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Monanthosin, a new megastigmane derivative from the leaves of *Monanthotaxis littoralis* (Annonaceae) with the antimicrobial and antioxidant activities of the chemical constituents

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Abstract: *2-Hydroxy-4-(1'-hydroxy-2',6',6'-triméthyl-4-oxo-cyclohex-2'-enyl)-but-3-enoic acid, a new megastigmane derivative, trivially named monanthosin (**1**), together with eight known compounds (chrysin (**2**), quercitrin (**3**), astilbin (**4**), heptulose (**5**), allantoin (**6**), heptitol (**7**), cis-N-p-coumaroyl tyramin (**8**) and trans-N-p-coumaroyl tyramin (**9**)) were isolated from the leaves of Monanthotaxis littoralis (Annonaceae). Structures were assigned by direct interpretation of their spectral data, mainly HR-TOFESIMS, 1D NMR (^{1}H and ^{13}C) and 2D NMR (^{1}H-^{1}H COSY, HSQC, HMBC and NOESY) and by comparison with reported values. The MeOH, EtOAc and n-BuOH extracts as well as compounds **1**, **2**, **4** and **8** exhibited variable antimicrobial and antioxidant activities. The ethyl acetate extract and compound **4** were the most active samples among extracts and compounds, respectively. The ethyl acetate extract and antibiotics (vancomycin and fluconazole) demonstrated synergistic effect against Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Candida tropicalis and Cryptococcus neoformans and additive effect against Staphylococcus aureus.*

Keyword*: Monanthotaxis littoralis; Leaves; Annonaceae; Monanthosin; Antimicrobial; Antioxidant.*

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I. Introduction

The Annonaceae family is characterized by the presence of flavonoïds, isoquinoline alkaloids and acetogenins [1-2]. Some members of *Monanthotaxis* species contain oxygenated cyclohexane epoxide derivatives [3-5], polyoxygenated cyclohexene [6], caryophyllene and caryophyllene_oxide [7], phenols and triterpenoids [8]. *Monanthotaxis littoralis* (Bagsh. & Baker F.) Verdc, is a persistent shrub with oblong elliptic leaves and solitary flowers [9]. It is a vascular plant widely distributed in some tropical African countries such as Cameroon, Central African Republic, Congo and Uganda [10]. Previous studies on this species reported the presence of flavonoids, essential oils [10], roseoside and its related compounds [11]. In our continuous search for potentially interesting novel and bioactive secondary metabolites from Cameroonian medicinal plants [12-13], we have examined the MeOH extract of the leaves of *M. littoralis*. In the present paper we report, the isolation and structural elucidation of a novel megastigmane derivative, together with eight known compounds (Figures 1, 3); the result of the antimicrobial and antioxidant activities of extracts and some of isolated compounds from *M. littoralis* (Tables 2–4) was also presented.

II. Material And Methods

2.1 General and experimental procedures:

^{1}H and ^{13}C-NMR spectra were performed in deuterated methanol on a Bruker AVANCE III. 600 spectrometer equipped with a cryoprobe (^{1}H at 600 MHz and ^{13}C at 150.91 MHz). 2D NMR (^{1}H-^{1}H COSY, HSQC, HMBC and NOESY) experiments were recorded by means of standard Bruker microprograms (Xwin-

NMR version 2.1 software TopSpin 3.2). All chemical shifts (δ) are given in ppm with reference to tetramethylsylane (TMS) as internal standard and the coupling constants (*J)* are in Hz. TOFESIMS and HR-TOFESIMS spectra were recorded using a Micromass Q-TOF micro instrument (Manchester, UK) equipped with an electrospray source. The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 μL min^{-1}. The IR spectra were recorded with a Thermo Scientific™ iD7 ATR spectrophotometer. The optical rotations were measured on a Bellingham & Stanley ADP 220 polarimeter (Bellingham + Stanley Ltd., United Kingdom). Column chromatography was run on Merck silica gel 60 (70-230 mesh) and gel permeation on Sephadex LH-20 while TLC was carried out on silica gel GF_{254} pre-coated plates with detection accomplished by spraying with 10% H_2SO_4 followed by heating at 90^{o}C, or by visual inspection under UV lamp at 254 and 365 nm.

2.2. Collection of plant sample:

The plant of *M. littoralis* (Bagsh. & Baker F.) Verdc was geolocated in Dschang, (Menoua Division, Western Region of Cameroon) according to the coordinates 5° 26' 0" N, 10° 4' 0" E. The leaves were collected in January 2016 and identified by Mr. Fulbert TADJOUTEU, a Botanist at the National Herbarium of Cameroon, where a voucher specimen (N^o 35048/HNC) has been deposited.

2.3 Extraction and isolation of Plant Material:

The air-dried plant material (3.0 Kg) was powdered and extracted at room temperature with methanol (18 L, 72 h). Evaporation of solvent under reduced pressure yielded 620.9 g of crude extract (ML). Part of this extract (613.9 g) was extracted with ethyl acetate and *n*butanol to give 206.9 g and 70.2 g dry fractions, respectively. The *n*-butanol extract (MLB) was fractionated by silica gel column chromatography (CC), eluted with gradient solvent system of EtOAc/MeOH (100:0; 95:5; 90:10; 80:20; 70:30; 60:40) to give seven fractions (MLB1-MLB7). Fraction MLB4 (3.57 g) was subjected to a silica gel CC and eluted with EtOAc/MeOH/H_2O (90/5/2, v/v/v) to provide three sub-fractions (MLB4.1-MLB4.3). Sub-fraction MLB4.3 (0.37 g) was submitted to silica gel CC, eluting with EtOAc/MeOH (98/2, v/v) to yield compound **1** (14.5 mg). Fraction MLB5 (10.5 g) was subjected to silica gel CC and eluted with EtOAc/MeOH (85/15, v/v) to give compounds **5** (19.4 mg) and **6** (20.5 g). The ethyl acetate extract (MLE) was fractionated by silica gel CC, eluting with gradient solvent system of hexane/EtOAc (85:15; 80:20; 60:40; 30:70; 10:90; 0:100) to give nine fractions MLE1-MLE9. Fraction MLE2 (9.6 g) was loaded to a silica gel CC and eluted with hexane/EtOAc (85/15, v/v) to give compound **2** (15 mg). Fraction MLE5 (14.5 g) was purified by silica gel CC, eluting with hexane/EtOAc (60/40, v/v) to give compounds **8** (4.5 mg) and **9** (15 mg). Fraction MLE7 (8.0 g) was subjected to sephadex LH-20 and eluted with MeOH to give compound **4** (4.5 mg). Fraction MLE8 (15.3 g) was subjected to silica gel CC, eluted with EtOAc to provide six sub-fractions (MLE8.1MLE8.6). Sub-fraction MLE8.6 (1.8 g) was separated by silica gel CC and eluted with EtOAc/MeOH (95/5, v/v) to give compound **3** (4.0 mg). Fraction MLE9 (8.5 g) was submitted by silica gel CC, eluted with hexane/EtOAc (85/15, v/v) to provide eigth subfractions (MLE9.1-MLE9.8). Sub-fraction MLE9.8 (2.45 g) was separated by silica gel CC and eluted with EtOAc/MeOH/H_2O (90/5/5, v/v/v) to give six sub-fractions (MLE9.8aMLE9.8f). Subfraction MLE9.8c (0.35 g) was subjected to silica gel CC and eluted with EtOAc/MeOH (98/2, v/v) to give compound **7** (17.0 mg).

2.4. Antimicrobial assay:

2.4.1 Microorganisms:

The studied microorganisms were one Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923), two Gram-negative bacteria (*Escherichia coli* S2 (1) and *Pseudomonas aeruginosa* PA01) and three strains of yeasts (*Candida tropicalis* PK233, *Candida albicans* ATCC10231 and *Cryptococcus neoformans* H99) taken from our laboratory collection. The bacterial and fungal species were grown at 37 °C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants respectively.

2.4.2 Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC):

MIC values were determined by a broth micro-dilution method as described earlier [14] with slight modifications. Each test sample was dissolved in dimethylsulfoxide (DMSO) and the solution was then added to Mueller Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth (SDB) for yeasts to give a final concentration of 8192 μg/mL. This was serially diluted twofold to obtain a concentration range of 0.125–4096 μg/mL. Then, 100 μL of each concentration were added in each well (96-well microplate) containing 95 μL of MHB or SDB and 5 μL of inoculum for final concentrations varying from 0.0625–2048 μg/mL. The inoculum was standardized at 2.5 x 10^5 cells/mL for yeasts and 10^6 CFU/mL for bacteria using a JENWAY 6105 UV/Vis spectrophotometer. The final concentration of DMSO in each well was <1% [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms]. The negative control well consisted of 195 μL of

MHB or SDB and 5 µL of the standard inoculum. The cultured micro plates were covered; then, the contents of each well were mixed thoroughly using a plate shaker (Flow Laboratory, Germany) and incubated at 35 °C for 24 h (bacteria) and 48 h (yeasts) under shaking. The assay was repeated three times. The MIC values of samples were determined by adding 50 µL of a 0.2 mg/mL *p*-iodonitrotetrazolium violet solution followed by incubation at 35 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC values were defined as the lowest sample concentrations that prevented this change in color indicating a complete inhibition of microbial growth. For the determination of MMC values, a portion of liquid (5 µL) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar or SDA and incubated at 35 °C for 24 h (for bacteria) or 35 °C for 48 h (for yeasts). The lowest concentrations that yielded no growth after this subculturing were taken as the MMC values. Vancomycin (Sigma-Aldrich, Steinheim, Germany) and fluconazole (Merck, Darmstadt, Germany) were used as positive controls for bacteria and yeasts, respectively.

2.4.3 Combined effect of the ethyl acetate extract and antibiotics:

The antimicrobial effects of a combination of the ethyl acetate extract of *M.littoralis* (MLE), which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [15]. The antimicrobial combinations assayed included MLE with antibiotics, vancomycin and fluconazole. Serial dilutions of three different antimicrobial agents were mixed in Mueller-Hinton broth. After 24-48 h of incubation at 37 °C, the MICs were determined as described above. The fractional inhibitory concentration (FIC) index was calculated according to the equation: FIC index = FICA + FICB = MIC of drug A in combination / MIC of drug A alone + MIC of drug B in combination / MIC of drug B alone. The FIC indices are the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0. All the experiments were performed in triplicate.

2.5 Antioxidant assay:

2.5.1 DPPH free radical scavenging assay:

The free radical scavenging activity of extracts as well as most of their isolated compounds was performed according to [16] with slight modifications. Briefly, different concentrations (10 to 2000 µg/mL) of extracts/compounds and vitamin C (positive control) were thoroughly mixed with 3 mL of methanolic DPPH solution (20 mg/L) in test tubes and the resulting solution was kept standing for 30 minutes at room temperature before the optical density (OD) was measured at 517 nm. The percentage radical scavenging activity was calculated from the following formula: % scavenging [DPPH] = $[(A_0 - A_1)/A_0] \times 100$ [17] where A_0 was the absorbance of the negative control (methanolic DPPH solution) and A_1 was the absorbance in the presence of the samples. EC_{50} value was determined from the graph obtained using standard vitamin C by using the "y = mx + c" formula from the slope of the graph. All the analyses were carried out in triplicate.

2.5.2 Gallic acid equivalent antioxidant capacity (GEAC) assay:

The GEAC test was done as previously described by Rice-Evans and Miller with slight modifications [18]. In a quartz cuvette, to 950 µL acetate buffer (pH = 5.0, 100 mM), the following were added: 20 µL laccase (1 mM stock solution), 20 µL test sample, 10 µL ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (74 mM stock solution). The laccase were purified from *Sclerotinia sclerotiorum* according to the protocol described by [19]. The sample concentrations in the assay mixture were 800, 400, 200, 100, 10 µg/mL for the extracts and 200, 100, 50, 25, 12.5 µg/mL for the isolated compounds. The content of the generated $ABTS^{\bullet+}$ radical was measured at 420 nm after 240 s reaction time and was converted to gallic acid equivalents by the use of a calibration curve (Pearson's correlation coefficient: $r = 0.996$) constructed with 0, 4, 10, 14, 28, 56, 84 µM gallic acid standards rather than Trolox. Experiments were done in triplicate.

2.6 Statistical analysis

Data were analyzed by one-way analysis of variance followed by Waller-Duncan Post Hoc test. The experimental results were expressed as the mean ± Standard Deviation (SD). Differences between groups were considered significant when $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

III. Result

3.1 Characterization of compound 1:

Compound **1** (figure 1) was obtained as a yellowish gum; $[\alpha]_D$: + 26 (*c* 0.25, MeOH). Its molecular formula $C_{13}H_{18}O_5$, corresponding to five degrees of unsaturation was determined from HRTOFESIMS (negative

ion mode) at *m/z* 253.1077 $[M-H]^-$ (Calcd for $C_{13}H_{17}O_5$, 253.1076). The peaks observed at *m/z* 507.2228 $[2M-H]^-$, 153.0915 $[M-H-C_4H_5O_3]^-$ are in agreement with this molecular formula. The IR spectrum indicates the presence of hydroxyl (3428 cm^{-1}), carbonyl groups (1645 and 1692 cm^{-1}) and carbon-carbon double bonds (1596 cm^{-1}).

The ^{13}C NMR spectrum combined with the HSQC spectroscopic analysis displays 13 carbon (table 1) two carbonyl at δ_C 199.6 (C-4') and 177.7 (C-1), four olefinic carbons at δ_C 166.2 (C-2'), 130.6 (C-4), 131.3 (C-3) and 125.7 (C-3'), one sp^3 methine bearing oxygen at δ_C 73.5 (C-2), one methylene at δ_C 49.3 (C-5'), three methyls at δ_C 22.0 ($\alpha\underline{CH_3}$-C-6'), 23.1 ($\beta\underline{CH_3}$-C-6') and 18.2 ($\underline{CH_3}$-C-2'), and two quaternary aliphatic carbon at δ_C 41.2 (C-6') and δ_C 78.8 (C-1'). The downfield shift observed for C-1', indicated that they are substituted by hydroxyl group.

The ^1H NMR data (table 1) confirmed the presence of three singlet methyls of which one is vinylic (δ 1.93, $\underline{CH_3}$-C-2'), and the two others were attached to the same sp^3 quaternary carbon (δ 1.06, $\underline{(CH_3)_2}$-C-6'). The vinyl protons appear at δ 5.89 (1H, s, H-3'), and 5.97 (2H, m, H-3 and H4). Signals observed as doublet at δ 2.17 and 2.56 (J = 16.5 Hz) were attributable to one methylenic protons (H-5'α, and H-5'β respectively). In the COSY spectrum, the two vinyl protons were correlated each other's and with the sp^3 methine proton bearing and oxygen at δ 4.66 (1H, brs, H-2).

Positions of methyl groups were deduced from its HMBC spectrum on which correlation between the protons at δ_H 1.06 (6H, $\underline{(CH_3)_2}$-C-6') with carbons at δ_C 41.2 (C-6'), 49.3 (C-5') and 78.8 (C-1'), and proton at δ_H 1.93 (3H, $\underline{CH_3}$-C-2') with carbons at δ_C 78.8 (C-1'), 125.7 (C-3') and 166.2 (C-2') were observed. The location of the carbonyl at C-4' position (δ_C 199.6) was deduced from its HMBC correlations (figure 2) with the methylenic protons H-5', and the vinylic proton H-3', thus indicating a conjugated ketone group. These correlations allowed us to build, in addition with the ^1H-^1H COSY spectrum, the carbon skeleton of the molecule.

Comparison of ^1H and ^{13}C data of monanthosin **1** with those of vomifoliol [20] and cucumegastigmane I [21] indicates that compound **1**, possess an intact fragment of cucumegastigmane I (Table 1). The difference was in the presence of a carbonyl group (δ_C 177.7) instead of an oxymethylene group in cucumegastigmane I. Thus, suggesting that compound **1** is the carbonyl derivative of cucumegastigmane I. This is confirmed in the MS spectrum giving an ion fragment at *m/z* 153.0915 ($C_9H_{13}O_2$) corresponding to the loss of the lateral chain in $C_4H_5O_3$ (C_2H_2-CHOH-COOH).

NOESY experiment gave no conclusive information on the absolute configurations around the C-1' and C-2 carbons. Only the correlations between H-3' and H-4, $\underline{CH_3}$-C-2'and $\underline{(CH_3)_2}$C-6'were observed on this spectrum. Usually, cucumegastigmane and its derivatives have (1'*S, 2R*)-configuration and the side chain double bond C-3(4) has *trans* stereochemistry [11, 21-22]. The oxidation of C-1 carbon to carboxylic acid function in compound **1** would therefore reverse the absolute configuration around the C-2 carbon. This would allow us to suggest that the absolute configurations around the two stereocenters would be *(1'S,2S)*. Horeau's method [23-24] was applied to **1** in order to confirm the configuration at C-2. A mixture of **1** with an excess of 2-phenylbutyric anhydride and DMAP in chloroform showed an immediate evolution of the optical rotation in the (-) sense, thus including the preferential esterification by the (+) antipode of the acid. Silica gel column chromatography coupled with an optical rotation detector (Chiral detector: Knauer France, reference: 1000) allowed the isolation of levorotatory 2-phenylbutyric acid. According to the Horeau's method, when (-)*(R)*-2-phenylbutyric acid accumulates in the mixture (i.e. when the (+)-(*S*)-acid is the preferential esterifying acid), the C-2 secondary hydroxyl has the *(S)* configuration. On the basis of aforementioned information, the structure of **1** was elucidated as 2-Hydroxy-4-(1'-hydroxy-2',6',6'-trimethyl-4-oxo-cyclohex-2'-enyl)-but-3-enoic acid named monanthosin.

Table 1: ^1H and ^{13}C NMR data of compound 1

Pos.	δ_C [a,b]	δ_H [a,c] (mult.; *J* in Hz)	COSY	HMBC
1	179.1	-		H-2
2	75.1	4.60 (br s)	H-3	C-1
3	132.0	5.91 (m)	H-4,2	C-4, 2
4	132.6	5.91 (m)	H-3	C-3, 2
1'	80.1	-		
2'	167.6	-		
3'	127.2	5.83 (s)	CH_3-$C_{2'}$	C-5', 1', CH_3-$C_{2'}$
4'	201.2	-		H-5', 3'
5'	50.7	2.11 (d, 16.5, H-5'α) 2.50 (d, 16.5, H-5'β)	H-5'β H-5'α,	C-6', 4', αCH_3-$C_{6'}$, βCH_3-$C_{6'}$

6’	42.6	-		
αCH_3-$C_{6'}$	23.4	1.01 (s)		C-6’, 5’, 1’, βCH_3-$C_{6'}$
βCH_3-$C_{6'}$	24.5	1.01 (s)		C-6’, 5’, 1’, βCH_3-$C_{6'}$
CH_3-$C_{2'}$	19.7	1.87 (s)		C-3’, 2’, 1’

[a]Recorded in CD_3OD, [b] 150MHz, [c]600 MHz

Fig. 1. Structure of compound **1**.

Fig. 2. The key HMBC and COSY correlation of **1**.

3.2 Identification of known compounds

Structures of compounds **2**, **3**, **4**, **5**, **6**, 7, **8** and **9** (Fig. 3) were determined by means of spectroscopic data and by comparative analysis of their spectral data with those reported in the literature as known chrysin (**2**) [25], quercitrin (**3**) [26], astilbin (**4**) [27], heptulose (**5**) [28], allantoin (**6**) [29], heptitol (**7**) [30], *cis*-*N*-*p*-coumaroyl tyramine (**8**) [14] and *trans*-*N*-*p*-coumaroyl tyramine (**9**) [31].

Fig. 4. Structures of the known compounds from *M. littoralis*.

3.4 Antimicrobial activity:

In the present work, the extracts as well as four compounds isolated from the leaves of *M. littoralis* were tested for their antimicrobial activities against three bacterial (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* S2 (1) and *Pseudomonas aeruginosa* PA01) and three fungal strains (*Candida albicans* ATCC10231, *Candida tropicalis* PK233 and *Cryptococcus neoformans* H99) using broth microdilution method [31] (Table 2). The MIC results indicated that the MeOH, *n*-BuOH and EtOAc extracts inhibited the growth of all tested bacterial species. The most active extract was the EtOAc extract (MIC = 64-256 µg/mL). Compound **1** displayed weak antibacterial activity (MIC = 128-256 µg/mL) and no antifungal activity (MIC = > 256 µg/mL). Compounds **2**, **4** and **8** inhibited the growth of all tested bacterial and fungal strains. Compound **4** (MIC = 8-16 µg/mL) was the most active with lowest MIC value of 8 µg/mL on *Staphylococcus aureus*, *Candida albicans*, *Candida tropicalis* and *Cryptococcus neoformans*, highlighting some medicinal potential for this compound. As shown in Table 2, vancomycin and fluconazole used as standard drugs were more potent than the tested samples against yeasts, Gram-positive and Gram-negative bacteria with the exception against *E. coli* and *P. aeruginosa* where the antibacterial activity of compound **4** was equal to or higher than that of vancomycin. The antibacterial activities of chrysin and astilbin are highest compared to those of the early reports [32-34]. Indeed, the relative antibacterial activity (MIC_{50} = 36.72 µg/ml) was recorded for chrysin against *E. coli* ATCC25922 [32] whereas astilbin had MIC values of 225 µg/ml against *Streptococcus sobrinus* [33]. The minimal inhibitory quantity (MIQs) of astilbin ranged from 50 to 100 µg against bacterial strains representative of skin microflora [34]. The antimicrobial activity of phenolic conjugate coumaroyl tyramine can be explained by the fact that it has been suggested to have two possible roles in plant defence, as direct antimicrobial agents and in cell-wall reinforcement [32-34]. However the antifungal activities of chrysin, astilbin and *cis*-*N*-*p*-coumaroyl tyramin are reported here for the first time. The microbicidal activities of extracts and isolated compounds against susceptible strains were analysed by the minimum microbicidal concentration (MMC) assay and summarized as MMC/MIC ratios in Table 2. Indeed, an antimicrobial agent is considered microbicidal if the MMC is not more than fourfold higher than the MIC, i.e. MMC/MIC ≤ 4 [35]. The MeOH, EtOAc and *n*-BuOH extracts as well as compounds **2, 4** and **8** were shown to be microbicidal (MMC/MIC ≤ 2) against the susceptible microorganisms whereas compound **1** displayed the bacteriostatic/fungistatic character (MMC/MIC > 4) against all the tested microorganisms.

The combination of the ethyl acetate extract (EtOAcMLE), which exhibited the highest antimicrobial activity, and antibiotics (vancomycin and fluconazole) were assessed by the checkerboard test as previously described [15]. EtOAcMLE and antibiotics demonstrated synergistic effect against *E. coli*, *P. aeruginosa*, *C. albicans*, *C. tropicalis* and *C. neoformans* and additive effect against *S. aureus* (Table 3).

Table 2. Antimicrobial activity (MIC and MMC in µg/mL) of extracts, isolated compounds and reference antimicrobial drugs.

Extracts/ Compounds	Inhibition parameters	*E. coli*	*P. aeruginosa*	*S. aureus*	*C. tropicalis*	*C. albicans*	*C. neoformans*
MeOH extract	MIC	256	512	256	512	512	256
	MMC	512	512	512	1024	1024	512
	MMC/MIC	2	2	2	2	2	2
EtOAc extract	MIC	64	64	64	256	256	256
	MMC	64	64	64	512	512	256
	MMC/MIC	1	1	1	2	2	1
n-BuOH extract	MIC	128	128	128	512	512	512
	MMC	128	128	256	1024	512	512
	MMC/MIC	1	1	2	2	1	1
1	MIC	256	256	128	>256	>256	>256
	MMC	>256	>256	>256	/	/	/
	MMC/MIC	/	/	/	/	/	/
2	MIC	64	32	32	64	64	32
	MMC	128	64	64	64	64	32
	MMC/MIC	2	2	2	1	1	1
4	MIC	16	16	8	8	8	8
	MMC	16	16	8	16	16	8
	MMC/MIC	1	1	1	2	2	1
8	MIC	64	32	16	64	32	16
	MMC	64	32	16	64	32	16
	MMC/MIC	1	1	1	1	1	1
Ref*	MIC	32	16	0.5	0.5	1	2
	MMC	32	16	0.5	0.5	1	2
	MMC/MIC	1	1	1	1	1	1

/: not determined; MIC: Minimum Inhibitory Concentration; MMC: Minimum Microbicidal Concentration; *: fluconazole for yeasts and vancomycin for bacteria; compounds **1, 5** and **6** were not active at concentrations up to 256 µg/mL; compounds **3, 7** and **9** were not tested.

Table 3. Checkerboard assay of EtOAc extract and reference antibiotics against pathogenic strains.

Strains	Agent	MIC (µg/mL)		FIC	FICI	Outcome
		Alone	Combination			
E. coli	EtOAcML	64	16	0.25	0.375	Synergistic
	Vancomycin	32	4	0.125		
P. aeruginosa	EtOAcML	64	8	0.125	0.25	Synergistic
	Vancomycin	16	2	0.125		
S. aureus	EtOAcML	64	32	0.50	0.75	Additive
	Vancomycin	0.5	0.125	0.25		
C. tropicalis	EtOAcML	256	32	0.125	0.25	Synergistic
	Fluconazole	0.50	0.062	0.125		
C. albicans	EtOAcML	256	64	0.25	0.375	Synergistic
	Fluconazole	1	0.125	0.125		
C. neoformans	EtOAcML	256	8	0.0312	0.0937	Synergistic
	Fluconazole	2	0.125	0.0625		

3.4 DPPH radical scavenging activity

In this study, free radical scavenging capacities were measured using DPPH radical and ABTS radical cation. The results are expressed as gallic acid equivalent antioxidant capacity of tested samples (Table 4) and as equivalent concentrations of test samples scavenging 50% of DPPH radical (Table 4). In all, the DPPH and ABTS scavenging activities in this study indicated that the MeOH, EtOAc and *n*-BuOH extracts were potent antioxidants. On order to identify compounds responsible to this activity, the antioxidant properties of the flavonoids **2**, **4** and the *cis*-*N*-*p*-coumaroyl tyramine (**8**) as well as the new compound **1** were measured. No antioxidant activity was observed with compound **1** (results not shown), while compounds **2, 4** and **8** were potent antioxidants. Compound **4** (EC_{50} = 3.68 µg/mL; GEAC= 96.71 µg/mL) exerted the greatest activity whereas compound **8** (EC_{50} = 58.44 µg/mL; GEAC= 51.27 µg/mL) displayed the lowest antioxidant activity in both assays ($p < 0.05$); suggesting that the ability of these compounds to scavenge DPPH could also reflect their ability to inhibit the formation of $ABTS^{\cdot+}$. However, their antioxidant activities are lesser than that of vitamin C (EC_{50} = 1.96 µg/mL).

The antioxidant properties of chrysin and astilbin are in agreement to those of the literature [38-39]. Indeed, Vijayalakshmi et al. (2011) [39] demonstrated significant antioxidant activity of astilbin with IC_{50} values of 7.50, 21.50 and 24.10 µg/mL against DPPH, nitric oxide and lipid peroxide radicals, respectively. A study conducted by Pushpavalli et al. (2010) [38] showed that the treatment of D-galactosamine in toxication rats with chrysin (25, 50 and 100 mg/Kg body weight) increased the activities of free-radical scavenging (enzymes superoxide dismutase, catalase and glutathione peroxidase) and the levels of non-enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E); suggesting that chrysin acts as antioxidant agent. Chemical properties of chrysin, due to lack of oxygenation on B and C-ring are linked with various pharmacological properties that varies from antioxidant to anticancer properties [40] (Habtemariam, 1997). Though, differences in the structure of flavones have been revealed to persuade the antioxidant property. Astilbin, which has 3',4'-hydroxylation demonstrated more antioxidant activity than chrysin. This finding foresees potential applications of astilbin as an antioxidant. The presence of 3',4'-hydroxylation, a double bond between carbons 2 and 3, and the presence of a carbonyl group on carbon 4 have been demonstrated to be crucial to generate antioxidant activity [41].

Table 4. Antioxidant activities of extracts and compounds 2, 4, 8

Extracts/compounds	DPPH (EC_{50})	GEAC
MeOH extract	65.18 ± 0.29^{a}	62.02 ± 0.54^{a}
n-BuOH extract	69.07 ± 1.22^{b}	43.17 ± 0.56^{b}
EtOAc extract	74.21 ± 0.63^{c}	48.09 ± 0.36^{c}
2	27.41 ± 0.79^{d}	80.53 ± 0.82^{d}
4	3.68 ± 0.32^{e}	96.71 ± 0.41^{e}
8	58.44 ± 1.62^{f}	51.27 ± 0.72^{f}
Vitamin C	1.96 ± 0.14^{g}	NA

EC_{50}: Equivalent concentrations of test samples scavenging 50% of DPPH radical. Data represent the mean ± SD of three independent experiments carried out in triplicate. In the same column, values affected by different superscript letters (a-f) are significantly different according to one way ANOVA and Waller Duncan test; $p < 0.05$.

nd Waller Duncan test; $p < 0.05$.

IV. Conclusion

Finally, the phytochemical study of the MeOH, EtOAC and *n*-BuOH extracts of *Monanthotaxis littoralis* afforded nine compounds including monanthosin (1), chrysin (2), quercitrin (3), astilbin (4), heptulose (5), allantoin (6), heptitol (7), *cis*-*N*-*p*-coumaroyl tyramin (8) and *trans*-*N*-*p*-coumaroyl tyramin (9). The MeOH, EtOAC and *n*-BuOH extracts as well as compounds 1, 2, 4 and 8 exhibited variable antimicrobial and antioxidant activities. They may be used as phytomedicines at low cost and easily affordable by the target population with caution of hemolytic activity and clinical studies currently going on in our laboratory.

Conflict of interest

The author has no conflict of interest

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