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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

LC-MS guided isolation of antibacterial and cytotoxic constituents from *Clausena anisata*

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Abstract Phytochemical investigation of leaves and stem bark extracts of *Clausena anisata* monitored by liquid chromatography high-resolution mass spectrometry (LC-HR-MS) analysis led to the isolation and characterization of twenty-one secondary metabolites: four carbazole alkaloids (**1–4**) including one new name clausamine H (**1**), fourteen coumarins (**5–18**), two porphyrin derivatives (**19–20**), and one limonoid (**21**). Crude extracts were analyzed first by LC-HR-MS, and target compounds were isolated by a multi-step separation procedure using column chromatography and preparative high-performance liquid chromatography (*prep*-HPLC) monitored by LC-HR-MS analysis. The structures of isolates were determined by means of spectroscopic and spectrometric data, as well as by comparison with literature values. The isolates showed weak to high antibacterial activities with imperatorin (**14**) being the most active one. Cytotoxic activities against HeLa and monkey Vero cells were also investigated, and murrayamine-A (**4**), 3-(1,1-dimethyl allyl) xanthyletin (**5**) gravelliferone (**7**), excavatin D (**10**), 7-[(E)-7-hydroxy-3,7-dimethylocta-2,5-dienyloxyl]-coumarin (**13**), phellopterin (**15**), and 1-*O*-methylclausenolide (**21**) were found active with LC_{50} values ranged from 1.14 to 3.26 µg/mL and a good selectivity index values (SI 38.20–231.58) against the HeLa cells. However, these compounds were non-toxic to normal cells indicating their high potential to be used as anticancer drug.

Keywords *Clausena anisata* · Coumarin · Carbazole alkaloid · Porphyrin · Antibacterial · Cytotoxicity

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Introduction

Clausena anisata (Will.) Hook. f. ex.benth. (Rutaceae) is a tropical shrub or tree growing up to ten-meters high in and on the margins of evergreen forests. The leaves are densely dotted with glands and have a strong smell when crushed (Letouzey, 1963). Various parts of the plant are used alone or in association with other plants in folk medicine. In Nigeria, a mixture of *Clausena anisata*, *Afraegle paniculata*, and *Azadirachtha indica* is taken against gut disturbance (Uwaifo, 1984). In Tanzania, traditional healers use *C. anisata* against oral candidiasis and fungal infections of the skin (Hamza *et al.*, 2006), whereas in the Temeke district (Daressalam, Tanzania) it is employed against epilepsy and as an anticonvulsant (Moshi *et al.*, 2005). A root decoction is taken to control convulsions in children and as a tonic by pregnant women (Ngadjui *et al.*, 1989a). In some parts of Africa, the burning of fresh leaves is utilized to repel mosquitoes (Songue *et al.*, 2012). *Clausena anisata* has been widely studied and is well known to produce carbazole alkaloids (Songue *et al.*, 2012; Okorie,

1975; Ito *et al.*, 2000, 2009), coumarins (Okorie, 1975; Ngadjui *et al.*, 1989a, b), and limonoids (Ngadjui *et al.*, 1989c). Recently, two dipeptide derivatives and mixture of sterols were isolated from the roots of *C. anisata* (Songue *et al.*, 2012). In addition, carbazole alkaloids and coumarins from *Clausena* species have shown cytotoxic effect (Maneerat *et al.*, 2012a), antibacterial (Maneerat *et al.*, 2012b), anti-inflammatory (Shen *et al.*, 2012), and antitumoral (Ito *et al.*, 2000) activities. These wide ranges of biological activities and traditional uses have prompted more chemical and biological studies on *Clausena* species.

In continuation of our search for bioactive secondary metabolites from Cameroonian medicinal plants, LC-HR-MS directed fractionation of leaves and stem bark of *C. anisata* and their biological activities were carried out. We report herein the isolation and structure elucidation of one new and three known carbazole alkaloids, as well as 14 known coumarins, two known porphyrin derivatives, and one known limonoid. We also report the antibacterial and cytotoxic activities of the isolated compounds. In addition, this is the first report of isolation and characterization of porphyrin derivatives from *C. anisata.*

Materials and methods

General procedures

The high-resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 200–800) with nominal mass resolving power of 60,000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard, Bis(2-ethylhexyl)phthalate: *m/z* = 391.284286. The spectrometer was attached with an Agilent (Santa Clara, USA) 1200 HPLC system consisting of LC-pump, PDA detector ($\lambda = 205$ nm), auto sampler (injection volume 10 µL), and column oven (30 °C). The following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, and tube lens 70 V. Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (5 arbitrary units). Helium served as the collision gas. The separations were performed using a Phenomenex Gemini NX C18 column (150 × 2 mm, 3 µm particle size) with a H_2O (+0.1 % HCOOH) (A)/acetonitrile (+0.1 % HCOOH) (B) gradient (flow rate 300 µL min^{-1}). Samples were analyzed using a gradient program as follows: 90 % A isocratic for 2 min, linear gradient to 100 % B over 13 min, after 100 % B isocratic for 5 min, the system returned to its initial condition (90 % A) within 0.5 min, and was equilibrated for 4.5 min.

NMR spectra were measured on Bruker DRX 500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR, with TMS as internal standard; chemical shifts are given in δ values (ppm). IR spectra were recorded with Nexus FT-IR spectrometer.

Flash column chromatography was performed using silica-gel 60 (Merck, 0.040–0.063 mm). Preparative reversed-phase HPLC was carried out with a Gilson system consisting of pump 322 with a UV detector 152 ($k = 250$ nm) using a Nucleodur Gravity column from Macherey-Nagel (Düren, Germany) (250 × 16 mm, 5 µm particle size). Separation was achieved by a H_2O (A)–MeOH (B) gradient program as follows (detection at 250 nm, flow rate 5 mL/min): 60 % A isocratic for 2 min, 60 % A linear to 100 % B for 18 min, following by 100 % B isocratic for 14 min. Afterwards, the system returned to its initial condition (60 % A) within 1 min and finally was equilibrated for 2 min.

Plant material

Leaves and stem bark of *C. anisata* were collected in Mount Cameroon, Buéa, Cameroon in May 2013. It was identified by Mr. Victor Nana, botanist at the National Herbarium, Yaoundé where a voucher specimen 43526HNC describing that the plant is deposited.

Extraction and isolation

The air-dried stem bark (2.7 kg) and leaves (3.9 kg) of *C. anisata* were powdered and extracted thrice with MeOH at room temperature for 48, 96, and 164 h, respectively. The filtrate was evaporated to give 374.7 and 143.6 g of methanol extract of leaves and stem bark, respectively. The obtained extracts were partitioned with hexane to afford 76.9 and 17.7 g of hexane extracts, and 251.7 and 116.9 g of MeOH residue extracts of leaves and stem bark, respectively.

The hexane extract (17.7 g) of stem bark was subjected to Si gel flash column chromatography eluted with cyclohexane-EtOAc gradient [10:0 (1.25 L), 95:5 (2.5 mL), 9:1 (2.75 L), 85:15 (1.5 mL), 4:1 (2.25 L), 65:35 (1.25 L), and 1:1 (2 L)] to give 55 fractions of 250 mL each. These fractions were combined into eight main fractions [F_1 (1–7), F_2 (8–16), F_3 (17–19), F_4 (20–24), F_5 (25–29), F_6 (30–42), F_7 (42–48), and F_8 (49–55)] based on their LC-MS profile. F_2 was subjected to Si gel column chromatography eluted with cyclohexane-EtOAc gradient [10:0 (900 mL), 98:2 (1,400 mL), 96:4 (2,400 mL), and 90:10 (300 mL)] to give 49 fractions of 100 mL each. These fractions were also combined into four fractions [F_{1A} (1–17), F_{1B} (18–22), F_{1C} (23–42), and F_{1D} (43–49)] based on LC-MS profile. Compound **3** (300 mg) crystallized in fractions 23–26 as a white powder. F_{1A}, F_{1C}, F_3, F_4, $F_{5,}$ and F_6 were submitted to preparative HPLC with the solvent

Table 1 ^{1}H (500 MHz, $CDCl_3$) ^{13}C (125 MHz) and HMBC data of clausamine H (**1**)

Position	δ_C (ppm)	δ_H (ppm)	HMBC
1	143.3[a]		
1a	128.9[a]		
2	109.3	6.75, s	C_1, C_{1a}, C_4, CH_3 on C_3
3	126.9		
4	127.3[a]		
4a	125.6		
5	123.2	8.14, d, $J = 7.5$ Hz	C_{8a}, C_{4a}
5a	124.1		
6	119.5	7.22, t, $J = 7.5$ Hz	C_{5a}, C_8
7	125.3	7.41, t, $J = 7.5$ Hz	C_{8a}, C_5
8	111.1	7.47, t, $J = 8.23$ Hz	C_{5a}, C_6
8a	139.9		
9	–	8.24, NH, brs	
1′	29.1	3.90, d, $J = 6.20$ Hz	$C_{3'}$, C_3, $C_{2'}$
2′	122.9	5.27, m	$C_{3'}$
3′	132.2		
4′	26.1	1.72, s	$C_{3'}$, $C_{2'}$, $C_{5'}$
5′	18.7	1.93, s	$C_{3'}$, $C_{2'}$, $C_{4'}$
3-CH_3	19.8	2.47, s	C_3, C_2
1-OCH_3	55.9	4.00, s	C_1

[a] These values were obtained from HMBC spectrum

system H_2O (B)–MeOH (A) with gradient program as described above. F_{1A} gave **1** (23.8 mg) and **5** (20 mg). F_{1C} yielded **8** (6 mg); F_3 and F_4 gave **2** (17 mg), **7** (10 mg), **6** (8 mg), and **9** (5 mg); F_5 afforded **14** (2.20 mg) and **15** (2.3 mg); and F_6 gave **18** (34 mg).

MeOH residue extracts of leaves and stem bark followed the same process. Two compounds, **4** (8 mg) and **21** (13 mg), were isolated from stem bark; and eight compounds, **10** (1000 mg), **11** (50 mg), **12** (2 mg), **13** (22 mg), **16** (250 mg), **17** (11 mg), **19** (25 mg), and **20** (1.4 mg), were obtained from leaves. Compounds **8** and **12** were obtained impure, while **20** was very small. Therefore, they were not submitted to biological tests.

Clausamine H (**1**): Orange oil; IRν_{max}: 3317, 2843, 1747, 1652, 1465, 1382 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz), see Table 1; HRAPCIMS (280.1699 $[M+H]^+$, calcd for $C_{19}H_{22}NO$ 280.1701); MS/MS of *m/z* 280: 265, 224, 212; MS/MS of *m/z* 265: 250, 222, 209 (100), 197; MS/MS of *m/z* 212: 197(100), 180.

Antibacterial assays

Microbial growth conditions

A total of six bacterial species kindly provided by Prof. Prasanta K. Bag, University of Calcutta, India, were tested for their susceptibility to the extracts and compounds. Among the clinical strains of *Vibrio cholerae* used in this study, strains NB2, SG24, and CO6 belonged to O1 and O139 serotypes, respectively. All these strains were able to produce cholera toxin, hemolysin, and multi-drug resistant (MDR). The other strains used in this study were *V. cholerae* non-O1, non-O139 (strain PC2), and *Shigella flexneri* 2a. The MDR *V. cholerae* non-O1 and non-O139 strain isolated from aquatic environment was positive for hemolysin production but negative for cholera toxin production (Bag *et al.*, 2008). The American Type Culture Collection (ATCC) strain, *Staphylococcus aureus* ATCC 25923, was used for quality control. The bacterial strains were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar plates 24 h prior to antibacterial test. The Mueller-Hinton Agar (MHA) was used for the activation of bacteria. The Mueller-Hinton Broth (MHB) and nutrient agar were used for the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) determinations, respectively.

Inoculum preparation

Suspensions of bacteria were prepared in MHB from cells arrested during their logarithmic phase growth (4 h) on MHB at 37 °C. The turbidity of the microbial suspension was read spectrophotometrically at 600 nm and adjusted to an OD of 1.0 with MHB, which is equivalent to 2×10^8 CFU/mL. From this prepared solution, other dilutions were made with MHB to yield 1×10^6 CFU/mL.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of the extracts and compounds **1–21** were assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999) with slight modifications. The 96-well round bottom sterile plates were prepared by dispensing 180 µL of the inoculated broth (1×10^6 CFU/mL) into each well. A 20 µL aliquot of the compounds was added. The plant extracts and pure compounds were tested at concentrations 0.125, 0.25, 0.50, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 µg/mL. Ampicillin and chloramphenicol served as positive controls, while MHB with 20 µL of DMSO 10 % was used as negative control. The *Staphylococcus aureus* ATCC 25923 strain was included for quality assurance purposes. Plates were covered and incubated for 24 h in ambient air at 37 °C. After incubation, minimum inhibitory concentrations (MIC) were read visually; all wells were plated to nutrient agar (Hi-Media) and incubated. The minimal bactericidal concentration (MBC) was defined as a 99.9 % reduction in

CFU from the starting inoculums after 24 h incubation interval.

The time-kill kinetic study (for antimicrobial drugs) against Vibrio cholerae SG24

Time-kill dynamic assay was performed using broth microdilution method as previously described (Avila *et al.*, 1999) with minor modifications. Cultures of bacteria in MHB (1×10^6 CFU/mL) were incubated separately at 37 °C for 0, 2, 4, 6, 10, and 24 h in the absence (control) and in the presence of the drug/extract at MIC and MBC of each sample. Compound **14** and ampicillin were used in the time-kill dynamic experiment. The final concentration of DMSO was 1 %. A control sample was made using DMSO 1 % and the inoculum. At each incubation time point, liquids (50 µL) were removed from the test solution for ten-fold serial dilution. Thereafter, a 100 µL liquid from each dilution was spread on the surface of the MHA plates and incubated at 37 °C for 24 h, and the number of CFU/mL was counted. Experiments were carried out in triplicate. Time-kill curves were constructed by plotting the surviving $\log_{10}$ of number of CFU/mL against time (h).

Cytotoxicity assays

HeLa (Human cervical cancer cell line) and monkey Vero cells (African green monkey kidney cells, normal non-cancer cells), obtained from the American Type Culture Collection (ATCC), were used in this study. Cytotoxicity activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay reported by Mosmann (1983). This cell viability assay is based on living cells' property to transform the MTT dye tetrazolium ring into a purple-colored formazan structure due to the action of mitochondrial and other dehydrogenases inside the cell. The color intensity yielded by the cell population is directly proportional to the number of viable cells. The extracts and pure compounds were prepared from the stock solutions by serial dilution in RPMI 1640 to give a volume of 100 µL in each well of a microtiter plate (96-well). Each well was filled with 100 µL of cells at 2×10^5 cells/mL. The assay for each concentration of extract and pure compounds was performed in triplicates, and the culture plates were kept at 37 °C with 5 % (v/v) CO_2 for 24 h. After removing the supernatant from each well and washing twice by PBS, 20 µL of MTT solution (5 mg/mL in PBS) and 100 µL of medium were then introduced. After 4 h of incubation, 100 µL of DMSO was added to each well to dissolve the formazan crystals and the absorbance values at 490 nm were measured with a microplate reader (Bio-RAD 680, USA). The relative cell viability was expressed as a percentage relative of treated cells to the untreated control cells (TC/UC × 100). The rate of cell inhibition was calculated using the following formula: inhibition rate = $[1 - (OD_{test}/OD_{negative\ control})] \times 100\ \%$. Paclitaxal served as positive control.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Science (SPSS, version 12.0). The experimental results were expressed as the mean ± standard deviation (SD). Group comparisons were performed using One Way ANOVA followed by Waller-Duncan Post Hoc test. A *p* value of ±0.05 was considered statistically significant.

Results and discussion

Phytochemical analysis

Hexane and methanol extracts of stem bark and leaves of *Clausena anisata* were analyzed by LC-HR-MS. Their analyses indicated that leaves extracts were rich in coumarins, and stem bark extracts are consisted of both coumarins and carbazole alkaloids. These extracts were submitted to a series of column chromatography (CC) and preparative high-performance liquid chromatography (HPLC) monitored by LC-HR-ESI-MS. One new carbazole alkaloid, clausamine H (**1**), was isolated together with three known carbazoles, ekeberginine (**2**), girinimbine (**3**), and murrayamine-A (**4**) (Songue *et al.*, 2012); fourteen known coumarins, 3-(1,1-dimethyl allyl) xanthyletin (**5**) (Nayar *et al.*, 1973), gravelliferone (**7**)(Kumar *et al.*, 1995), gravelliferone methyl ether (**8**), xanthoxyletin (**6**) (Ngadjui *et al.*, 1989a), 5,7-dimethoxy-8-(3′-methylbut-2′-enyl) coumarin (**9**) (Chang *et al.*, 1977), excavatin D (**10**) (Thuy *et al.*, 1999), 7-methoxy-6(2'-oxo-3'-methyl butyl) coumarin (**11**) (Gonzalez *et al.*, 1977), (R)-(+)-6-(2′-hydroxy-3′-methyl-3′-butenyl)-7-methoxycoumarin (**12**) (Burke and Parkins, 1979), 7-[(E)-7-hydroxy-3,7-dimethylocta-2,5-dienyloxyl]-coumarin (**13**) (Abdul *et al.*, 1992), imperatorin (**14**) (Ngadjui *et al.*, 1989a), phellopterin (**15**) (Lee and Soine, 1969), bergapten (**16**), isooxypeucedanin (**17**) (Harkar *et al.*, 1984), and chalepin (**18**) (Okorie, 1975); two chlorophyll derivatives, $13^2(R)$-hydroxypheophyton a (**19**) and $13^2(S)$-pheophyton a (**20**) (Lin *et al.*, 2011); and one limonoid, 1-*O*-methylclausenolide (**21**) (Wu *et al.*, 1993) (Fig. 1). The structures were characterized by spectroscopic methods, including IR, MS, UV, 1D and 2D NMR, and by comparison with literature data. Most of these compounds were tested for antibacterial activities against *vibrio cholerae* SG24, *Vibrio cholerae* CO6, *Vibrio*

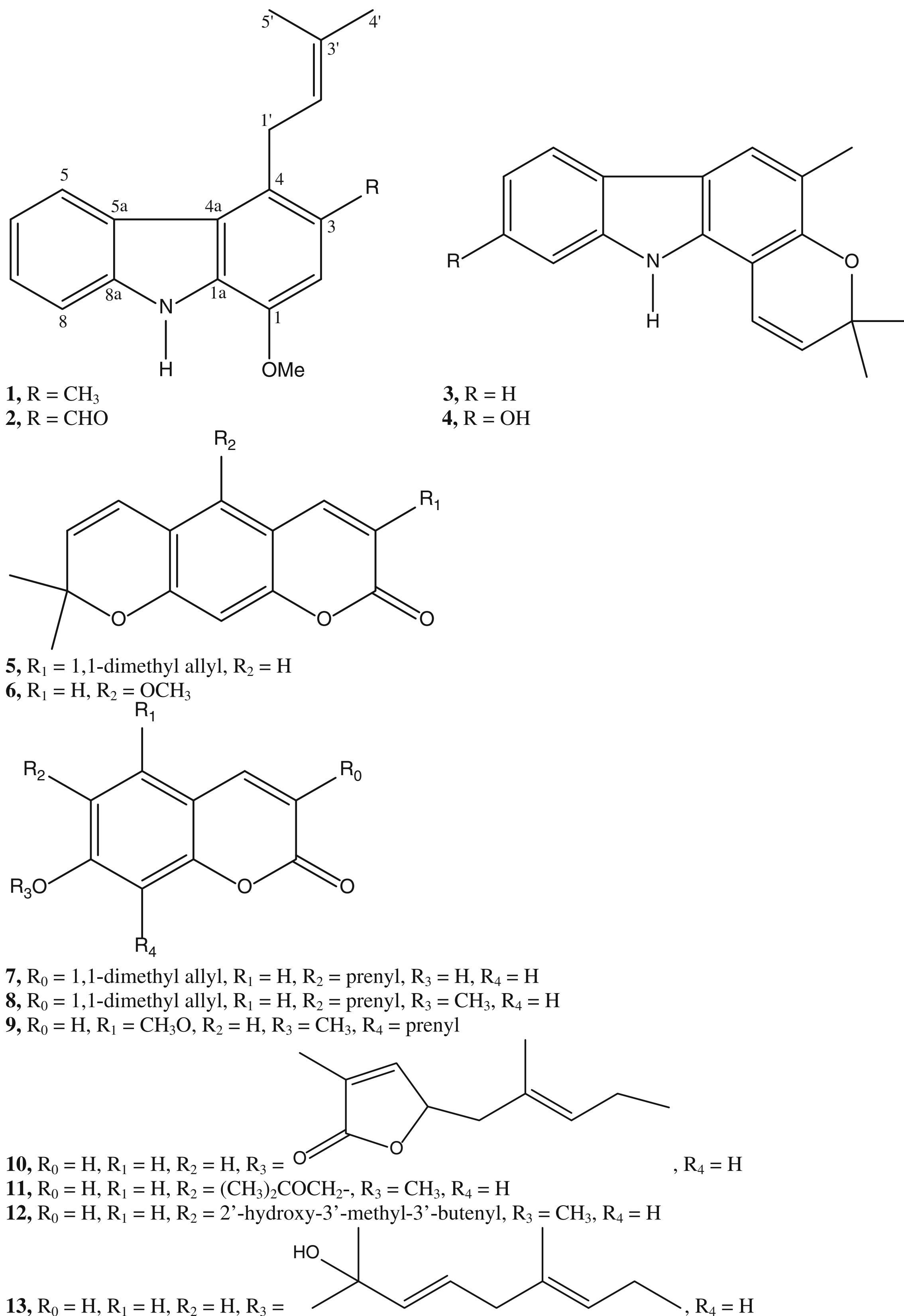

Fig. 1 Chemical structures of isolated compounds from leaves and stem bark of *C. anisata*

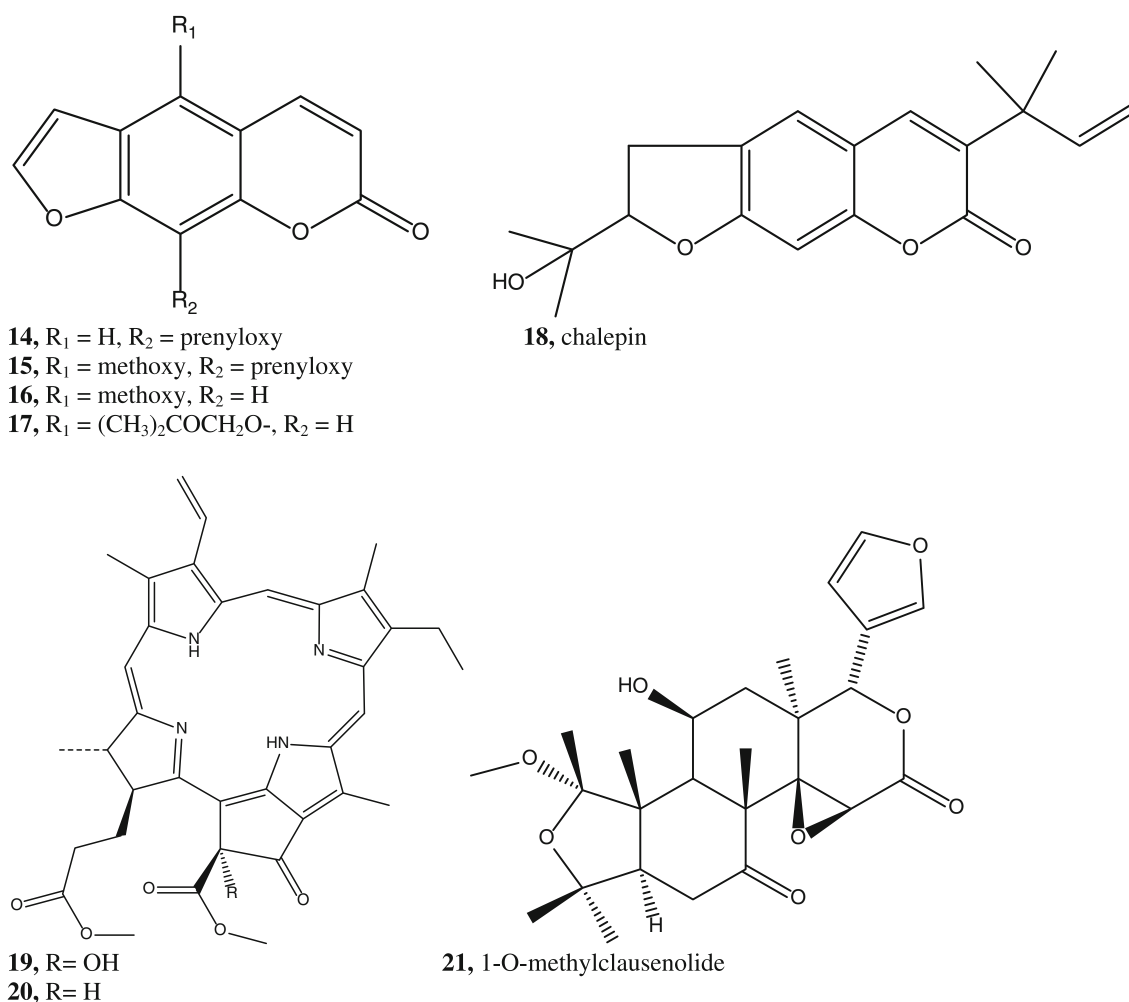

Fig. 1 continued

cholerae NB2, *Vibrio cholerae PC2*, *Staphylococcus aureus* and *Shigella flexneri,* and cytotoxicity against HeLa and monkey Vero cells.

Clausamine H (**1**) was obtained as orange oil. The molecular formula, $C_{19}H_{21}NO$, was established by HRMS (280.1699 $[M+H]^+$, calcd for $C_{19}H_{22}NO$ 280.1701). The IR spectrum displayed absorption bands characteristic of amino (3317 cm^{-1}) and aromatic ring (1747, 1652, 1465 cm^{-1}) groups, and the UV spectrum showed absorbances at λ_{max} 228, 288, and 332 nm. The ^{1}H-NMR of **1** indicated a set of four coupled aromatic protons at δ_H 8.14 (1H, d, $J = 7.5$ Hz), 7.47 (1H, d, $J = 7.5$ Hz), 7.41 (1H, t, $J = 7.5$ Hz), and 7.22 (1H, t, $J = 7.5$ Hz). These couplings were confirmed with correlations between protons at δ_H 8.14 and 7.22, 7.47, and 7.41 observed on COSY spectrum, suggesting an ABCD ring system. Furthermore, the ^{1}H-NMR showed a characteristic broad singlet at δ_H 8.24 for NH, one aromatic proton at δ_H 6.75(s), one methoxy group at δ_H 4.00 (s), one aromatic methyl group at δ_H 2.47 (s), and characteristic signals for a prenyl moiety at δ_H 5.27 (1H, m, vinyl proton), 3.90 (2H, d, $J = 6.2$ Hz, benzylic protons), 1.72 (3H, s), and 1.93 (3, s). The above information, associated with biogenetic considerations and literature references (Songue *et al.*, 2012; Ito *et al.*, 2000), indicated the presence of a 1-oxygenated 3-substituted carbazole skeleton having no substituent on ring A. In addition, the HMQC spectrum showed correlation of the proton at δ_H 6.75(s) with the carbon at δ_C 109.3. The HMBC spectrum showed correlations of that proton with carbons at δ_C 143.3, 128.9, 127.3, and 19.9, the methoxy protons with carbon at δ_C 143.3, the aromatic methyl proton at δ_H 2.47 with carbons at δ_C 126.9 and 109.3, and the benzilic protons with carbon at δ_C 126.9. This suggested that the methoxy, methyl, and prenyl moieties are located at positions C-1, C-3, and C-4, respectively. Therefore, clausamine H was deduced to be **1** (Fig. 1). Detailed assignments of protons, carbons, and 2D data are shown in Table 1. This structure was further confirmed

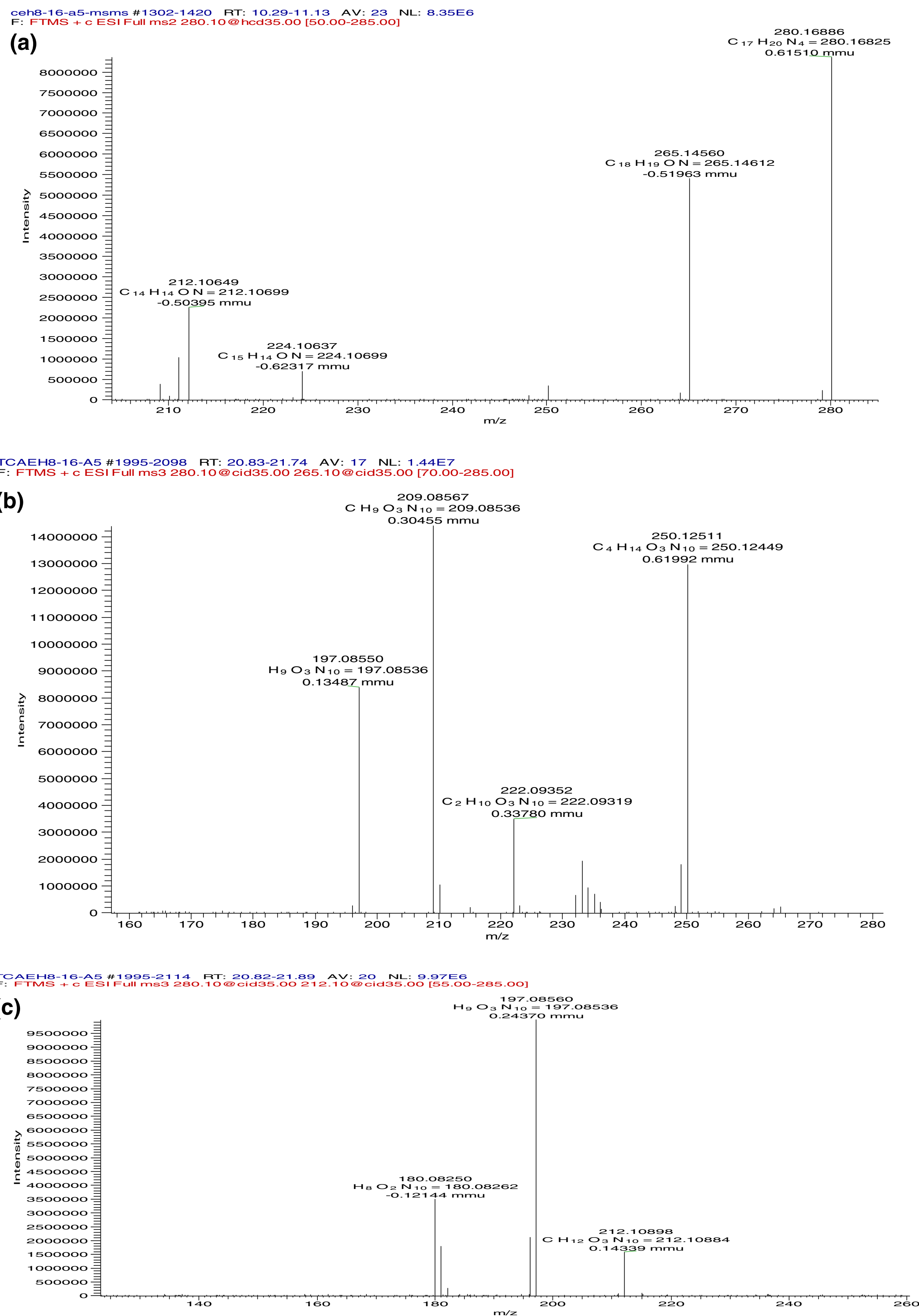

Fig. 2 MS/MS spectra of compound **1** (**a** *m/z* 280; **b** *m/z* 265; and **c** *m/z* 212)

Table 2 Antimicrobial activity (MIC and MBC in µg/mL) of *C. anisata* extracts and isolated compounds

Samples	Inhibition parameters	*Vibrio cholerae* SG24	*Vibrio cholerae* CO6	*Vibrio cholerae* NB2	*Vibrio cholerae* PC2	*Shigella flexneri* 2a	*Staphylococcus aureus ATCC 25923*
Leaves MeOH residue	MIC	2048	2048	2048	1024	512	512
	MBC	2048	2048	>2048	1024	512	512
	MBC/MIC	1	1	–	1	1	1
Stem bark hexane	MIC	128	64	256	512	128	128
	MBC	256	128	256	512	128	128
	MBC/MIC	2	2	1	1	1	1
Stem bark MeOH residue	MIC	64	64	128	128	128	128
	MBC	64	128	128	256	128	128
	MBC/MIC	1	2	1	2	1	1
(**1**)	MIC	128	256	256	512	128	128
	MBC	128	512	512	>512	128	256
	MBC/MIC	1	2	2	–	1	2
(**2**)	MIC	32	64	128	256	256	128
	MBC	32	64	256	512	256	128
	MBC/MIC	1	1	2	2	1	1
(**3**)	MIC	32	64	256	128	256	128
	MBC	64	64	256	128	256	128
	MBC/MIC	2	1	1	1	1	1
(**4**)	MIC	32	32	64	128	64	64
	MBC	32	64	128	128	128	128
	MBC/MIC	1	2	2	1	2	2
(**5**)	MIC	512	512	64	512	256	256
	MBC	512	>512	128	>512	256	256
	MBC/MIC	1	–	2	–	1	1
(**6**)	MIC	64	128	128	128	256	256
	MBC	128	128	128	128	256	256
	MBC/MIC	2	1	1	1	1	1
(**7**)	MIC	128	512	128	512	128	256
	MBC	128	>512	128	>512	128	256
	MBC/MIC	1	–	1	–	1	1
(**9**)	MIC	64	128	>512	>512	256	512
	MBC	128	256	>512	>512	512	512
	MBC/MIC	2	2	–	–	2	1
(**10**)	MIC	128	128	256	>512	512	256
	MBC	128	256	512	>512	512	256
	MBC/MIC	1	2	2	–	1	1
(**11**)	MIC	16	16	64	64	128	128
	MBC	16	32	64	64	256	256
	MBC/MIC	1	2	1	1	2	2
(**13**)	MIC	16	32	128	64	128	128
	MBC	16	64	128	64	128	128
	MBC/MIC	1	2	1	1	1	1
(**14**)	MIC	8	8	32	64	64	32
	MBC	8	8	32	128	128	64
	MBC/MIC	1	1	1	2	2	2
(**15**)	MIC	16	32	64	64	64	128
	MBC	64	32	64	128	64	128
	MBC/MIC	4	1	1	2	1	1

Table 2 continued

Samples	Inhibition parameters	*Vibrio cholerae* SG24	*Vibrio cholerae* CO6	*Vibrio cholerae* NB2	*Vibrio cholerae* PC2	*Shigella flexneri* 2a	*Staphylococcus aureus ATCC 25923*
(16)	MIC	64	64	256	512	256	256
	MBC	64	128	256	512	512	512
	MBC/MIC	1	2	1	1	2	2
(17)	MIC	64	64	128	256	128	256
	MBC	64	128	128	512	256	256
	MBC/MIC	1	2	1	2	2	1
(18)	MIC	64	64	32	128	64	64
	MBC	64	128	32	128	64	64
	MBC/MIC	1	2	1	1	1	1
(19)	MIC	128	64	64	256	128	128
	MBC	128	256	128	256	128	256
	MBC/MIC	1	4	2	1	1	2
(21)	MIC	256	128	64	256	128	128
	MBC	256	128	128	256	128	128
	MBC/MIC	1	1	2	1	1	1
Ampicillin	MIC	16	16	>512	>512	>512	16
	MBC	16	16	>512	>512	>512	16
	MBC/MIC	1	1	–	–	–	1
Chloramphenicol	MIC	4	16	8	1	64	8
	MBC	8	64	32	4	>512	32
	MBC/MIC	2	4	4	4	–	4

with fragments at *m/z* 265 [M+H-CH_3], 224 [M+H-$(CH_3)_2C=CH_2$], and 212 [M+H-$(CH_3)_2C=CH–CH_2$+H] observed on MS and MS–MS (of *m/z* 280) spectra (Fig. 2).

Antibacterial activity

Results of the antibacterial study showed that the stem bark hexane, leaves and stem bark MeOH residue extracts, and compounds **1**–**21** of *C. anisata* significantly inhibited the growth of the tested bacteria (Table 2). The stem bark hexane and MeOH residue extracts were more active than MeOH residue extract of leaves indicating that the antibacterial activity of *C. anisata* is more concentrated in the stem bark. The antibacterial activity of stem bark hexane and MeOH residue extracts were in some cases higher than that of their isolated compounds indicating the broad spectrum antibacterial activities against the corresponding bacterial strains. In addition, the crude extract might also contained varieties of active ingredients, which showed synergistic activities against the tested bacterial spp. The high antimicrobial activities of *C. anisata* leaf extracts were also reported previously by Senthilkumar and Venkatesalu (2009), who attributed these activities to the presence of the major chemical compounds β-pinene, 1,8-cineole, pulegone, estragole, and sabinene.

Total activity is the volume at which test extract can be diluted with the ability to kill microorganisms. It is calculated by dividing the amount of sample from 1 g plant material by the MIC of the same sample and expressed in mL/g (Eloff, 2004). Among all the isolated compounds, compound **14** had the highest antibacterial activity with an average MIC (aMIC) of 34.66 µg/mL and average total activity (aTA) of 2.77 mL/g followed by compounds **15** (aMIC = 61.33 µg/mL and aTA = 1.56 mL/g), **4** (aMIC = 64 µg/mL and aTA = 0.83 mL/g), **11** (aMIC = 69.33 µg/mL and aTA = 1.38 mL/g), **18** (aMIC = 69.33 µg/mL and aTA = 1.38 mL/g), **13** (aMIC = 82.66 µg/mL and aTA = 1.16 mL/g), **19** (aMIC = 128 µg/mL and aTA = 0.75 mL/g), **2** (aMIC = 144 µg/mL and aTA = 0.66 mL/g), **3** (aMIC = 144 µg/mL and aTA = 0.66 mL/g), **17** (aMIC = 149.33 µg/mL and aTA = 0.64 mL/g), **6** (aMIC = 160 µg/mL and aTA = 0.60 mL/g), **21** (aMIC = 160 µg/mL and aTA = 0.33 mL/g), **1** (aMIC = 235.33 µg/mL and aTA = 0.40 mL/g), **16** (aMIC = 256 µg/mL and aTA = 0.37 mL/g), **7** (aMIC = 277.33 µg/mL and aTA = 0.34 mL/g), and **5** (aMIC = 352 µg/mL and aTA = 0.27 mL/g).

No activity was observed for ampicillin against *V. cholerae* NB2, *V. cholerae* PC2, and *S. flexneri* 2a at concentrations up to 512 µg/mL, while these bacterial strains were found to be sensitive to most of the tested

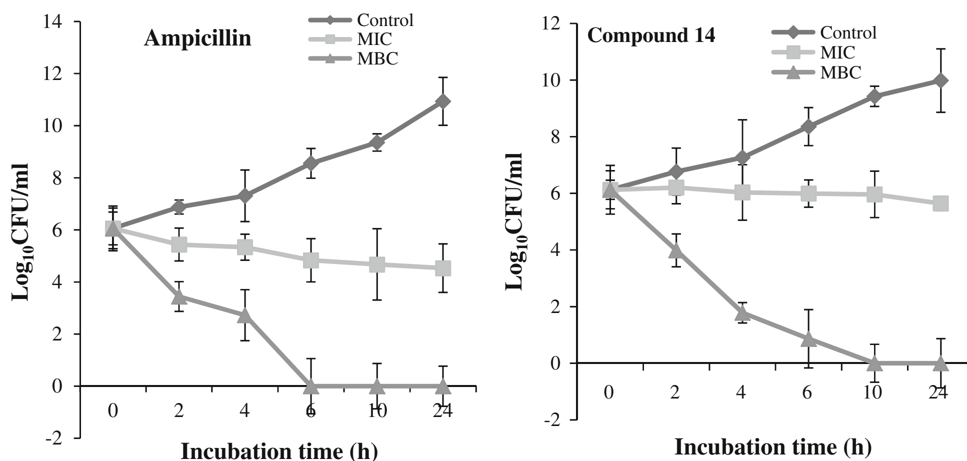

Fig. 3 Survival curves for *Vibrio cholerae* CO6 cells exposed to the compound **14** and ampicillin. Control: MHB medium with DMSO 1 % + inoculums

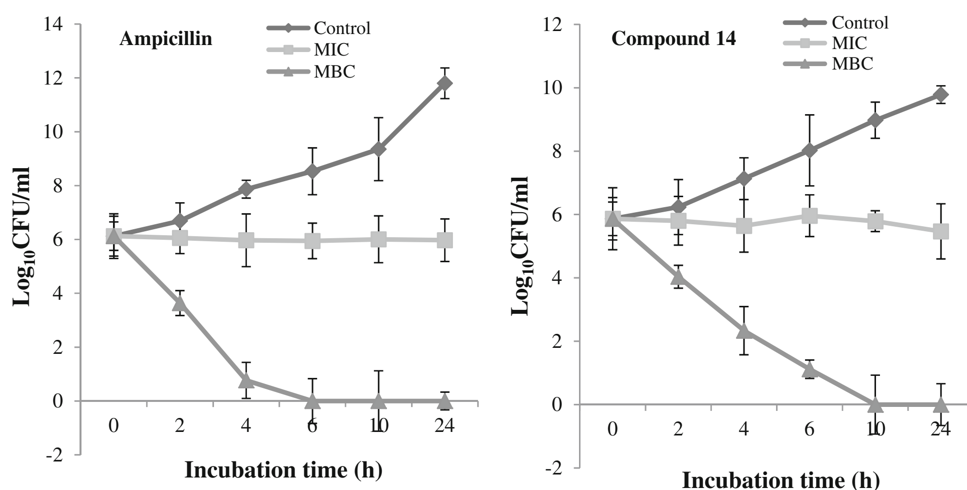

Fig. 4 Survival curves for *Vibrio cholerae* SG24 cells exposed to the compound **14** and ampicillin. Control: MHB medium with DMSO 1 % + inoculums

compounds. These findings propose the antibacterial potencies of these compounds in particular for the treatment of multi-drug resistant strains of *Vibrio cholerae* and *Shigella flexneri*. A Keen look at the MBC values indicates that most of them are equal to their corresponding MICs. This proves that the killing effects of many tested samples could be expected on the sensitive strains (Tamokou *et al.*, 2012). The present study demonstrated the significant antibacterial activities (MIC and MBC ranging from 16 to 256 µg/mL) of the compounds isolated from C. *anisata*, especially the compounds **4**, **11**, **13**, **14**, **15**, and **18**, against multi-drug resistant enteropathogenic bacteria including the clinical MDR isolates of toxigenic *V. cholerae*, the causative agents of dreadful disease cholera, and *Shigella* sp., the causative agent of shigellosis. These compounds also showed significant antibacterial activities against Gram-positive bacteria, *S. aureus*, indicating their potential broad spectrum properties. In addition, these compounds were non-toxic to normal Vero cells (LC_{50} is much higher than 256 µg/mL) indicating their promising therapeutic potential. Although phenolic compounds have been reported to possess interesting activity against a wide range of microorganisms (Tamokou *et al.*, 2013), no study has been reported on the activity of these compounds against these types of pathogenic strains.

The time-kill kinetic study

The time-kill kinetic study for compound **14** against *V. cholerae* CO6 and *V. cholerae SG24* (as a function of incubation time) is shown in Figs. 3 and 4. It can be noted that significant reduction (~6-log reduction in growth, compared to the untreated control) of the bacterial population was observed with the compound **14** and ampicillin at a concentration corresponding to their MBC values within 6–10 h (Fig. 3). At this concentration, all the bacterial population was completely killed after 6 and 10 h of incubation with ampicillin and compound **14**, respectively.

Table 3 Cytotoxic activity and selective index (SI) of the most active compounds

Compounds	Cytotoxicity (LC_{50} in μg/mL)		Selectivity index*
	HeLa cells	Vero cells	HeLa cells
4	3.26 ± 0.14^{a}	434.78 ± 2.59^{c}	133.36
5	1.14 ± 0.16^{b}	111.35 ± 3.91^{a}	97.67
7	1.81 ± 0.09^{c}	69.15 ± 3.67^{b}	38.20
10	2.98 ± 0.22^{a}	–	–
13	1.27 ± 0.03^{b}	294.11 ± 1.99^{d}	231.58
15	19.45 ± 0.65^{d}	–	–
21	2.36 ± 0.08^{e}	290.69 ± 4.32^{d}	123.17
Paclitaxal	>40 nM	>40 nM	–

– not determined, *SI = LC_{50} on monkey Vero cells/LC_{50} on HeLa cells; Each LC_{50} value represents the mean ± SD ($n = 3$). In the same column, LC_{50} value marked with different superscript letters are significantly different ($P < 0.05$)

Cytotoxic activity

The crude extracts and isolated compounds from the leaves/stem bark of *Clausena anisata* were evaluated for their cytotoxicity against human cancer cells (HeLa cells) and normal non-cancer cells (Vero cells) in vitro using the MTT assay, and the results are presented in Table 3. The cytotoxicity test revealed that all the tested samples ($LC_{50} = 1.14$–19.45 μg/mL) were most cytotoxic on HeLa cells when compared with Vero cells ($LC_{50} = 69.15$–434.78 μg/mL). Some of the isolated compounds (**1**, **2**, **3**, **6**, **9**, **11**, **14**, **16**, **17**, **18**, and **19**) did not show cytotoxicity against HeLa cells. However, compounds **15** had LC_{50} value greater than 10 μg/mL, while the others isolated compounds (**4**, **5**, **7**, **10**, **13**, **15**, and **21**) had cytotoxic activity against the HeLa cells with LC_{50} values ranging from 1.14 to 3.26 μg/mL. In addition, these compounds were non-toxic to normal Vero cells ($LC_{50} = 69.15$–434.78 μg/mL, which are much higher than that for HeLa cells), indicating their high potential to be used as anticancer drug. Selectivity is important because most anticancer drugs currently in use induce serious adverse effects. In the present study, SI index of the compounds (**4**, **5**, **7**, **13**, and **21**) were in the range between 38.20 and 231.58 for HeLa cells in comparison to the Vero cells indicating that it is significantly specific for cancer cells, which will be useful for cancer treatment. Compounds **4**, **5**, **7**, **10**, **13**, **15**, and **21**, could be considered relatively less toxic than the positive control paclitaxal (LC50 > 40 nM). However, their cytotoxicity can be considered more important when taking into consideration the criterion of the American National Cancer Institute (NCI) regarding the cytotoxicity of pure compounds ($LC_{50} < 4$ μg/mL) (Tanamatayarat *et al.*, 2003).

Selectivity is important because most anticancer drugs currently in use induce serious adverse effects. Apart from compound **4** on *V. cholerae* SG24 and *V. cholerae* CO6, and compound **13** on *V. cholerae* SG24, the selectivity index (SI) values of the tested samples against the bacterial strains ranged from 0.13 to 9.19 and could be considered as poor when taking in consideration that the ratio for a good therapeutic index for a remedy or drug should be ≥10 (Caamal-Fuentes *et al.*, 2011). However, the selectivity index (SI) values of the tested compounds against the HeLa cells ranged from 38.20 to 231.58 and could be considered as good. This is the first report on the cytotoxicity of compounds **4**, **5**, **7**, **10**, **13**, **15**, and **21** from *C. anisata* against Vero cells and HeLa cells. These results are consistent with the use of these compounds for treating breast cancer and give support to *C. anisata* use in Cameroonian folk medicine.

Conclusion

Compounds **4**, **5**, **7**, **10**, **13**, **15**, and **21** had good cytotoxic activity against human cancer HeLa cells with no toxicity to normal cells, and may be useful in topical applications to combat cancer. The stem bark hexane and MeOH residue extracts, compounds **4**, **11**, **13**, **14**, **15**, and **18**, possess potent antibacterial activities (MIC and MBC ranging from 16 to 256 μg/mL) against MDR clinical isolates of enteropathogenic bacteria with no toxicity to Vero cells (LC_{50} is much higher than 256 μg/mL) that may lead to new drug development for the treatment of severe infectious diseases.

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