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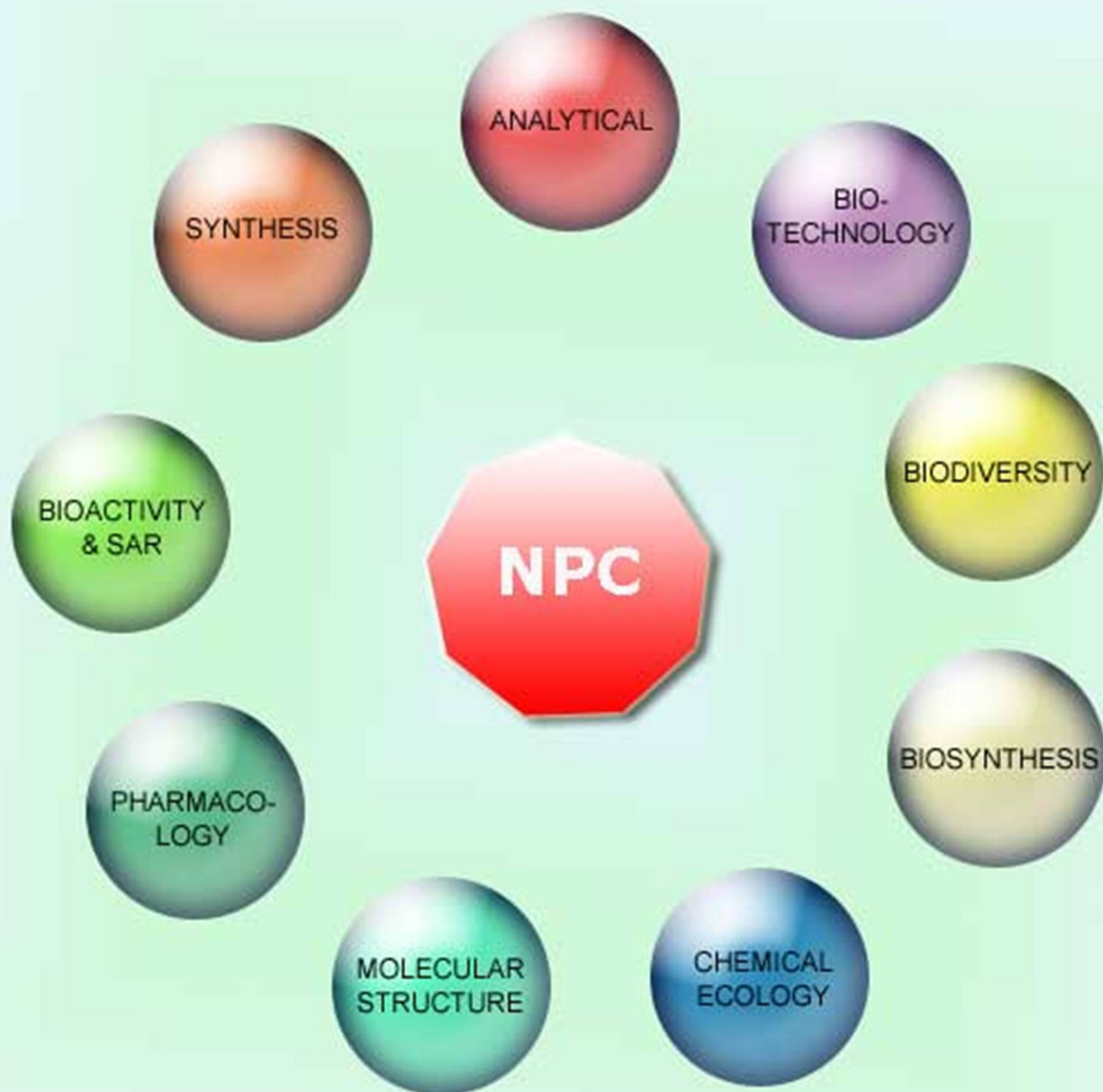

Volume 7. Issue 9. Pages 1117-1258. 2012
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2012
Vol. 7
No. 9
1123 - 1126

Antiparasitic and Anticancer Carvotacetone Derivatives of *Sphaeranthus bullatus*

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Received: May 31st, 2012; Accepted: July 10th, 2012

The CH_2Cl_2-MeOH (1:1) extract of the aerial parts of *Sphaeranthus bullatus*, an annual herb native to tropical East Africa, showed activity against chloroquine sensitive D6 (IC_{50} 9.7 µg/mL) and chloroquine resistant W2 (IC_{50} 15.0 µg/mL) strains of *Plasmodium falciparum*. Seventeen secondary metabolites were isolated from the extract through conventional chromatographic techniques and identified using various spectroscopic methods. The compounds were evaluated for their *in vitro* antiplasmodial, antileishmanial and anticancer activities revealing activity of four carvotacetone derivatives, namely 3-acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**), 3,7-dihydroxy-5-tigloyloxycarvotacetone (**2**), 3-acetoxy-5,7-dihydroxycarvotacetone (**3**) and 3,5,7-trihydroxy-carvotacetone (**4**); with antiplasmodial IC_{50} values of 1.40, 0.79, 0.60 and 3.40 µg/mL, respectively, against chloroquine sensitive D6 strains of *P. falciparum*; antiplasmodial activity of IC_{50} 2.00, 0.90, 0.68 and 2.80 µg/mL, respectively, against chloroquine resistant W2 strains of *P. falciparum*; antileishmanial IC_{50} values of 0.70, 3.00, 0.70 and 17.00 µg/mL, respectively, against the parasite *L. donovanii* promastigotes, and anticancer activity against human SK-MEL, KB, BT-549 and SK-OV-3 tumor cells, with IC_{50} values between <1.1 - 5.3 µg/mL for **1-3**. In addition, cytotoxic effects of the active compounds were evaluated against monkey kidney fibroblasts (VERO) and pig kidney epithelial cells ($LLC-PK_{11}$). The structures of carvotacetone derivatives were determined by 1D and 2D NMR spectroscopy; the absolute stereochemical configuration of 3-acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**) was determined as 3*R*, 4*R*, 5*S* by circular dichroism, specific rotation, ^{1}H NMR and 2D NMR ROESY and NOESY experiments.

Keywords: *Sphaeranthus bullatus*, Asteraceae, Antiplasmodial, Antileishmanial, Anticancer, Carvotacetones.

Historically, medicinal plants have proven to be a major source of drugs and novel template compounds. In the light of this notion, and the observed antiparasitic and anticancer activities of the extract, we have analyzed the compounds isolated from an African medicinal plant, *Sphaeranthus bullatus*, for antimalarial, antileishmanial and anticancer properties. Malaria, predominant in the tropics, is transmitted by female *Anopheles* mosquitos, which infect blood with parasites of the genus *Plasmodium*. Clinical malaria is manifested by a range of symptoms such as fever, vomiting, joint pain and convulsions [1]. Besides contributing to over a million deaths yearly, malaria is known to be a cause of anemia and its various complications, including miscarriages, brain damage, decreased cognition and irreversible disabilities [2].

Leishmaniasis on the other hand is transmitted by a bite of some species of sand flies which infect the blood with parasites of the genus *Leishmania* [3]. Two common forms of leishmaniasis are known; cutaneous leishmaniasis (CL), which causes a sore at the bite site, and visceral leishmaniasis (VL), which affects vital organs. Leishmaniasis is spread in tropical and subtropical regions of the world, with estimated numbers of new cases of CL and VL of 1.5 million and 500,000 annually, respectively [4]. Cancer, which is slightly neglected in Africa and the developing world, is a disease resulting from uncontrolled cell growth. According to GLOBOCAN estimates, the number of new cancer cases in 2008 was over twelve million and the number of new cancer deaths was over seven million [5].

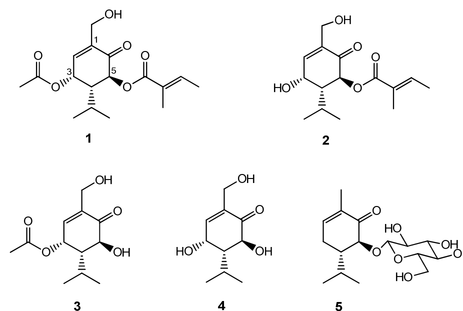

Figure 1: Chemical structures of compounds **1** – **5**.

Sphaeranthus bullatus Mattf. (synonym: *S. gallensis* Sacleux.: family: Asteraceae) is an annual herb, 0.2–1 m tall with aromatic leaves and purple flowers, native to tropical East Africa [6]. The leaves of this plant are used against malaria [7]. *Sphaeranthus* contains about forty species distributed mainly in tropical areas of Africa, southern Asia and Australia [8]. Previously reported compounds from *S. bullatus* are carvotacetone derivatives, *p*-cymene derivatives, thiophene derivatives, a sesquiterpene, α-humulene, and a triterpene, squalene [8]. The carvotacetone derivatives are known to possess antibacterial and antifungal activities [9], but nothing is known about their antimalarial, antileishmanial and anticancer properties against solid tumor cells. We are, therefore, reporting the first account of antiplasmodial, antileishmanial and anticancer properties of four carvotacetone

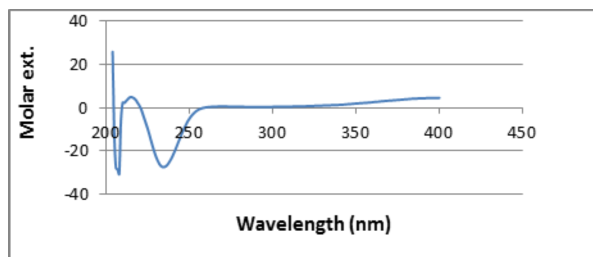

Figure 2: CD spectrum of compound **1**.

derivatives (**1-4**, Figure 1) from *S. bullatus*, identified as 3-acetoxy-7-hydroxy-5-tigloyloxy- carvotacetone (**1**) [10,11], 3,7-dihydroxy-5-tigloyloxy- carvotacetone (**2**) [8], 3-acetoxy-5,7-dihydroxy-carvotacetone (**3**) [12], 3,5,7-trihydroxycarvotacetone (**4**) [8] and 5-*O-β*-glucopyranosylcarvotacetone (**5**) [14].

The availability of 3-acetoxy-7-hydroxy-5-tigloyloxy-carvotacetone (**1**) offered the opportunity to assess the potential of circular dichroism for defining the absolute configuration of the C-3 stereocenter by application of the α,β-unsaturated cyclohexanone chromophore system [13]. Compound **1** has previously been reported from *S. confertifolius* [10] and *S. ukambaensis* [11], but the absolute stereochemical configuration at C-3, C-4 and C-5 has not been assigned. The CD spectrum of the compound {$[\alpha]_D$ -318 at 25^0C} showed strong absorption at 236 nm, with a negative Cotton effect corresponding to π-π* transitions of the α,β-unsaturated carbonyl chromophore (Fig 2) defining the configuration of C-3. Comparison was made to CD studies of a related cyclohexenone, woodwardine C [13], which showed similar absorption, but with an opposite Cotton effect, implying that the absolute configuration of **1** at C-3 was opposite to that of woodwardine C, leading to a conclusion that **1** has a 3*R* configuration. Accordingly, the absolute configurations of C-4 and C-5 were established as 4*R* and 5*S*, respectively, from 2D ^{1}H-^{1}H NMR ROESY analysis and ^{1}H NMR ^3J values (*cis* geometry of H-3 and H-4 observed from their NOE interactions and their equatorial-axial ^3J of 3.8 Hz; *trans* geometry of H-4 and H-5 observed from their axial-axial ^3J of 12.6 Hz). However, a weak pseudo nOe correlation was observed between H-4 and H-5 due to their close spatial proximity in a twisted cyclohex-1-en-6-one ring system containing three bulky substituents at C-3, C-4 and C-5. Collectively, these arrangements enable the protons H-3, H-4, H-5, Me-9 and Me-10 in close spatial proximity, as observed from their nOe correlations in both ROESY and NOESY spectra (see NMR spectra; Supplementary Information) by placing acetyl, isopropyl and tigloyl substituents, respectively.

The other known compounds isolated from *S. bullatus* were thymol 3-*O*-*β*-glucopyranoside [8], zataroside A [15], zataroside B [15], quercetin [16], quercetin-3,7-dimethylether [17], penduletin [18], crysoplenol D [19], 4-hydroxylonchocarpin [20], caffeic acid [21], coniferaldehyde [22], dammara-20,24-dienylacetate [23] and boehmery acetate [24].

The antiplasmodial and antileishmanial activities of compounds **1-4** are shown in Table 1, whereas the anticancer activities are tabulated in Table 2. The antiplasmodial and antileishmanial activities seemed to be enhanced by the presence of acetyl or tigloyl substituents at 3-OH and 5-OH, respectively, as witnessed by the weaker activity of **4** as compared with **1**, **2** and **3**, while **5** was inactive. Compound **3** (3-acetoxy-5,7-dihydroxy- carvotacetone) was the most active, having antiplasmodial IC_{50} values of 0.60 and 0.68 µg/mL against chloroquine sensitive and chloroquine resistant strains of *P. falciparum*, respectively, as well as antileishmanial activity of IC_{50} 0.70 µg/mL against *L. donovanii* promastigotes.

Table 1: *In-vitro* antiplasmodial and antileishmanial activity.

	P. falciparum				VERO	*L. donovanii*	
	D6 (µg/mL)		W2 (µg/mL)		µg/mL	µg/mL	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	IC_{50}	IC_{90}
Extract	9.70	NT	15.0	NT	NT	NT	NT
1	1.40	2.0	2.00	1.4	2.80	0.70	1.30
2	0.79	>6.2	0.90	>5.3	NC	3.00	6.90
3	0.60	21.7	0.68	19.1	0.013	0.70	1.40
4	3.40	>1.4	2.80	>1.7	NC	17.00	>40
Chloroquine	0.01	NT	0.14	NT	NT	NT	NT
Artemisinin	0.004	NT	0.005	NT	NT	NT	NT
Pentamidine	NT	NT	NT	NT	NT	0.1	NT

NC = Not Cytotoxic; NT = Not Tested, IC_{50} = concentration that affords 50% inhibition of growth, SI = Selectivity index (IC_{50} D6 or W2 / IC_{50} VERO).

However, compound **1** was assayed against *L. donovanii* promastigotes in THP1 macrophage cultures giving an IC_{50} value of 1.80 µg/mL. These results are supportive of the observed antiplasmodial activity of the plant extract (IC_{50} 9.7 and 15.0 against D6 and W2 clones of *P. falciparum*, respectively) and justify the use of the plant in traditional medicine for malaria [7].

The *in vitro* anticancer activity was evaluated against solid tumor cells of SK-MEL, KB, BT-549 and SK-OV-3, as well as against non-cancer monkey kidney fibroblast (VERO) and pig kidney epithelial cells (LLC-PK_{11}). Among the carvotacetone compounds, strong anticancer activity was observed for 3-acetoxy-5,7-dihydroxy-carvotacetone (**3**), with IC_{50} values between <1.1 – 1.7 µg/mL against these cells, followed by 3-acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**) and 3,7-dihydroxy-5-tigloyloxy-carvotacetone (**2**), with IC_{50} values between <2.75 – 5.3 µg/mL.

Table 2: *In vitro* anticancer and cytotoxicity activity.

	Anticancer activity (IC_{50}, µg/mL)					Vero
	SK-MEL	KB	BT-549	SK-OV-3	PK-1	TC_{50}, µg/mL
Extract	34.0	38.0	52.0	43.0	37.0	50.0
1	<2.75	3.8	4.5	4.0	3.3	3.0
2	3.5	3.8	5.3	4.5	2.8	4.0
3	<1.1	1.7	1.6	<1.1	1.3	1.7
Doxorubicin	0.65	1.4	0.9	1.1	0.55	>5

NA = Not Active (up to the maximum dose tested 10.0 µg/mL; 50 µg/mL for extract); IC_{50} = concentration that affords 50% inhibition of growth; SK-MEL = Human malignant melanoma; KB = Human epidermal carcinoma, oral; BT-549 = Ductal carcinoma, Breast; SK-OV-3 = Human ovarian carcinoma; Vero = Monkey kidney fibroblast

This appears to be the first report of 3*R*-acetoxy-7-hydroxy-5*S*-tigloyloxy-4*R*-carvotacetone (**1**) from *S. bullatus* and glucopyranosylcarvotacetone (**5**) from the genus *Sphaeranthus*. In addition, it is also the first report of the antimalarial and anticancer (i.e., against solid tumor cells) activities of carvotacetone derivatives.

Experimental

General: Optical rotations were measured in MeOH using an AUTOPOL IV® instrument at ambient temperature. Circular Dichroism (CD) spectra were recorded in MeOH using an Olis DCM 20 CD spectrometer at ambient temperature. ^{1}H- and ^{13}C NMR spectra were recorded on either a Bruker AVANCE 600 spectrometer or a 600 MHz Varian Spectrometer at 600 (^{1}H) and 150 MHz (^{13}C). EIMS were recorded on a GCMS TRACE DSQII single quadrupole mass spectrometer, and HRESIMS were obtained by direct injection using a Bruker Bioapex-FTMS with electrospray ionization. Column chromatography used Merck Silica gel 60 (0.063-0.200 mm) and Fluka Sephadex LH-20 as stationary phases.

Analytical TLC was carried out using factory prepared aluminum plates (0.25 mm) coated with silica gel (60 F254, Merck) and compounds were visualized by observing under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-H_2SO_4 spray reagent and heating.

Plant material: The aerial parts of *Sphaeranthus bullatus* Mattf. were collected from Ngong forest, Nairobi, in November 2007 and identified by Mr Patrick Mutiso of the School of Biological Sciences, University of Nairobi, where a voucher specimen (JMFM/2007/14) was deposited. The aerial parts were air dried in shade and pulverized.

Extraction and isolation: The shade dried and pulverized aerial parts of *S. bullatus* (2.3 Kg) were extracted by cold percolation at room temperature using CH_2Cl_2/MeOH, 1:1 (3×5 L, 24 h each), followed by 100% methanol (1×4 L, 24 h). The filtrates were concentrated and combined to give 168 g of black-brown gummy extract, of which 100 g was suspended in methanol/water (2:8) and partitioned successively with *n*-hexane, CH_2Cl_2 and EtOAc to give 31.5 g of *n*-hexane extract, 26 g of CH_2Cl_2 extract and 9.2 g of ethyl acetate extract. The *n*-hexane soluble part (30 g) was adsorbed on 40 g of silica gel and chromatographed over silica gel (300 g, 5 x 35 cm) eluted with *n*-hexane containing increasing amounts of EtOAc to afford boehmery acetate (37 mg), dammaradienyl acetate (26 mg) and 5-acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**) (114 mg). The dichloromethane soluble fraction of the original extract (25 g) was adsorbed on 30 g of silica gel and chromatographed over silica gel (300 g, 5 x 35 cm) eluted with *n*-hexane/CH_2Cl_2, followed by CH_2Cl_2/MeOH mixtures in varying proportions to give 5-acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**) (326 mg), 3,7-dihydroxy-5-tigloyloxycarvotacetone (**2**) (46 mg), 3-acetoxy-5,7-dihydroxycarvotacetone (**3**) (13 mg), penduletin (93 mg), quercetin-3,7-dimethylether (42 mg), 4-hydroxylonchocarpin (18 mg), chrysoplenol D (177 mg), 3,5,7-trihydroxycarvotacetone (**4**) (18 mg), and quercetin (12g). The ethyl acetate extract (9 g) was chromatographed over silica gel (100 g, 2.5 x 30 cm) eluted with CH_2Cl_2/MeOH mixtures in varying proportions to give coniferaldehyde (13 mg), caffeic acid (18 mg), 5-*O*-*β*-glucopyranosylcarvotacetone (**5**) (8 mg), thymol 3-*O*-*β*-glucopyranoside (28 mg), and zataroside B (283 mg), which contained zataroside A as a minor component.

Identification of the compounds: The isolated compounds **1**-**5** were identified using spectroscopic methods (^{1}H NMR, ^{13}C NMR, DEPT, COSY, NOESY, HMBC, HSQC), EI-MS, and direct comparison with published spectral data and structures [8-12].

3-Acetoxy-7-hydroxy-5-tigloyloxycarvotacetone (**1**)
Colorless gum.
R_f: 0.30 (CH_2Cl_2/MeOH 98:2).
$[\alpha]_D^{25}$: 318 (*c* 1.0 mg/mL, CH_3OH), Lit. (not reported).
CD (c 0.00008M, MeOH λmax (Δε) 236 nm (-27.05)
^{1}H and ^{13}C NMR, see Supporting Information.
HRESIMS: *m*/*z* 325.1651 $[M+H]^+$ (calcd for $C_{17}H_{24}O_6$ + H, 325.1573).

Antiplasmodial assay: The antiplasmodial activity was measured *in vitro* by a colorimetric assay that determines the parasitic lactate dehydrogenase (pLDH) activity [25,26]. The assay was performed in a 96-well microplate and included two *P. falciparum* strains [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant)]. A suspension of red blood cells infected with *P. falciparum* (D6 or W2) strains (200 µL, with 2% parasitemia and 2% hematocrit in RPMI - 1640 medium supplemented with 10% human serum and 60 µg/mL amikacin) was added to the wells of a 96-well plate containing 10 µL of test samples at various concentrations. The plate was flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2, in a modular incubation chamber (Billups-Rothenberg, 4464 M) and incubated at 37°C, for 72 h. Plasmodial LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR). The IC_{50} values were computed from the dose response curves generated by plotting percent growth against test concentrations. DMSO, artemisinin and chloroquine were included in each assay as vehicle and drug controls, respectively.

Antileishmanial assay: Antileishmanial activity of the compounds was tested *in vitro* on a culture of *Leishmania donovani* promastigotes. In a 96 well microplate assay, compounds with appropriate dilution were added to the *Leishmania* promastigotes culture (2×106 cells/mL). The plates were incubated at 26°C for 72 h and growth of *Leishmania* promastigotes was determined by Alamar blue assay [27]. Pentamidine and amphotericin B were used as standard antileishmanial agents. IC_{50} values for each compound were computed from the growth inhibition curve.

Cytotoxicity assay: The *in vitro* cytotoxic activity was determined against 4 human cancer cell lines (SK-MEL, KB, BT-549 and SK-OV-3), monkey kidney fibroblasts (VERO) and pig kidney epithelial cells (LLC-PK_{11}) (Table 5). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The assay was performed in 96-well tissue culture-treated microplates. Cells were seeded at a density of 25000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined using Neutral Red according to a modification of the procedure of Borenfreund *et al*. [26, 28]. IC_{50} values were determined from dose response curves of percent growth inhibition against test concentrations. Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

In vitro *macrophage amastigote assay*: In this assay, the THP1 cells (human acute monocytic leukemia cell line) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were prepared prior to each assay and suspended in RPMI1640 medium with 10% FBS at the cell density of $2.5X10^5$ cells/mL, and the assay was performed using a 96 well microplate, using a previously described method developed in our laboratory [29]. Each compound was tested in duplicate at 6 concentrations, IC_{50} and IC_{90} values were computed from the dose response curves.

Acknowledgments**:** The authors sincerely thank Mr Frank Wiggers and Dr Bharathi Avula, NCNPR, for recording MS and NMR spectra, and Mr Surendra K. Jain and Mr John Trott for assistance in biological work. This work was supported by the US Department of Defense CDMRP grant # W81XWH-09, and the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-2-0009. FM is thankful to the German Academic Exchange Service (DAAD) and the Natural Products Research Network for Eastern and Central Africa (NAPRECA) for a Ph. D. Scholarship.

Supporting information: The spectroscopic data for compound **1**, including 1D (^{1}H NMR and ^{13}C NMR) and 2D (ROESY and NOESY) NMR spectra, and HRESIMS.

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