc 40%], [(311-340), *n*-hexane/EtOAc 50%], [(341-360), *n*-hexane/EtOAc60%], [(361-400), *n*-hexane/EtOAc 70%], [(401-450), *n*-hexane/EtOAc 80%], [(451-485), EtOAc 100%] and [(486-515) EtOAc/MeOH 5%]. Those sub-fractions were combined on the basis of their TLC profiles to give 5 main fractions (B1-B5) as follows: B1 (1-130, 2.05 g), B2 (131-285, 2.80 g), B3 (286-365, 3.53 g), B4 (366-455, 1.08 g) and B5 (456-515, 2.48 g).

Globimetulin C (**6**; 3.05 mg) and pentacosanoic acid (**7**; 4.02 mg) were both obtained from main fraction B1 by purification on a silica gel column Merck 60 F254 (0.1-0.5 mm) eluting with *n*-hexane/EtOAc (95/5) as white needles and white powder respectively. From B2 through column

chromatography using silica gel Merck 60 F254 (0.1-0.5 mm), methyl gallate (**3**; 20.12 mg) was isolated as white powder eluting with *n*-hexane/EtOAc (80/20). Dodoneine (**2**; 70.34 mg) and (-)-brunneusine (**1**; 30.23 mg) were isolated through purification of main fraction B3 on silica gel column Merck 60 F254 (0.1-0.5 mm) with *n*-hexane/EtOAc (50/50) and *n*-hexane/EtOAc (45/55) as white amorphous solid. Quercetin 3-*O*-α-l-rhamnopyranoside (**13**; 1.50 g) and 3-*O*-β-D-glucopyranoside of β-sitosterol (**10**; 2.31 mg) were also obtained from B4 using a silica gel column Merck 60 F254 (0.1-0.5 mm) respectively as yellow powder and white needles with *n*-hexane/EtOAc (30/70) for **13** and *n*-hexane/EtOAc (20/80) for **10**.

Similar methods were applied to fraction F5 (12.5 g) which was also purified over a silica gel column Merck 60 F254 (0.1-0.5 mm) by eluting with *n*-hexane, *n*-hexane/EtOAc, EtOAc and EtOAc/MeOH of increasing polarity to yield 490 sub-fractions of 100 mL each. These sub-fractions were collected as follows: [(1-60), *n*-hexane/EtOAc 5%], [(61-100), *n*-hexane/EtOAc 10%], [(101-139), *n*-hexane/EtOAc 15%], [(140-165), *n*-hexane/EtOAc 20%], [(166-190), *n*-hexane/EtOAc 25%], [(191-230), *n*-hexane/EtOAc 30%], [(231-261), *n*-hexane/EtOAc 35%], [(262-295), *n*-hexane/EtOAc 40%], [(296-335), *n*-hexane/EtOAc 50%], [(336-371), *n*-hexane/EtOAc60%], [(372-415), *n*-hexane/EtOAc 70%], [(416-445), *n*-hexane/EtOAc 80%], [(446-470), EtOAc 100%] and [(471-490) EtOAc/MeOH 5%]. The sub-fractions were combined on the basis of their TLC profiles to give 6 main fractions (C1-C6) as follows: C1 (1-140, 2.18 g), C2 (142-195, 1.42 g), C3 (196-300, 2.83 g), C4 (301-372, 0.98 g), C5 (373-450, 1.50 g) and C6 (451-490, 2.13 g).

Sub-fraction C3 was chromatographed on a silica gel column Merck 60 F254 (0.1-0.5 mm) with *n*-hexane/EtOAc (65 /35) and *n*-hexane/EtOAc (55 /45) respectively to yield rel-(1R,5S,7S)-7-[2-(4-hydroxyphenyl)ethyl]-2,6-dioxabicyclo[3.3.1]nonan-3-one (**8**; 500.12 mg; greenish crystals) and gallic acid (**9**; 90.52 mg; white powder). Further purifications were applied to sub-fractions C4 and C5 to yield quercetin (**11**; 35.12 mg; yellow powder), quercetin 3-*O*-α-l-arabinofuranoside (**12**; 650.32 mg; yellow powder), quercetin 3-*O*-α-l-rhamnopyranoside(**13**;2,51g) and quercetin 3-*O*-α-arabinopyranoside (**14**; 28 mg) respectively with *n*-hexane/EtOAc (50 /50), *n*-hexane/EtOAc (40 /60), *n*-hexane/EtOAc (30 /70) and *n*-hexane/EtOAc (20 /80) on a silica gel column Merck 60 F254 (0.1-0.5 mm).

3.4. Antibacterial assays

The reference antibiotics used was gentamicin obtained from Sigma-Aldrich (St Quentin Fallavier, France). DMSO-MHB was used to dissolve the tested samples; the microbial growth indicator used was p -iodonitrotretrazolium chloride ≥ 97% (INT, Sigma-Aldrich). Four strains bacterial species isolated from well water intended for human consumption were investigated in this work. These are *Escherichia coli, Salmonella typhi, Vibrio cholerea* and *Streptococcus*

feacalis. Those bacterial species were chosen because of their importance in hygiene and public health in the indication of the microbiological quality of human pathogenic germs CHU-PS. (2003) [35]. Prior to the test, bacteria were cultured on Ordinary agar (Plate Count Agar) for the culture of pure bacterial strains and on The Mueller Hinton Broth (Mueller Hinton Broth: MHB) for sensitivity tests of both samples and antibiotic. The MICs of the different products were determined by the liquid microdilution method using the M07-A9 protocol as described by the CLSI (2012) [36]. Crude extracts, fractions, compounds and reference drug were dissolved in DMSO-MHB. The bacterial inoculum used was 2 x 10^6 CFU/mL and the incubation conditions at 37°C for a period of 18 hours to 24 hours. Column 12 containing only Mueller Hinton broth was used as negative control and antibiotic as positive control. The assays were in triplicate. The MICs of samples were detected following addition (40 µL) of 0.2 mg/ mL p-iodonitrotetrazolium chloride and incubation at 37 °C for a period of 18 hours to 24 hours. MIC was defined as the lowest concentration that can inhibit any visible growth of a microorganism after incubation at 37°C for 18 to 24 hours.

The minimal bactericidal concentrations (MBCs) of samples were determined following the addition of a 150 µL of culture broth into new plates, then the volume was completed to 200 µL by adding a volume of 50 µL of the wells' contents with a concentration greater than or equal to the MIC. These plates were then incubated for 24 hours at 37°C followed by a revelation with p-iodonitrotetrazolium chloride (INT). All the concentrations at which no pink coloration was observed were taken as bactericidal and the smallest of these was noted as MBC. The tests were carried out in triplicates. The MBC/MIC ratios were calculated and allowed the characterization of the activity of a given antibiotic [37].

3.5. (-)-Brunneusine (1)

White amorphous solid. $[\alpha]^{22.1}_D = -37.1$ (c = 0.014, CH_2Cl_2); IR (neat) 3338 (O-H), 2920 (C-H), 1699 and 1615 (C=O), 1516 (C=C), and 1081.5 (C-O-C) cm^{-1}; ^1H ($CD_3OD/CDCl_3$; 500MHz) and ^{13}C-NMR ($CD_3OD/CDCl_3$; 125 MHz) : see table 1; HR-ESI-MS: 263.1308 ($[M+H-H_2O]^+$, $C_{15}H_{19}O_4^+$; calc. 263.1283).

4. Conclusion

Chemical investigations of leaves and flowers of *Agelanthus brunneus* (Eng.) Tiegh (Loranthaceae) led to the isolation of one new phenolic compound namely (–)-brunneusine (**1**) together with thirteen known compounds: dodoneine (**2**), methyl gallate (**3**), lupeol (**4**), taraxerol (**5**), globimetulin C (**6**), pentacosanoic acid (**7**), rel-(1*R*,5*S*,7*S*)-7-[2-(4-hydroxyphenyl)ethyl]-2,6-dioxabicyclo[3.3.1]nonan-3-one (**8**), gallic acid (**9**), 3-*O*-β-D-glucopyranoside of β-sitosterol (**10**), quercetin (**11**), quercetin 3-*O*-α-l-arabinofuranoside (**12**), quercetin 3-*O*-α-l-rhamnopyranoside (**13**) and quercetin 3-*O*-α-arabinopyranoside (**14**). Crude extracts, fractions,

and some isolated compounds (**1-3**; **8-9**; **11-14**) and standard drug Gentamicin were evaluated for their antibacterial properties against four Gram+ and Gram- bacterial species using rapid *p*-iodonitrotetrazolium chloride ≥ 97% (INT) microdilution technique. All samples except compound **3** showed antibacterial activity on tested bacterial strains with MICs ranging from 0.43 to $8.88.10^3$ µg/mL and MBCs from 0.43 to $3.55.10^3$ µg/mL. **9** and **14** showed better activity on all bacterial species tested with MICs ranging from 0.43 to 27.77 µg/mL. The samples exert either a bactericidal or a bacteriostatic effect on the bacterial species tested. Only CLE, EAEF and compounds **14**, **2**, **8**, and **9** showed bactericidal effects on all bacterial species tested, whilst compound **3** was not active. These results are consistent with strong antibacterial properties reported for phenolic compounds. All the results presented at the end of this study show that the traditional use of *A. brunneus* extracts in reducing the flow of polluting bacteria in water and in the treatment of infectious diseases could be an alternative in the fight against these scourges. However, it would be relevant to consider the role of their association with conventional antibiotics in the fight against the spread of microorganisms resistant to antibacterial agents.

Disclosure statement: The authors declare no conflict of interest.

Funding: This research was supported by the UEA Vice Chancellor's Global Challenges Research Fellowships Scheme; Code: CHE 21A3.

Acknowledgment

B. Ngameni, T. E. Storr and G. R. Stephenson acknowledge Open Lab Africa through this exchange research program. The authors are also grateful to the School of Chemistry, Faculty of Science, University of East Anglia, UK for research facilities and support with the experimental equipment and Dr. C.J. Macdonald, NMR Facility Manager, School of Chemistry for technical advice and training.

Appendix A.

HR-ESI-MS, IR as well as 1D and 2D NMR spectra of compounds 1–3; 6-14 are available as supplementary material.

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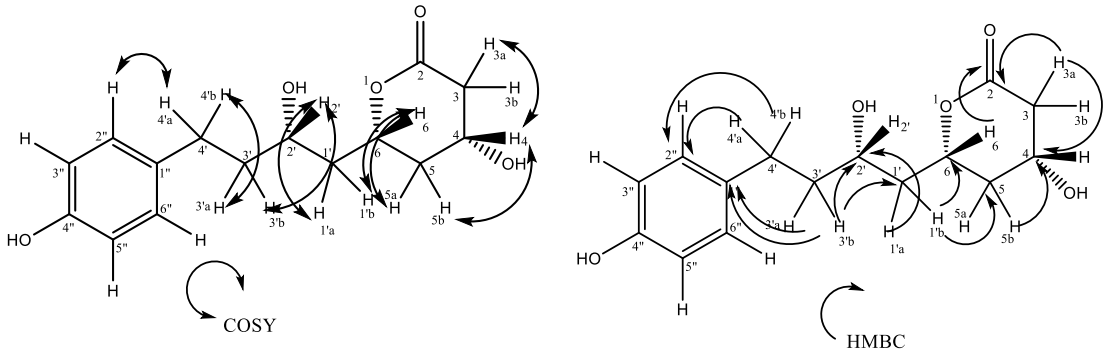

Figure 1: Selected HMBC and COSY correlations of compound **1**

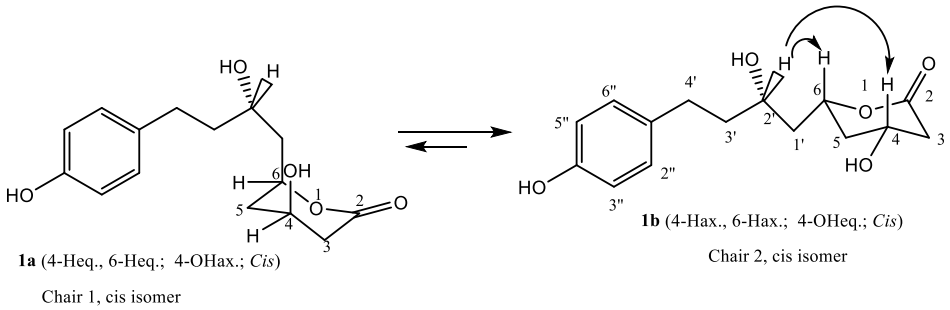

Figure 2: Interconverting structural conformations (chairs 1 and 2) of natural isolated *cis* isomers **1**

Table 1: ^{1}H and ^{13}C NMR data for compound **1** and signal correlation deduced from cross-peacks in HMBC and NOESY spectra.

Position[a)]	δ_H	δ_C	HMBC	NOESY
1	-	-	-	-
2 (C=O)	-	171.1	-	-
3	2.67 (d, *J* = 3.2, 2H)	36.2	C-1', C-4, C-2	-
4	4.24 (br.s,1H)	65.8	C-3, C-1', C-2 (weak)	-
5a	1.80 (m, 1H)	29.5	C-3, C-4, C-6 (weak)	H-5b
5b	1.86 (m, 1H)		C-5, C-4, C-6	H-5a
6	4.76 (dd, 1H, *J* = 3.6, 1.8)	73.6	C-5, C-1', C-4, C-2	H-4,
1'a	1.47 (ddd, *J* = 13.9, 11.7, 2.1,1H)	36.8	C-1', C-6, C-2'	H-1'b
1'b	1.84 (m, 1H)		C-1', C-6, C-2'	H-1'a
2'	3.56 (ddd, *J* =14.7, 7.3, 3.8, 1H)	64.8	C-5, C-4', C-3', C-6, C-4	H-6
3'a	1.54 (m, 1H)	37.7	C-4', C-2', C-1"	H3'b
3'b	1.66 (m, 1H)		C-4', C-2', C-1"	H3'a
4'a	2.40 (ddd, *J* = 13.8, 9.4, 7.2, 1H)	30.4	C-3', C-2', C-2", C-6", C-1"	H4'b
4'b	2.53 (ddd, *J* = 14.5, 9.8, 5.2, 1H)		C-3', C-2', C-2", C-6", C-1"	H4'a
1"	-	132.4	-	-
2"; 6"	6.84 (d, *J* = 8.4, 2H)	129.2	C-4', C-3", C-5", C-1", C-4"	H3",H5"
3"; 5"	6.59 (d, *J* = 8.4, 2H)	115.2	C-2", C-6", C-1", C-4"	H2",H6"
4"	-	154.8	-	-
a) Atom numbering as indicated in Figure 1				

Table 2: Minimal inhibitory concentrations (MIC) in µg/mL of the samples on the bacterial species tested.

Extracts, fractions, secondary metabolites and antibiotic	Bacterial species tested			
	Escherichia coli	***Enterococcus feacalis***	***Salmonella typhi***	***Vibrio cholerea***
CLE	8888.88	2222.20	8888.88	4444.44
EAFL	555.55	555.55	138.88	69.44
CFLE	468.75	1875.00	3750.00	29.29
EAFFL	7361.10	460.06	1840.27	920.13
1	125.00	3.90	31.25	250.00
2	229.16	57.29	57.29	114.58
3	-	-	-	-
8	911.45	1822.91	3645.83	7291.66
9	6.94	0.43	0.86	0.86
11	83.33	41.66	83.33	20.83
12	48.61	97.22	12.15	6.07
13	13.88	111.11	27.77	55.55
14	3.47	0.86	27.77	6.94
Gentamicin	5000.00	625.00	1250.00	625.00

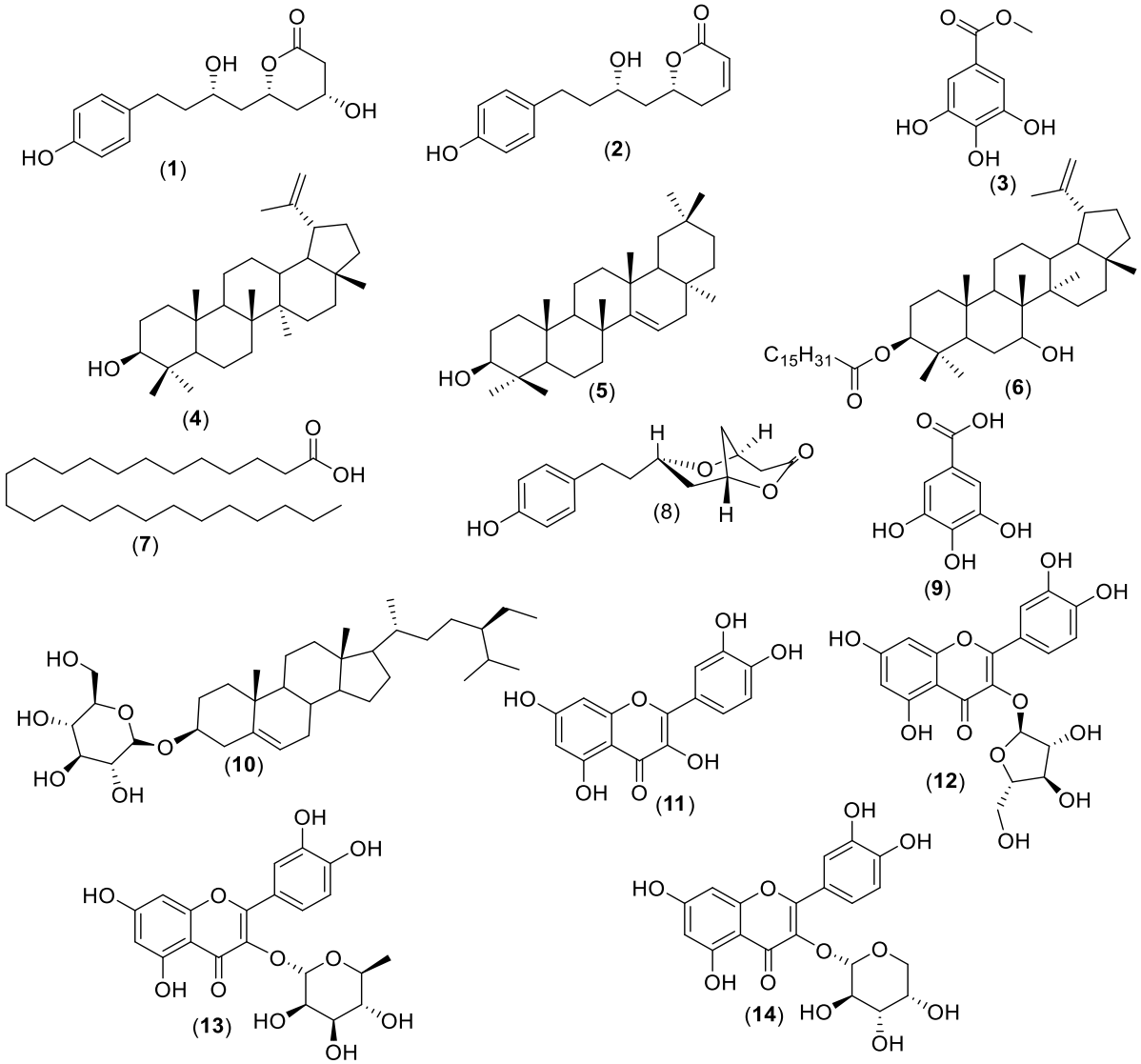

Figure 3: Structures of the isolated compounds **1**-**14**.

Table 3: Minimal bactericidal concentrations (MBC) in µg/mL of the samples on the bacterial species tested.

Extracts, fractions, secondary metabolites and antibiotic	Bacterial species tested			
	Escherichia coli	***Enterococcus faecalis***	***Salmonella typhi***	***Vibrio cholerea***
CLE	35555.52	2222.20	35555.52	17777.60
EAFL	1111.10	1111.10	555.52	69.44
CFLE	937.50	11250.00	15000.00	58.58
EAFFL	29444.40	812.12	11041.62	920.13
1	500.00	3.90	64.50	2500.00
2	916.64	57.29	57.29	458.32
3	-	-	-	-
8	911.45	3645.82	29166.64	29166.64
9	13.88	0.43	1.72	1.72
11	333.32	166.64	333.32	124.98
12	194.44	583.32	12.15	12.14
13	52.32	888.88	27.77	222.20
14	3.47	1.72	111.08	13.88

Gentamicin	ND	ND	ND	ND

ND: Not Determined; CLE: Crude Leaf Extract; EAFL: Ethyl Acetate Fraction of Leaves; CFLE: Crude FLowers Extract; EAFFL: Ethyl Acetate Fraction of FLowers

Table 4: MBC/MIC reports of samples

Extracts, fractions and secondary metabolites	Bacterial species tested			
	Escherichia coli	***Enterococcus faecalis***	***Salmonella typhi***	***Vibrio cholerea***
CLE	4	1	4	4
EAFL	2	2	4	1
CFLE	2	6	4	2
EAFFL	4	2	6	1
1	4	1	2	10
2	4	1	1	4
3	-	-	-	-
8	1	2	4	4
9	2	1	1	2
11	4	4	1	6
12	4	6	2	2
13	4	8	1	4
14	1	2	4	2