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Bioactive compounds from the leaves of *Eugenia uniflora*

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ABSTRACT

From the MeOH extract of the leaves of *Eugenia uniflora* eight compounds were isolated, including one sterol, β-sitosterol (**1**); two triterpenes, betulinic acid (**2**) and centelloside C (**8**); three flavonoids, myricetrin (**3**), myricetin 3-*O*-(4''-*O*-galloyl)-α-L-rhamnopyranoside (**4**), and myricetin 3-*O*-β-D-glucopyranoside (**5**); and two megastigmanes, actinidioionoside (**6**), and (6*S*,9*R*)-roseoside (**7**). All the isolated compounds were obtained for the first time from this plant. The structures of isolated compounds were determined through a combination of spectroscopic and chemical analyses. All of the isolated compounds were evaluated for their antifungal, antibacterial, anti-leishmania, DPPH radical-scavenging and cytotoxic activities by means of MTT assay. Compounds **3** and **4** had antibacterial activity against *S. aureus.* Compounds **3**–**5** showed potent IC_{50} in the DPPH radical scavenging activity. Compounds **4** and **5** exhibited moderate growth inhibitory activity toward A549.

Keywords: *Eugenia uniflora*; *Myrtaceae;* Phytochemical; Biological activity.

INTRODUCTION

Eugenia uniflora L. is one of the 14 species of the genus growing in subtropical North and Northeastern Argentina, Brasil, Uruguay and Paraguay. Its common names are pitanga and Brazilian cherry. Used as antihypertensive agent in folk medicine, as well as in the treatment of digestive disorders, used as a diuretic, antiinflamatory, antidiarrheic, antirheumatic, antifebrile, eupeptic and carminative and also to lower blood cholesterol levels, to control uric acid levels and to reduce weight (Consolini, et al., 1999; Schapoval, et al., 1994).

To the best of our knowledge, little studies were focusing on the phytochemical and biological activity of *E. uniflora*, and this is the first study

describing in details the chemistry of the constituents as well as the potential biological activities of the isolated compounds.

In the course of our ongoing research activities towards the isolation of biologically active compounds from plants growing in Egypt either wild or cultivated, in particular the species of diverse chemical constituents with various reported biological activity, we had the opportunity to work on leaves of *E. uniflora* to investigate its chemical constituents and potential biological activities.

In the present study, we report the isolation and structural elucidation of eight compounds (Figure 1) from *E. uniflora* for the first time in addition to biological evaluation of the isolated compounds; including antifungal, antibacterial, anti-leishmania, DPPH radical-scavenging and cytotoxic activities by means of MTT assay.

MATERIALS AND METHODS

General Experimental Procedures: Optical rotation data were measured on a JASCO P-1030 polarimeter. IR spectrum was obtained on a Horiba FT-710 Fourier transform infrared spectrophotometer. ^{1}H- and ^{13}C-NMR spectrum was recorded on a JEOL JNM α-400 spectrometer with tetramethylsilane as an internal standard. HR-ESI mass spectrum was taken on a LTQ Orbitrap XL mass spectrometer. Silica gel column chromatography (CC) was performed on silica gel 60 [(E. Merck, Darmstardt, Germany), 70–230 mesh]. Reversed-phase [octadecylsilanized silica gel (ODS)] open CC (RPCC) was performed on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) (*Φ*=2cm, *L*=40cm, 10g fractions being collected). High-performance liquid chromatography (HPLC) was performed on an ODS column [Inertsil ODS-3; GL Science, Tokyo, Japan; (*Φ*=6mm, *L*=25cm, flow rate: 1.0ml/min), using a refractive index and/or a UV detector. Precoated silica gel 60 F_{254} plates (E. Merck; 0.25 mm in thickness) were used for TLC analyses, visualized by spraying with a 10%H_2SO_4 solution in EtOH and heating to around 150°C on a hotplate.

Plant Material: The leaves of *E. uniflora* (Family- *Myrtaceae*) were collected in May 2010 from Aswan Botanical Garden, Egypt. A voucher specimen of the plant is deposited in the Herbarium of Faculty of Pharmacy, Minia University, Egypt (Minia-10-May-EU).

Microorganisms: The microorganisms used in this study, *Mucor racemousus*, methicillin-resistant *Staphylococcus aureus*, and *Leishmania major*, were from Medical mycology research center, Chiba university, Japan collection of microorganisms, and Institute of tropical medicine, Nagasaki university, respectively. Human lung cancer cell, A549, was obtained from National institute of biomedical innovation JCRB cell bank.

Extraction and Isolation: The air-dried powdered leaves (1.30kg) of *E. uniflora* was extracted with methanol (5L×5) till exhaustion and then concentrated under reduced pressure to yield a viscous gummy material (202g). This residue was dissolved in 500ml of water and defatted with *n*-hexane (1L×5). The aqueous layer was evaporated to remove a trace amount of organic solvent, and then extracted with EtOAc and 1-BuOH, successively (1L×5 each).The EtOAc and 1-BuOH fractions were concentrated under reduced pressure to give 36.3g and 7.5g of residues, respectively.

The remaining aqueous layer was concentrated to furnish a water-soluble fraction (80g).

The EtOAc fraction (36.3g) was subjected to silica gel CC (720g), (Φ=60 mm, L=60cm). The column was eluted initially with *n*-hexane (5L), then with *n*-hexane-EtOAc gradient mixture, 500ml fractions being collected. The similar fractions have been combined, affording 17 fractions. The first fraction E-1 gave compound **1** (18.8mg). The second fraction E-2 afforded compound **2** (61.2mg). Fraction E-15 (1.45g) was purified on RPCC, affording nine fractions. The eighth fraction E-15-8 (98.7mg) was purified by HPLC (50% MeOH) to produce compound **3** (8.66mg). Fraction E-17 (3.7g) was applied to RPCC, giving 13 fractions. The ninth fraction E-17-9 (86.2mg) was purified by HPLC (40% MeOH) to furnish compound **4** (4.5mg).

The 1-BuOH fraction (7.5g) was chromatographed over silica gel (200g), (*Φ*=50 mm, *L*=30 cm), using $CHCl_3$-MeOH gradient system, 200ml fractions being collected and the similar fractions were combined to yield nine fractions. Fraction B-8 (2.35g) was purified on RPCC, affording 15 fractions. The third fraction B-8-3 (72.3mg) was purified by HPLC (40% MeOH) to produce compound **6** (16.5mg). The fourth fraction B-8-4 (40.8mg) was purified by HPLC (30% MeOH) to give compound **7** (4.32mg). The eighth fraction B-8-8 (85.5mg) was purified by HPLC (40% MeOH) to afford compound **5** (3.45mg). The thirteenth fraction B-8-13 (23.4mg) was purified by preparative TLC ($CHCl_3$-MeOH-H_2O, 15:6:1) to furnish compound **8** (4.55mg).

Analysis of the sugar moiety: About 1mg of compound **8** was hydrolyzed with 1MHCl (1.0ml) at 80°Cfor 2h. The reaction mixture was neutralized with Amberlite IRA96SB (OH^-), then partitioned with an equal amount of EtOAc (1.0ml), and the water layer was analyzed for its sugar component. The sugar was determined by HPLC on an amino column [ShodexAsahipak NH_2P-50 4E (4.6mm×250mm), CH_3CN-H_2O (4:1), 1ml/min], using chiral detector (JASCO OR-2090*plus*), in comparison with authentic sugar (D-glucose). Compound **8** gave a peak for D-glucose at retention time of 9.55min with a positive rotation sign.

Antibacterial susceptibility assay: Susceptibility tests were performed using a broth micro-dilution assay according to National Committee for Clinical Laboratory Standards (NCCLS) reference methods. Assays were performed using Müller-Hinton broth (Difco). The bacterial inocula were adjusted to yield a density of 5×10^5 colony forming units (CFU)/ml. Samples were diluted directly in 96-well microtiter plates by serial 2-fold dilution using a multichannel pipette. Microtiterplates were incubated during 24h at 37°C and were read using a Molecular Device Versamex tunable microplate reader at 620nm as well as by visual observation. The MIC_{50} was determined as a 50% decrease in the optical density. Amphotericin B and oxacillin were used as positive controls (Phan, et al., 2006).

Anti-leishmania assay: The leishmanicidal activities of isolated compounds were performed using the colorimetric MTT assay. Medium 199 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100µg/ml of kanamycin was used as the cell culture medium. The test compounds were dissolved in DMSO and added to the each well of the 96-well microtitration plates at 1% as the final concentration. *Leishmania major* cells (2×10^5 cells /well) were cultured in a CO_2

incubator at 25°C for 72h and then MTT solution was added to each well and the plates were incubated overnight at 25°C.The absorbance was measured at 540nm using a Molecular Device Versamex tunable microplate reader. Amphotericin B was used as a positive control (Takahashi, et al., 2004).
The inhibition % was calculated using the following equation:

$$\% \text{ Inhibition} = [\, 1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank}) \,] \times 100$$

- $A_{control}$ is the absorbance of the control reaction mixture (containingDMSO and all reagents except for the test compounds).
- IC_{50} was determined as the concentration of sample required to inhibit the formation of MTT formazan by 50%.

DPPH radical scavenging activity: The absorbance with various concentrations of the test extracts and compounds dissolved in MeOH (100µl) in a 96-well microtiter plate was measured at 515nm as A_{blank}. Then, 200µM DPPH solution (100µl) was added to each well, followed by incubation at room temperature for 30min. The absorbance was measured again as A_{sample}.
The % inhibition was calculated using the following equation:

$$\% \text{ inhibition} = [1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$$

- Where $A_{control}$ is the absorbance of the control reaction mixture (containing DMSO and all reagents except for the test extracts and compounds.
- IC_{50} was determined as the concentration of sample required to inhibit the formation of the DPPH radical by 50% (Matsunami, et al., 2001).

Human cancer cell growth inhibition assay*:* This assay was performed using human lung cancer cell line (A549) and the viability was estimated by the colorimetric MTT assay. Dulbecco's modified Eagle medium (DMEM) supplemented with FBS and 100µg/ml of kanamycin and 5.6µg/ml of amphotericin B was used as the cell culture medium. The test compounds were dissolved in DMSO and added to the each well of the 96-well micro-titration plates at 1% as the final concentration. A549 cells (5×10^3 cells/well) were cultured in a 5% CO_2 incubator at 37°C for 72h and then MTT solution was added to each well and the plates incubated for a further 1.5h, then the formazan precipitates were dissolved in DMSO and then the optical density values for each well was measured at 540nm with a microplate reader. Doxorubicin was used as a positive control.
The cell growth inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = [\, 1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank}) \,] \times 100$$

- $A_{control}$ is the absorbance of the control reaction mixture (containingDMSO and all reagents except for the test compounds).
- IC_{50} was determined as the concentration of sample required to inhibit the formation of MTT formazan by 50% (Phan, et al., 2006).

RESULTE AND DISSCUSSION

Air-dried leaves of *E. uniflora* were extracted with MeOH three times and the concentrated MeOH extract was partitioned with solvents of increasing polarity. The EtOAc and 1-BuOH-soluble fraction were separated by means of various chromatographic procedures including column chromatography (CC) on a highly-porous synthetic resin (Diaion HP-20), normal silica gel CC and reversed-phase octadecyl silica gel (ODS) CC, and high-performance liquid chromatography (HPLC), to afford eight compounds (**1**–**8**).The structures of known compounds were

determined to be β-sitosterol (**1**) (Kojima, et al., 1990), betulinic acid (**2**) (Cichewicz, et al., 2004), myricetrin (**3**) (Chung, et al., 2004), myricetin 3-*O*-(4''-*O*-galloyl)-α-L-rhamnopyranoside (**4**) (Nicoluer, et al., 1983), myricetin 3-*O*-β-D-glucopyranoside (**5**) (Scharbert, et al., 2004), actinidioionoside(**6**) (Otsuka, et al., 2003), (6*S*,9*R*)-roseoside (**7**)(Otsuka, et al., 1995) and centelloside C (**8**) (Weng, et al., 2011) by comparing their spectroscopic data with those reported in literature.

β-Sitosterol (1): ^{1}H-NMR spectrum data of compound **1** exhibited two singlet methyls at δ_H 0.68 and 1.05, three doublet methyls at δ_H 0.78, 0.85 and 0.88 and one triplet methyl at δ_H 0.85. ^{13}C-NMR revealed the presence of 29 carbon signals were attributable to the steroidal aglycone consisted of 6 methyls, 11 methylenes, 9 methines and 3 quaternary carbons. By comparing the ^{1}H- and ^{13}C- NMR data of this compound with the reported data, can be concluded that compound **1** was elucidated as β-sitosterol.

^{1}H-NMR (400 MHz, pyridine-d$_6$): 0.68(3H, s, H_3-18),0.78(3H, d, J=7.8 Hz, H_3-27), 0.85 (3H, t, J=7.8, 8 Hz, H_3-29), 0.85(3H, d, J=8 Hz, H_3-26),0.88(3H, d, J=6.4 Hz, H_3-21),1.05 (3H, s, H_3-19);**^{13}C-NMR (100 MHz, pyridine-d$_6$):** 12.0 (C-18),12.1 (C-29), 19.0 (C-21), 19.2 (C-27),19.6 (C-19),19.9 (C-26), 21.3 (C-11),23.4 (C-28),24.5 (C-15), 26.5 (C-23), 28.5 (C-16), 29.5 (C-25),32.2 (C-2),32.2 (C-8),32.5 (C-7),34.2 (C-22), 36.4 (C-20),36.9 (C-10), 37.8 (C-1),40.0 (C-12), 42.5 (C-13),43.4 (C-4),46.1 (C-24),50.4 (C-9),56.3 (C-17),56.9 (C-14),71.3 (C-3),121.2 (C-6),142.0 (C-5).

Betulinic acid (2): ^{1}H-NMR spectrum data of compound **2** showed six singlet methyls at δ_H 0.83, 0.99, 1.05, 1.07, 1.20 and 1.66, and two broad singlet signals at δ_H 4.75 and 4.92.^{13}C-NMR revealed the presence of 30 carbon signals were attributable to the triterpenea glycone. By comparing the ^{1}H- and ^{13}C- NMR data of this compound with the reported data, can be concluded that compound **2** was identified as betulinic acid.

^{1}H-NMR (400 MHz, pyridine-d$_6$): 0.83 (3H, s, H_3-24), 0.99 (3H, s, H_3-25), 1.05 (3H, s, H_3-23), 1.07 (3H, s, H_3-26), 1.20 (3H, s, H_3-27), 4.75 (1H, br s, H-29a), 4.92 (1H, br s, H-29b), 1.66 (3H, s, H-30); **^{13}C-NMR (100 MHz, pyridine-d$_6$):** 14.9 (C-27),15.3 (C-24), 16.3 (C-26), 16.4 (C-25),18.8 (C-6), 19.5(C-30), 21.8 (C-11), 26.1 (C-12), 28.2 (C-2),28.5 (C-23), 30.3 (C-21), 31.2 (C-15), 32.9 (C-16), 34.9 (C-7),37.5 (C-22), 37.6 (C-10), 38.6 (C-13), 39.3 (C-1), 39.5 (C-4), 41.1 (C-8), 42.9 (C-14),47.7 (C-18), 49.8 (C-19), 51.0 (C-9), 56.0 (C-5), 56.6 (C-17), 78.2 (C-3), 109.9 (C-29), 151.3 (C-20), 178.8 (C-28).

Myricetrin (3): The ^{1}H-NMR spectrum of **3** displayed one proton singlet signal at δ_H 6.94 and two doublet signals at δ_H 6.19 (1H, d, J=2 Hz) and 6.35 (1H, d, J=2 Hz), together with one methyl signal at δ_H 0.94 (3H, d, J=6.2 Hz), one anomeric proton signals at δ_H 5.31 (1H, br s). The ^{13}C-NMR spectrum exhibited six signals assignable to β-rhamnopyranosyl moiety, and the remaining carbon signals for the aglycone myricetin. Therefore, the structure of compound **3** was elucidated to be myricetin 3-*O*-α-L-rhamnopyranoside, namely myricetrin.

^{1}H-NMR (400 MHz, CD$_3$OD): 0.94 (3H, d, J=6.2 Hz, H_3-6''), 5.31 (1H, br s, H-1''), 6.19 (1H, d, J=2 Hz, H-6), 6.35 (1H, d, J=2 Hz, H-8), 6.94 (2H, s, H-2',6'); **^{13}C-NMR (100 MHz, CD$_3$OD):**17.6 (C-6''), 71.9 (C-2''), 72.0 (C-3''), 72.1(C-5''), 73.3 (C-4''), 94.6 (C-8), 99.8 (C-6), 103.6 (C-1''), 105.9 (C-10),109.6 (C-2',6'),121.9 (C-

1'),136.3 (C-3),137.8 (C-4'), 146.8 (C-3',5'),158.5 (C-9),159.4 (C-2),163.2 (C-5), 165.8 (C-7),179.7 (C-4).

Myricetin 3-*O*-(4"-*O*-galloyl)-α-L-rhamnopyranoside (4): Compound **4** was analogous compound to **3**, except for the presence of galloyl moiety attached to C-4" of rhamnopyranose.

^{1}H-NMR (400 MHz, CD_3OD): 1.003 (3H, d, J=6.2 Hz, H_3-6"), 5.29 (1H, br s, H-1"), 6.20 (1H, d, J=2 Hz, H-6), 6.36 (1H, d, J=2 Hz, H-8), 6.99 (2H, s, H-2"', 6"'), 7.17 (2H, s, H-2',6'); **^{13}C-NMR (100 MHz, CD_3OD):** 17.7 (C-6"), 69.9 (C-5"),70.9 (C-3"), 72.3 (C-2"), 75.4 (C-4"),94.7 (C-8),99.8 (C-6),103.7 (C-1"), 105.9 (C-10),109.6 (C-2"',6"'),110.5 (C-2',6'),121.7 (C-1'),121.9 (C-1"'),136.4 (C-3),137.9 (C-4'), 139.9 (C-4"'),146.4 (C-3"',5"'), 146.8 (C-3',5'), 158.5 (C-9), 159.4 (C-2), 163.2 (C-5), 165.9 (C-7), 168.4 (C-7"'), 179.6 (C-4).

Myricetin3-*O*-β-D-glucopyranoside (5): Compound **5** was analogous compound to **3**, except for the presence of β-glucopyranosyl moiety, instead of rhamnopyranose.

^{1}H-NMR (400 MHz, CD_3OD): 5.15 (1H, d, J=7.6 Hz, H-1"), 6.19 (1H, d, J=2 Hz, H-6), 6.38 (1H, d, J=2 Hz, H-8), 7.36 (2H, s, H-2',6'); **^{13}C-NMR (100 MHz, CD_3OD):** 61.9 (C-6"), 70.0 (C-4"), 75.1 (C-2"), 77.2 (C-5"),78.2 (C-3"),94.7 (C-8),99.9 (C-6),104.6 (C-1"), 105.6 (C-10),110.0 (C-2',6'),121.7 (C-1'),136.0 (C-3),138.0 (C-4'),146.4 (C-3',5'), 158.4 (C-2), 158.4 (C-9), 162.9 (C-5), 166.2 (C-7), 179.4 (C-4).

Actinidioionoside (6): ^{1}H-NMR spectrum showed an anomeric proton signal at δ_H 4.33 (1H, d, *J*=7.6 Hz) corresponding to a β-glucopyranosyl moiety, two olefinic proton signals at δ_H 5.81 (1H, dd, J=15.9, 7.3 Hz) and6.07 (1H, dd, J=15.9, 0.75 Hz), along with three singlet methyls at δ_H 0.83 (3H, s), 1.151 (3H, s), 1.158 (3H, s),and one doublet methyl at δ_H 1.30 (3H, d, J=6.2 Hz).The ^{13}C-NMR displayed 19 carbon signals, six of which were attributable to a β-glucopyranosyl moiety, the remaining 13 carbon signals comprising those of four methyls, two methylenes, two methines, a di-substituted *trans* double bond and three quaternary carbons. Consequently, compound **6** was established as (*3S, 5R, 6R, 9R*)-actinidioionoside.

^{1}H-NMR (400 MHz, CD_3OD): 0.83 (3H, s, H_3-12), 1.151 (3H, s, H_3-11), 1.158 (3H, s, H_3-13),1.30 (3H, d, J=6.2 Hz, H_3-10), 1.41(1H, ddd, J=11.9, 4.5, 1.6 Hz, H-2a), 1.60 (1H, t, J=11.9 Hz, H-2b), 1.70 (1H, t, J=11.9 Hz, H-4a), 1.74 (1H, ddd, J=11.9, 4.5, 1.6 Hz, H-4b),3.57 (1H, dd, J=11.7, 6 Hz, H-6'a),3.80 (1H, dd, J=11.5, 1.2 Hz, H-6'b), 4.00 (1H, m, H-3),4.33 (1H, d, J=7.6 Hz, H-1'),4.37(1H, quintet, J=6.4 Hz, H-9), 5.81 (1H, dd, J=15.9, 7.3 Hz, H-8), 6.07 (1H, dd, J=15.9, 0.75 Hz, H-7); **^{13}C-NMR (100 MHz, CD_3OD):** 21.7 (C-10), 26.2 (C-11), 27.4 (C-12), 27.5 (C-13), 40.6 (C-1), 45.6 (C-4), 46.3 (C-2), 62.8 (C-6'), 65.2 (C-3), 71.8 (C-4'), 75.3 (C-2'), 77.7 (C-5), 77.9 (C-3'), 78.1 (C-5'), 78.8 (C-9), 78.9 (C-6), 102.6 (C-1'), 132.9 (C-7), 134.6 (C-8).

(6*S*, 9*R*) - Roseoside (7): ^{1}H-NMR spectrum showed an anomeric proton signal at δ_H 4.32 (1H, d, *J*=7.8 Hz) corresponding to a β-glucopyranosyl moiety, three olefinic proton signals at δ_H 5.84 (1H, d, *J*=1.5 Hz), 5.85 (1H, d, *J*=3.6 Hz) and 5.86 (1H, br s) were assignable to H-7, H-8 and H-4, respectively, along with two singlet methyls at δ_H 1.02 (3H, s) and 1.04 (3H, s) and two doublet methyls at δ_H 1.27 (3H, d, *J*=4.6 Hz) and 1.91(3H, d, *J*=1.2 Hz).

The ^{13}C-NMR spectral data, displayed 19 carbon signals, six of which were attributable to a β-glucopyranosyl moiety. The remaining 13 carbon signals consisted of four quaternary carbon signals, one of which for olefinic carbon at δ_C 167.2 was assignable to C-5, another for carbonyl carbon at δ_C 201.2 was attributable to C-3, three olefinicmethines, one methine, one methylene and four methyls.

The number of carbons as well as the presence of four methyl moieties indicated that the aglycone was of a megastigmane skeleton. From the above data, compound **7** could be identified as (6*S*, 9*R*)-roseoside.

^{1}H-NMR (400 MHz, CD_3OD): 1.02 (3H, s, H_3-11), 1.03 (3H, s, H_3-12), 1.90 (3H, s, H_3-13), 1.27 (3H, d, J=6.4 Hz, H_3-10), 2.12 (1H, d, J=16.8 Hz, H-2a), 2.48 (1H, d, J=16.8 Hz, H-2b), 4.32 (1H, d, J=7.8 Hz, H-1'), 4.35 (1H, m, H-9),5.84 (1H, d, J=1.1 Hz, H-7), 5.85 (1H, d, J=3.1 Hz, H-8),5.86 (1H, br s, H-4); **^{13}C-NMR (100 MHz, CD_3OD):**19.5 (C-13), 21.1 (C-10), 23.4 (C-11), 24.6 (C-12), 42.4 (C-1), 50.7 (C-2), 62.8 (C-6'), 71.7 (C-4'), 75.2 (C-2'), 77.2 (C-9), 78.0 (C-5'),78.1 (C-3'), 80.0 (C-6),102.7 (C-1'), 127.2 (C-4), 131.5 (C-7), 135.3 (C-8), 167.2 (C-5), 201.2 (C-3).

Centelloside C (8): $[\alpha]_D^{20}$+73.3°, was obtained as a white amorphous powder, having the molecular formula $C_{36}H_{58}O_{11}$ as determined by positive-ion mode HR-ESI-MS. The IR spectrum of **8** showed the bands at 3392 and 1735 cm^{-1}corresponding to hydroxy and carbonyl group absorptions, respectively. The ^{1}H-NMR spectrum of **8** displayed signals corresponding to four singlet methyls (δ_H 1.69, 1.71, 1.72 and 1.79), two doublet methyls (δ_H 0.87 and 0.93), an olefinic proton (δ_H 5.52), and an anomeric proton at δ_H 6.19 (1H, d, *J*=8.2 Hz). The ^{13}C-NMR spectrum showed 36 carbon signals including six primary, nine secondary, 14 tertiary, and seven quaternary carbons, which led to the conclusion that **8** is a triterpene monoglycoside. The presence of two secondary methyl groups (δ_C 17.3, 21.2) and the chemical shifts of the olefinic carbons (δ_C 126.5, 137.8) suggested that **8**is an ursane-type triterpene with a double bond at C-12. The signals at δ_C 66.3, 67.6, 69.1 and 78.3 indicating the presence of four hydroxy groups at C-23, C-6, C-2 and C-3, respectively. The further NMR analyses were performed with the aid of ^{1}H-^{1}H COSY, HSQC and HMBC spectroscopies. In the HMBC spectrum, both the methine protons H-2 and H-3 showed long-range correlations with C-4 and the oxymethylene protons (H_2-23) showed correlations with C-3, C-4, and C-5 and the methyl carbon (C-24). The sugar moiety of **8** was supposed to be β-glucopyranose based on the coupling constant of the anomeric proton (1H, d, *J*=8.2 Hz) and the ^{13}C-NMR chemical shifts, which was confirmed by HPLC analysis of the hydrolyzate of **8** and the absolute configuration of the sugar was simultaneously determined to be in D-series using a chiral detector. Furthermore, the chemical shifts of the anomeric proton (δ_H 6.19) and carbon (δ_C 95.7) revealed that the glucopyranose was attached to the carboxyl group (C-28). This was confirmed by a long-range correlation between the anomeric proton and the carboxyl carbon (δ_C 176.1) in the HMBC spectrum (Fig. 2). The stereochemistry of **8** was determined by analysis of its coupling constant (*J*=9.5 Hz) between H-3 and H-2 indicated the hydroxy groups to have a 2α and 3β orientation, which was further supported by ROESY spectrum (Fig. 3). It showed correlation between H-2 and the methyl protons (H_3-24 and H_3-25) and between H-3 and H-5, indicating the orientation of the hydroxy groups to be 2α, 3β and 4α-CH_2OH. The broad singlet of

H-6 showed correlation with H-5 indicated the configuration of 6-OH to be β. The configurations of the methyl groups at C-19 and C-20 were also determined to be 19β and 20α from the ROESY correlations between H-18 and H_3-29 and between H-18 and H-20. Thus, the structure of **8** was determined to be centelloside C

^{1}H-NMR (400 MHz, pyridine-d_6): 0.87 (3H, br s, H_3-30), 0.93 (3H, d, J= 6.3 Hz, H_3-29),1.69 (3H, s, H_3-27), 1.71 (3H, s, H_3-24), 1.72 (3H, s, H_3-26), 1.79 (3H, s, H_3-25), 3.92 (1H, d, J= 9.5 Hz, H-3),4.03 (1H, d, J=10.5 Hz, H-23a), 4.39 (1H, d, J =10.5 Hz, H-23b), 4.86 (1H, br s, H-12), 6.19 (1H, d, J=8.2 Hz, 1'); **^{13}C-NMR (100 MHz, pyridine-d_6):** 15.9 (C-24), 17.3 (C-29), 19.0 (C-26),19.2 (C-25), 21.2 (C-30), 23.8 (C-27), 24.8 (C-11),26.1 (C-16),29.7 (C-15),30.8 (C-21), 36.7 (C-22), 38.2 (C-10), 39.1 (C-20),39.4 (C-19),39.7 (C-7),41.3 (C-8), 43.1 (C-14), 44.5 (C-4), 48.4 (C-17), 48.7 (C-5), 48.7 (C-9),50.5 (C-1), 53.5 (C-18), 62.4 (C-6'), 66.3 (C-23),67.6 (C-6), 69.1 (C-2), 71.3 (C-4'), 74.1 (C-2'), 95.7 (C-1'), 78.3 (C-3), 78.7 (C-5'), 79.0 (C-3'), 126.5 (C-12), 137.8 (C-13), 176.1 (C-28).

Compounds **1**−**8** were examined for their antifungal, antibacterial, anti-leishmania activities, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities, and also tumor cell growth inhibitory activity toward A549 by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1).

Antimicrobial activity for all compounds was tested against gram +ve bacteria as well as fungi showing that compounds **3** and **4** exhibited inhibitions against *Staphylococcus aureus* (MIC_{50} 81.9±16.55 and 36.9±20.24µM, respectively). All compounds showed no activity against the tested fungal strain. None of the tested compounds showed activity against *L. major* at concentration of 100 µM.

For antioxidant activity, the direct measurement of DPPH radical scavenging activity was determined using MTT assay. The different compounds of *E. uniflora* were tested for their DPPH radical scavenging activity. Results indicated strong radical scavenging activity for compounds **3–5**towards DPPH• in comparison with trolox (positive control), while other compounds showed no scavenging activity at the same concentration. Compounds **3–5** showed remarkable IC_{50} of 11.3±0.93, 5.1±0.23 and 21.3±2.73µM, respectively.

The cytotoxicity of all compounds was comparable to IC_{50}value for doxorubicin (0.53±0.03µM). Compound **4** showed weak activity against A549 cell lines (IC_{50} 46.42±0.23µM), while **5** inhibited 50% cell growth only at the 79.44±2.36µM range.

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REFERENCES

Chung, S.K., Kim, Y.C., Takaya, Y., Terashima, K., Niwa, M., (2004): Novel flavonol glycoside, 7-*O*-methyl mearnsitrin, from *Sageretia theezans* and its antioxidant effect. *J. Agric. Food Chem.*, 52: 4664-4668.

Cichewicz, R.H., Kouzi, S.A., (2004): Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Med. Res. Rev.*, 24: 90-114.

Consolini, A.E., Baldini, O.A.N., Amat, A.G., (1999): Pharmacological basis for the empirical use of *Eugenia uniflora* L. (*Myrtaceae*) as antihypertensive. *J. Ethnopharm.*, 66:33–39.

Kojima, H., Sato, N., Hatano, A., Ogura, H., (1990): Sterol glucosides from *Prunella vulgaris. Phytochemistry*, 29: 2351–2355.

Matsunami, K., Otsuka, H., Takeda, Y., (2011): Myrseguinosides A-E, five new glycosides from the fruits of *Myrsine seguinii*. *Chem. Pharm. Bull.*, 59: 1274–1280.

Melo, R.M., Corrêa, V.F.S., Amorim, A.C.L., Miranda, A.L.P., Rezende, C.M., (2007): Identification of impact aroma compounds in *Eugenia uniflora* L. (Brazilian pitanga) leaf essential oil. *J. Brazil. Chem. Soc.*, 18: 179–183.

Nicoluer, G., Thompson, A.C., (1983): Flavonoids of *Desmanthus illioensis*. *J. Nat. Prod.*, 46: 112–114.

Otsuka, H., Yao, M., Kamada, K., Takeda, Y., (1995): Alangionosides G-M: glycosides of megastigmane derivatives from the leaves of *Alangium premnifolium*. *Chem. Pharm. Bull.*, 43: 754–759.

Otsuka, H., Hirata, E., Shinzato, T., Takeda, Y., (2003): Stereochemistry of mega stigmane glucosides from *Glochidionzey lanicum* and *Alangium premnifolium*. *Phyto chemistry*, 62: 763–768

Phan, M.G., Phan, T.S., Matsunami, K., Otsuka, H., (2006): Chemical and biological evaluation on scopadulane type diterpenoids from *Scoparia dulcis* of Vietnamese origin. *Chem. Pharm. Bull.*, 54: 546–549.

Schapoval, E.E.S., Silveira, S.M., Miranda, M.L., Alice, C.B., Henriques, A.T., (1994): Evaluation of some pharmacological activities of *Eugenia uniflora* L. *J. Ethnopharm.*, 44: 137–142.

Scharbert, S., Holzmann, N., Hofmann, T., (2004): Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.*, 52: 3498–3508.

Weng, X., Chen, Y., Shao, Y., Kong, D., (2011): A new ursane-type triterpene saponin from *Centella asiatica. Chin. J. Pharm.*, 3:187.

Takahashi, M., Fuchino, H., Satake, M., Agatsuma, Y., Sekita, S., (2004): In vitro screening of leishmanicidal activity in Myanmar timber extracts. *Biol. Pharm. Bull.*, 27:921–925.

Table-1: Antifungal, anti-bacterial, anti-leishmania, DPPH radical scavenging and cytotoxic activities of compounds 1−8.

	Mucor racemousus	***Staphylococcus aureus* (MRSA)**	***Leishmania major***	**DPPH**	**A549**
4*	NA	56.3±4.8 (81.9±16.6)	NA	94.6±0.16 (11.3±0.93)	NA
5*	NA	54.8±14.2 (36.9±20.24)	NA	93.7±1.86 (5.1±0.23)	93.4±3.54 (46.4±0.23)
6*	NA	NA	NA	91.6±3.36 (21.3±2.73)	65.0±10.4 (79.4±2.36)
Amphotericin B (1µM)	84.2±9.72 (0.5 ±0.2)	−	−	−	−
Oxacillin(20µM)	−	99.8±0.28 (10.3±4.52)	−	−	−
Amphotericin B (1 µM)	−	−	97.7±0.5 (0.39±0.5)	−	−
Doxorubicin (1µM)	−	−	−	−	64.9±3.8 (0.53±0.03)
Trolox (50µM)	−	−	−	95.2±0.31 (16.6±2.2)	−

- NA: Not active.
- The upper and lower values indicate % inhibition at 100 µM and IC_{50} (µM), respectively.
- For positive controls, % inhibition at the indicated concentration and IC_{50} (µM) were shown.
- Compounds **1**, **2**, **6**- **8** were not active.

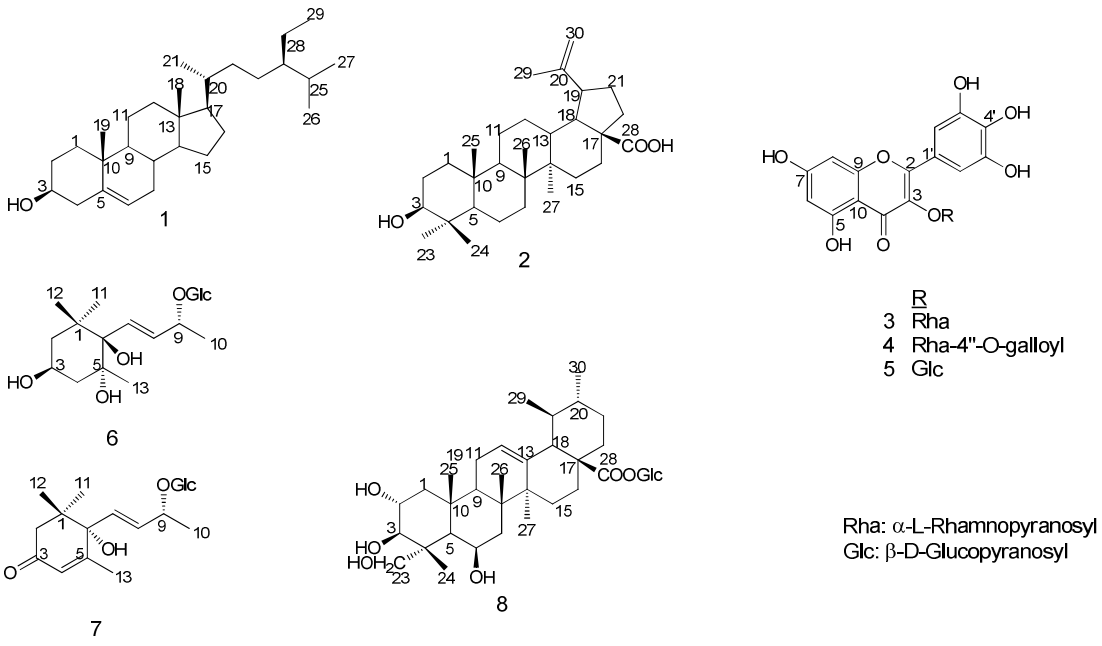

Figure- 1: Compounds 1-8.

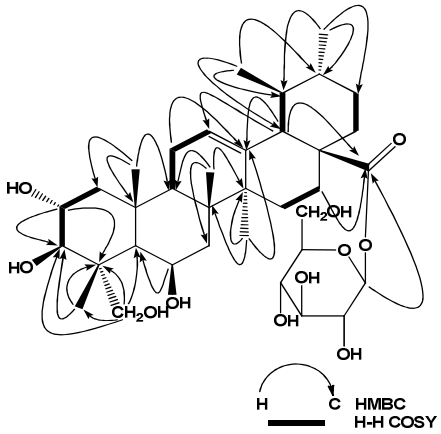

Figure- 2: Selected COSY and HMBC Correlations of 8.

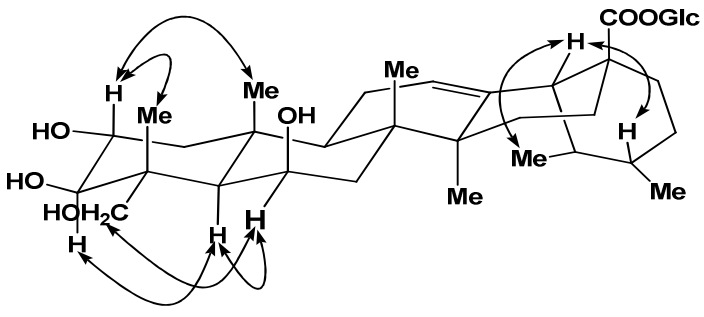

Figure-3: Selected ROESY Correlations of 8.

