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Immunomodulatory activities of methanolic extract and isolated compounds from the leaves of *Combretum aculeatum* Vent

Amadou Dawe, Fawai Yakai, Marius Mbiantcha, Angele Fanta, Gilbert Deccaux Wabo Fotso Kapche, Muhammad Shaiq Ali and Bonaventure Tchaleu Ngadjui

Abstract
Popularly known as *Lahugni* in Fulfuldeh and *Karkarbaw* in Tupuri, *Combretum aculeatum* Vent. is used in traditional medicine in the Northern regions of Cameroon as back pain, muscular-skeletal aches and eyes remedies; it is also used for the treatment of blennorrhoea, leprosy, rheumatic pains and other inflammatory diseases. This study was conducted to evaluate the immunomodulatory effect of crude methanolic extract and isolated compounds from *C. aculeatum* using luminol/zymosan and lucigenin/MPA based chemiluminescence assay. Isolation of secondary metabolites from the leaves of *C. aculeatum* was done by the means of usual chromatographic techniques and their structures were confirmed on the basis of spectroscopic data and those available in literature. The first phytochemical exploration of the leaves of *C. aculeatum* afforded ß-sitosterol (1) and its glucoside (2), oleanolic acid (3), maslinic acid (4), corosolic acid (5), alphitoli cacid (6), arjunolic acid (7), asiatic acid (8), 3,3'-di-O-methylellagic acid (9) and 3,3',4-tri-O-methylellagic acid-4'-O-D-glucopyranoside (10) (Figure 1). To the best of our knowledge, all these compounds are isolated and reported for the first time from this plant and, alphitolic acid is characterized for the first time from the genus *Combretum*. This study also demonstrated potent immunomodulatory activities of methanolic extract and compounds 3, 4, 5 and 9 in both luminol and lucigenin enhanced chemiluminescence assay compare to ibuprofen used as reference. These results are in agreement with the enthnobotanical uses of the plant and some previous reports on pharmacological activities such as anti-inflammatory, antioxidant, anticancer effects of some isolates and their related previously isolated and identified from several plants.

Keywords: Immunomodulatory, chemiluminescence, triterpenes, *C. Aculeatum*

1. Introduction
Combretum aculeatum is a scrambling shrub up to 0.5-4 m in height with virgate branches, or scandent to 8 m and, grey or dark red bark. The recurved spines are up to 3 cm long formed by persistent enlarged petioles [1]. It is found in dry savannah, thickets on dry soils and sometimes riverine [2]. *C. aculeatum* popularly known as Lahugni (Fulfuldeh) or Karkarbaw (Tupuri) in the Northern regions of Cameroonhas diuretic properties. Water in which the leaves have been boiled is drunk in northwest Senegal to promote micturition in cases when venereal disease obstructs the urethra. Macerations of its roots are used to enhance wounds healing, as a purgative and as a poultice for skin tuberculosis. It is prescribed for blennorrhoea, helminthiasis, back pain, muscular-skeletal aches and loss of appetite. It is also used in Burkina Faso and Senegal for leprosy [3-5]. In Senegal, the *Soce* tribe claims that a root decoction has a well-established reputation in the treatment of catarrh; the *Serer* tribe uses sap from the centre of the stem for eye troubles. The boiled roots are taken in Kenya for stomach upsets [3]. Plants of the genus *Combretum* are known as a rich source of secondary metabolites such as triterpenes, phenanthrene, stilbenes, flavonoids, tannins and other aromatic compounds [6]. Several authors have demonstrated that some extracts or purified compounds of the species of this genus have a broad spectrum of biological activities including antiviral, antibacterial, antiprotozoal, anticancer, analgesic, anti-inflammatory, hepatoprotective and molluscicidal [7,8]. Miracidicidal and cercaricidal activity of the aqueous extract of the leaves of *C. aculeatum* have been demonstrated on *Schistosoma mansoni*. Some extracts (H_2O, MeOH and $CDCl_3$) of the leaves, roots and stem were also shown to possess strong antifungal activity [9, 10].
Worldwide, several laboratories are finding a growing interest in the search of natural substances with immunomodulatory activities that can be used in the treatment of some chronic diseases likes diabetes, rheumatoid arthritis, transplantation rejection, cancer and

atherosclerosis [11-13]. Polymorphonuclear neutrophils (PMNs), monocytes and macrophages constitute professional phagocytes used as fist line of defense during an innate immune response. Phagocytosis is therefore the processes by which pathogens are eliminated by oxidative burst. The eradication of infectious agents is effected by the production of mediators such as chemokines (cytokine and ROS) when the phagocytes migrate to the site of inflammation after being excited. Despite their protective roles, the anarchic production of these mediators can cause serious damage to body tissues and lead to chronic diseases such as rheumatoid arthritis, cancer and atherosclerosis. Thus, all less toxic compounds capable of modulating the immune response by inhibiting the production of ROS, TNF-α, IL-1β, etc. with less side effects have a very high therapeutic value [14-16].

To date, no phytochemical studies have been carried out to identify the active metabolites from *C. aculeatum*. In our systematic search for bioactive metabolites from plants, particularly those of the genus *Combretum*, we have earlier reported the antibacterial activity of *C. fragrans, C. micranthum and C. molle*. We also reported the isolation of two new cycloartanes triterpenes from the leaves of *C. fragrans*.

Here in, we report for the first time, the isolation of six related triterpenes: oleanic acid, maslinic acid, corosolic acid, alphitolic acid, arjunolic acid and asiatic acid along with ß-sitosterol and it's glucoside, 3,3'-di-O-methylellagic acid and 3,3',4-tri-O-methylellagic acid-4'-O-D-glucopyranoside from *C. aculeatum*. Although the compounds have been previously isolated from other plants including the leaves of *Ugnimolinae* and *Syzygiumkusukusense* (Myrtaceae) [17,18], *Ziziphusmauritiana* (Rhamnaceae) [19], *Ocimumbasilicum*(Lamiaceae) [20] and *Combretum albidum* [21], this is the first report on the isolation of secondary metabolites from C. *aculeatum*. It is also the first report on the remarkable chemiluminescence inhibition activity of the extracts and secondary metabolites from the leaves of *C. aculeatum*.

2. Material and Methods

2.1 Phytochemistry

Reagents, Chemicals and Equipments

Melting points were obtained on a Büchi M-560 melting point apparatus. 1D and 2D NMR spectra were run on Bruker spectrometer operating at 125 MHz (^{13}C) and 500 MHz (^{1}H). Chemical shifts (δ) were expressed in ppmwith the residual solvent peaks as internal references. Column chromatographies were performed on silica gel (230-400 mesh). Fractions were monitored by TLC using Merck pre-coated silica gel sheets ($60F_{254}$), and the identification of the spots on the TLC plate were carried out by spraying ceric sulfate reagent solution and heating the plate at about 80 °C.

Plant Material

The leaves of *C. aculeatum* were collected in February 2016 in Maroua, Far-North Region of Cameroon. The identification of the plant was performed by Mr. Victor Nana of the National Herbarium, Yaoundé where a voucher specimen (14900/H.N.C.) has been deposited.

Extraction and Isolation

Dried leaves of *C. aculeatum* (1.7 kg) were ground and extracted with MeOH (3x10 l) at room temperature. After removing the solvent, the residue (162 g) was suspended in H_2O and extracted with *n*-hexane, AcOEt and *n*-BuOH. The AcOEt-soluble fraction (36 g) was subjected to CC and eluted with the mixture of *n*-hexane/AcOEt and AcOE/MeOH of increasing polarity. Fraction obtained with *n*-hexane/AcOEt (90:10-80:20) were combined and subjected to column chromatography on a silica gel column (2 cm × 45 cm, 40 g), eluted with *n*-hexane/AcOEt gradient mixtures to afford ß-sitosterol(**1**) and oleanolic acid (**3**). Fractions obtained with *n*-hexane/AcOEt (70:30) were combined and re-chromatographed over silica gel column and eluted with *n*-hexane/AcOEt gradient mixtures to afford maslinic acid (**4**), corosolic acid (**5**), alphitolic acid (**6).** A mixture of arjunolic acid (**7**) and asiatic acid (**8**) together with ß-sitosterol glucoside (**2**), 3,3'-di-O-methylellagic acid (**9**) and 3,3',4-tri-O-methylellagic acid-4'-O-D-glucopyranoside (**10**) were obtained from fraction C (*n*-hexane/AcOEt60:40) after separation by repeated silica gel CC with *DCM*/MeOH (9:1) washed on sephadex*LH-20* (MeOH) and purified by preparative TLC respectively.

Beta-sitosterol (1): The crystalline material was further purified by recrystallization from methanol to get a colorless needle shape crystal.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.66 (3H, s, Me-18), 0.80 (3H, d, Me-27), 0.81 (3H, d, Me-26), 0.83 (3H, t, Me-29), 0.90 (3H, d, Me-21), 0.99 (3H, s, Me-19), 3.50 (1H, m, H-3), 2.26 (2H, m, H-4), 5.33 (1H, m, H-6).

^{13}C-NMR (125 MHz, $CDCl_3$) : δ 37.4 (C-1), 31.8 (C-2), 72.0 (C-3), 42.3 (C-4), 140.9 (C-5), 121.9 (C-6), 32.1 (C-7), 32.1 (C-8), 50.4 (C-9), 36.7 (C-10), 21.3 (C-11), 39.9 (C-12), 42.5 (C-13), 56.0 (C-14), 24.5 (C-15), 28.4 (C-16), 56.5 (C-17), 12.0 (C-18), 19.6 (C-19), 36.3 (C-20), 19.0 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 20.0 (C-26), 19.2 (C-27), 23.2 (C-28), 12.2 (C-29).

β-sitosterol glucoside (2): White crystalline solid, turned into pink color with ceric sulfate

^{1}H NMR (500 MHz C_5D_5N): δ 0.65 (3H, s, Me-18), 0.85 (3H, d, Me-29), 0.87 (3H, d, Me-27), 0.89 (3H, t, Me-26), 0.93 (3H, s, Me-19), 0.98 (3H, d, Me-21), 3.95 (1H, m, H-3), 5.34 (1H, t, H-6), 5.05 (1H, d, H-1'), 4.56 (1H, d, Ha-6'), 4.41 (1H, d, Hb-6'), 4.29 (1H, d, H-3'), 4.28 (1H, d, H-4'), 4.07 (1H, d, H-2'), 3.98 (2H, d, H-5');

^{13}C NMR (125 MHz, C_5D_5N): δ 37.5 (C-1), 30.3 (C-2), 78.6 (C-3), 39.4 (C-4), 140.9 (C-5), 121.9 (C-6), 39.4 (C-7), 32.1 (C- 8), 50.4 (C-9), 36.9 (C-10), 21.3 (C-11), 39.9 (C-12), 40.8 (C-13), 56.3 (C-14), 26.4 (C-15), 28.6 (C-16), 56.9 (C-17), 11.9 (C-18), 19.4 (C-19), 36.4 (C-20), 19.2 (C- 21), 34.2 (C-22), 32.2 (C-23), 46.1 (C-24), 29.5 (C-25), 19.9 (C-26), 19.4 (C-27), 23.4 (C-28), 12.2 (C-29) and 102.6, 78.5, 78.1, 75.4, 71.7, 62.9 ppm (for six carbons of glucose unit).

Oleanolic acid (3): White powder

^{1}H-NMR (500 MHz, CD_3OD): δ 0.77 (3H, s, Me-24), 0.81 (3H, s, Me-26), 0.90 (3H, s, Me-30), 0.93 (3H, s, Me-25), 0.94 (3H, s, Me-29), 0.97 (3H, s, Me-23), 1.52 (3H, s, Me-27), 2.84 (1H, d, H-18), 3.14 (1H, dd, H-3), 5.24 (1H, t, H-12);

^{13}C-NMR (125 MHz, CD_3OD): δ 39.9 (C-1), 27.8 (C-2), 79.7 (C-3), 38.2 (C-4), 56.8 (C-5), 19.5 (C-6), 34.0 (C-7), 40.6 (C-8), 49.2 (C-9), 33.8 (C-10), 24.1 (C-11), 123.6 (C-12), 145.3 (C-13), 42.9 (C-14), 27.8 (C-15), 24.5 (C-16), 47.7 (C-17), 42.8 (C-18), 47.3 (C-19), 31.6 (C-20), 28.9 (C-21), 34.9 (C-22), 28.7 (C-23), 16.3 (C-24), 15.9 (C-25), 17.7 (C-26), 26.4 (C-27), 180.4 (C-28), 33.5 (C-29), 23.9 (C-30).

Maslinic acid (4): White powder
^{1}H-NMR (500 MHz, CD_3OD): δ 5.24 (1H, t, H-12), 3.61 (1H, m, H-2), 2.90 (1H, d, H-3), 1.16 (3H, s, Me-27), 1.01 (3H, s, Me-23), 0.99 (3H, s, Me-25), 0.93 (3H, s, Me-29), 0.90 (3H,s, Me-29), 0.81 (3H, s, Me-26), 0.80 (3H, s, Me-24);
^{13}C-NMR (125 MHz, CD_3OD) : δ 48.1 (C-1), 69.5 (C-2), 84.5 (C-3), 39.3 (C-4), 56.7 (C-5), 19.6 (C-6), 33.8 (C-7), 40.6 (C-8), 48.7 (C-9), 38.1 (C-10), 24.6 (C-11), 123.4 (C-12), 145.4 (C-13), 42.9 (C-14), 28.8 (C-15), 24.1 (C-16), 47.7 (C-17), 42.7 (C-18), 47.3 (C-19), 31.7 (C-20), 33.9 (C-21), 34.9 (C-22), 29.3 (C-23), 17.1 (C-24), 17.4 (C-25), 17.7 (C-26), 26.4 (C-27), 180.4 (C-28), 33.6 (C-29), 23.9 (C-30).

Corosolic acid (5): White powder
^{1}H NMR (500 MHz, CD_3OD) δ 3.64 (1H, ddd, H-2), 2.93 (1H, H-3), 5.25 (1H, t, H-12), 2.23 (1H, d, H-18), 1.04 (3H, s, H-23), 0.83 (3H, s, H-24), 1.04 (3H, s, H-25), 0.87 (3H, s, H-26), 1.14 (3H, s, H-27), 0.91 (3H, d, J = 6.5 Hz, H-29), 0.99 (3H, s, H-30);
^{13}C NMR (125 MHz, CD_3OD) δ 42.6 (C-1), 67.1 (C-2), 80.1 (C-3), 39.5 (C-4), 49.3 (C-5), 19.1 (C-6), 31.8 (C-7), 40.9 (C-8), 48.7 (C-9), 39.4 (C-10), 24.4 (C-11), 126.7 (C-12), 139.8 (C-13), 43.4 (C-14), 29.2 (C-15), 25.3 (C-16), 48.5 (C-17), 54.4 (C-18), 40.4 (C-19), 40.4 (C-20), 31.8 (C-21), 38.1 (C-22), 29.2 (C-23), 17.0 (C-24), 22.5 (C-25), 17.8 (C-26), 24.2 (C-27), 181.7 (C-28), 17.6 (C-29), 21.6 (C-30).

Alphitolic acid (6): White powder
^{1}HNMR (500 MHz, CD_3OD) δ 4.70 (1H, d, H-29a), 4.59 (1H, d, H-29b), 3.60 (1H, ddd, H-2), 3.01 (1H, m, H-19), 2.87 (1H, d, H-3), 2.31 (1H, ddd, H-13), 1.67 (3H, s, H-30), 1.60 (1H, t, J = 11.3 Hz, H-18), 1.38 (1H, t, J = 11.3 Hz, H-9), 1.00 (3H, s, H-27), 0.98 (3H, s, H-23), 0.96 (3H, s, H-26), 0.91 (3H, s, H-25), 0.77 (3H, s, H-24).
^{13}CNMR (500 MHz, CD_3OD) δ 48.7 (C-1), 69.7 (C-2), 84.4 (C-3), 40.5 (C-4), 56.8 (C-5), 19.5 (C-6), 35.4 (C-7), 39.5 (C-8), 51.9 (C-9), 39.4 (C-10), 22.2 (C-11), 26.2 (C-12), 39.6 (C-13), 43.6 (C-14), 30.8 (C-15), 31.7 (C-16), 57.5 (C-17), 50.4 (C-18), 48.5 (C-19), 152.0 (C-20), 30.8 (C-21), 38.2 (C-22), 29.1 (C-23), 17.2 (C-24), 17.8 (C-25), 16.6 (C-26), 15.0 (C-27), 180.4 (C-28), 110.1 (C-29), 19.5 (C-30).

Arjunolic acid (7) and Asiatic acid (8) as a mixture: colorless amorphous powder
Compound 7
^{1}HNMR (500 MHz, CD_3OD): δ 5.24 (1H, t, J = 3.5 Hz, H-12), 3.68 (1H, m, H-2), 3.35 (1H, d, H-3), 3.49 (1H, m, H-23a), 3.25 (1H, m, H-23b), 2.84 (1H, d, H-18), 1.17 (3H, s, Me-27), 1.02 (3H, s, Me-25), 0.93 (3H, s, Me-30), 0.90 (3H, s, Me-29), 0.81 (3H,s, Me-26), 0.69 (3H, s, Me-24);
^{13}CNMR (125 MHz, CD_3OD): δ 48.0 (C-1); 69.7 (C-2); 78.2 (C-3); 43.4 (C-4); 48.2 (C-5); 19.1 (C-6); 33.7 (C-7); 40.8 (C-8); 48.9 (C-9); 39.1 (C-10); 24.5 (C-11); 123.4 (C-12); 145.4 (C-13); 43.4 (C-14); 29.2 (C-15); 25.3 (C-16); 47.7 (C-17); 42.8 (C-18); 47.3 (C-19); 31.6 (C-20); 34.9 (C-21); 33.9 (C-22); 66.3 (C-23); 13.9 (C-24); 17.7 (C-25); 17.9 (C-26); 26.5 (C-27); 181.9 (C-28); 33.6 (C-29); 23.9 (C-30).

Compound 8
^{1}HNMR (500 MHz, CD_3OD) δ 5.24 (1H, t, H-12), 3.68 (1H, m, H-2), 3.35 (1H, H-3), 3.49 (1H, m, H-23a), 3.25 (1H, m, H-23b), 2.19 (1H, d, H-18), 1.13 (3H, s, H-27), 1.04 (3H, s, H-25), 0.96 (3H, s, H-30), 0.88 (3H, s, H-29), 0.84 (3H, d, H-26), 0.69 (3H, s, H-24);
^{13}CNMR: (125 MHz, CD_3OD) δ 47.9 (C-1); 69.7 (C-2); 78.2 (C-3); 43.0 (C-4); 48.2 (C-5); 19.1 (C-6); 33.3 (C-7); 40.6 (C-8); 48.9 (C-9); 39.0 (C-10); 24.0 (C-11); 125.5 (C-12); 139.2 (C-13); 43.0 (C-14); 28.8 (C-15); 24.6 (C-16); 47.7 (C-17); 54.4 (C-18); 40.4 (C-19); 40.4 (C-20); 31.8 (C-21); 38.1 (C-22); 66.3 (C-23); 13.9 (C-24); 17.7 (C-25); 17.8 (C-26); 24.1 (C-27); 181.7 (C-28); 17.5 (C-29); 21.6 (C-30).

3, 3'-di-O-methylellagic acid (9): colorless amorphous powder
^{1}HNMR (500 MHz, DMSO-d_6): δ 7.51 (2H, s, H-5 and H-5), 4.03 (6H, s, 3-OCH_3 and 3'-OCH_3).
^{13}CNMR (125MHz, DMSO-d6): δ 111.5 (C-1 and C-1'), 141.2 (C-2 and C-2'), 140.3 (C-3 and C-3'), 152.3 (C-4 and C-4'), 111.5 (C-5 and C-5'), 112.2 (C-6 and C-6'), 158.6 (C-7 and C-7'), 60.9 (3-OCH_3 and 3'-OCH_3).

3, 3', 4-tri-O-methylellagic acid-4'-O-D-glucopyranoside (10): colorless amorphous powder
^{1}HNMR (500 MHz, DMSO-d_6): δ 7.67 (1H, s, H-5), 7.84 (1H, s, H-5'), 4.09 (3H, s, 3-OCH_3), 4.04 (3H, s, 3-OCH_3), 4.00 (3H, s, 4-OCH_3), 5.17 (1H, d, H-1"), 3.33 (1H, H-2"), 3.34 (1H, H-3"), 3.22 (1H, H-4"), 3.43 (1H, H-5"), 3.68 (2H, H-6a"), 3.50 (2H, H-6b").
^{13}CNMR (125 MHz, DMSO-d 6): δ 112.9 (C-1), 141.3 (C-2), 140.9 (C-3), 154.4 (C-4), 107.6 (C-5), 112.7 (C-6), 158.5 (C-7), 113.7 (C-1'), 141.3 (C-2'), 141.7 (C-3'), 151.9 (C-4'), 111.9 (C-5'), 112.4 (C-6'), 158.2 (C-7'), 61.4 (3-OCH3), 56.8 (4-OCH3), 61.7 (3-OCH3), 101.32 (C-1"), 73.3 (C-2"), 76.5 (C-3"), 69.5 (C-4"), 77.3 (C-5"), 60.5 (C-6").

2.2 Immunomodulatory activity
Reagents, Chemicals and Equipments
Luminol (3-aminophthalhydrazide) was purchase from Research Organics, while Hanks Balance Salts Solution (HBSS) were purchased from Sigma, Germany. Lymphocytes Separation Medium (LSM) was purchased from MP Biomedicals Inc. Germany. Zymosan-A/lucigenin was obtained from Fluka (Biochemika). Dimethylsulphoxide (DMSO), ethanol and ammonium chloride of analytical grades were purchased from Merck Chemicals, Darmstadt, Germany. The luminometer used was Luminoskan RS Finland.

Isolation of human polymorphoneutrophils (PMNs)
Venous blood was collected from healthy human volunteers of about 25-38 years age at the Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), Pakistan. It was then aseptically introduced (10 ml) into heparinized tubes and through the ficollhypaque density gradient centrifugation method and neutrophils were isolated [22]. In general, blood sample, lymphocyte separation medium (LSM) and HBSS were mixed-up at equivalent volume into all the 45 ml empty centrifugation tubes. After 30 minutes, the supernatant was collected and progressively introduce into new tube (15 ml empty tubes) previously filled with 5 ml of LSM. They were centrifuged at 400 grm for 20 minutes/room temperature and supernatant was collected, added to 1 ml distilled water for lysis blockage. Then, 5 ml of HBSS were further added into those tubes which were submitted to another centrifugation at 300 grm/10 minutes/4°C. After collection of supernatant, 1 ml of HBSS was equal introduce in tubes and they were maintained freezing on ice. A haemocytometer was then used for cells count using the method of trypan blue exclusion and finally, an adjustment of cells concentration to 1×10^6 cells/ml was performed.

Peritoneal macrophages isolation from mice

NMRI mice weighing between 18-25 g from the Animal house facility of PCDM were immunized by intraperitoneal (*i.p.*) injection of 1 ml Fetal Bovine Serum (FBS), kept for 72 hours in the animal house and then sacrificed by cervical dislocation. An injection of 10 % RPMI medium (10 ml, *i.p.*) was done after having dipped the whole animal body into 70 % ethanol. A sustained massage of the peritoneal cavity was conducted within 2 minutes and abdominal skin removed from the lower side for the peritoneal cavity exposure. Infected RPMI thus containing macrophage was collected using a sterile syringe and conducted to centrifugation (400 grm/20minutes/4°C). Pellet was washed after supernatant was discarded and put in suspension with 1 ml of incomplete RPMI media/HBSS. Cells count was determine by an haemocytometer and viable cells were calorimetrically identified through trypan blue dye exclusion method [23, 24].

Chemiluminescence assay

Modified luminol/lucigenin enhanced chemiluminescence assay was performed as previously described [23, 24]. 25 µl of diluted blood sample (dilution ratio 1:50 in sterile $HBSS^{++}$), PMNs (1×10^6) or macrophages (2×10^6) cells were incubated with 25 µl of plant extracts, previously diluted serially to give concentrations ranging from 100 µg/ml to 3.10 µl/ml. Control wells (of 96 wells plate) containing $HBSS^{++}$ and cells. Incubation of plate was made at 37°C for 20 minutes in a thermostated chamber of the luminometer. These wells were supplemented with 25 µl serum opsonized zymosan/PMA and 25 µl luminol (7×10^5 M) and ibuprofen used as standard drug. Results were expressed in relative light unit (RLU). Total integral values and peak were sat with repeated scan (30 s intervals and 1 s points measuring time) [24]. The following formula was used for inhibition percentage (%) calculation of each compound:

$$\text{Inhibition } (\%) = \frac{(RLU_{control} - RLU_{sample}) \text{ X } 100\ \%}{RLU_{control}}$$

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. The compared values were considered significantly different when $P < 0.05$.

3. Results

Phytochemistry

The ethyl acetate soluble part of the methanol extract of the leaves of *C. aculeatum* was separated and ten compounds were obtainedand purified including two steroids: ß-sitosterol (1) and its glucoside (2), six related pentacyclic triterpenes: oleanolic acid (3), maslinic acid (4), corosolic acid (5), alphitolic acid (6), arjunolic acid (**7**) and asiatic acid (8) and two ellagic acid derivatives: 3,3'-di-O-methylellagic acid (9) and 3,3',4-tri-O-methylellagic acid-4'-O-D-glucopyranoside (10) (Fig. 1). The structures of all the isolated compounds were confirmed on the basis of spectroscopic and available literature data [17, 20, 21].

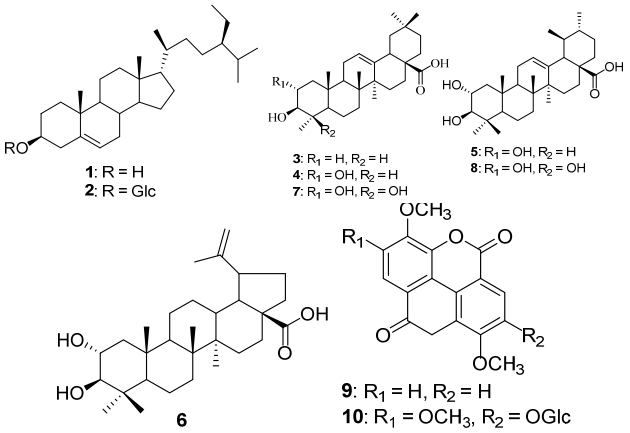

Fig 1: Structures of the isolated compounds from the leaves of *C. aculeatum*

Effect of extract and compounds on intracellular ROS production

Myeloperoxidase dependent effect was studied using human peripheral whole blood, neutrophils as well as macrophages from mice peritoneum, where the luminol is used as probe. The preliminary screening results on human whole blood phagocytes showed that some compounds possess significant inhibitory activity at the initial tested concentration (3.1-100 µg/ml). Methanolic extract and isolated compounds 3, 4, 5 and 9 were found to inhibit the production of intracellular ROS from whole blood with IC_{50} values of 11.24, 45.81, 27.82, 22.31 and 81.22 µg/ml, respectively (table 1).

Concerning neutrophils, methanolic extract and isolated compounds also showed potent inhibitory activity with IC_{50} value of 1.84, 29.12, 27.47, 9.09 and 70.39 µg/ml, respectively for crude extract, compounds 3, 4, 5 and 9 (table 1). When tested on ROS produced from the mice peritoneal macrophages, crude methanolic extract, compounds 3, 4, 5 and 9 showed significant inhibitory activity with IC_{50}value of 4.82, 39.29, 28.55, 10.91 and 77.81 µg/ml respectively (table 1). Ibuprofen, used as reference product shows IC_{50} of 11.2, 2.50 and 16.90 µg/ml, respectively for whole blood, neutrophils and mice peritoneal macrophages.

Effect of compounds on extracellular ROS production

Myeloperoxidase independent ROS production was monitored using the lucigenin as probe on human neutrophils and mice peritoneal macrophages. The results showed that, on both neutrophils and macrophages, crude methanolic extract, compounds 3, 4, 5 and 9 showed a significant inhibition of ROS and the IC_{50} values observed were 1.75, 67.66, 21.10, 8.18 and 87.22 µg/ml, respectively on neutrophils compared to the ibuprofen (IC_{50} = 13.69 µg/ml) (table 1). Similarly on mice macrophages the extract and isolated compounds showed a potent inhibitory activity with IC_{50} of 4.55, 93.23, 22.30, 9.37 and 90.56 µg/ml respectively, compared to ibuprofen IC_{50} = 14.30 µg/ml (table 1).

Table 1: Effect of methanolic extract and compounds from *C aculeatum* on myeloperoxidase dependent (luminol/zymosan) and independent (lucigenin/PMA) oxidative burst

Compouds	IC_{50} (µg/ml)				
	Luminol			Lucigenin	
	Whole blood	PMNs	Macrophages	PMNs	Macrophages
M. Extract	11.24 ± 0.35	1.84 ± 0.61	4.82 ± 0.17	1.75 ± 0.46	4.55 ± 0.21
1	240.22	202.45	>250	233.10	>250
2	>250	>250	>250	>250	>250
3	45.81	29.12	39.20	67.66	93.23
4	27.82	27.47	28.55	21.10	22.30
5	22.31	9.09	10.91	8.18	9.37
6	>250	>250	>250	>250	>250
7 and 8	145.71	160.31	166.23	190.49	192.44
9	81.22	70.39	77.81	87.22	90.56
10	>250	>250	>250	>250	>250
Ibuprofen	11.2	2.5	16.9	13.69	14.30

The IC_{50} values were obtained using various concentrations of test compounds and reading are presented as mean of triplicates

4. Discussion

It has been mentioned in actual research that natural products constitutes an enriched source of compounds with anti-inflammatory properties and thus can be used for treating tissue damage and inflammation in RA and other related autoimmune diseases by new mechanism. This study aims to isolate secondary metabolites from the leaves of *C. aculeatum* and evaluate their immunomodulatory activity. In the innate immune system, inhibition of ROS can be a pathway for inflammatory responses regulation. Luminol and lucigenin are used in demonstrating *in vitro* immunomodulatory activities, since luminol have a capacity to cross the cell membrane and react both with the extra and intracellular ROS (OH, O_2, H_2O_2 and HOCl for example), while lucigenin act specifically on extracellular ROS(O_2^-). These ROS thus have a main role in a number of inflammatory disorders (atherosclerosis, and ischemic heart diseases, cancer). The results of phytochemical study revealed the presence of pentacyclic triterpenes, steroids and ellagic acid derivatives. Initially, themethanolic extract and isolated compounds were tested in oxidative burst assay by using whole-blood, isolated neutrophils and mice macrophages. The results demonstrate that the methanolic extract has been found to significantly inhibit the production of ROS and,the isolated compounds 3, 4, 5 and 9 were found to cause a significant inhibition of the intra- and extra-cellular ROS production for luminol/zymosan amplified chemiluminescence assay on whole blood, neutrophils and mice macrophages. In another set of experiment, using lucigenin as a probe and PMA as an activator to monitor, the inhibition of extracellular ROS production, such as superoxide radicals, was monitored. Again, the methanolic extract and the isolated compounds 3, 4, 5 and 9 were found to be the most active on neutrophils and mice macrophages. The significant immunomodulatory activity observed could be a result of the presence of pentacyclic triterpenes. Triterpenes are known as very good anti-inflammatory, anticancer, antibacterial, antifungal, antidiabetic etc. [25-27]. This finding corroborates earlier reports, which presented anti-inflammatory and anticancer activities of oleanic, maslinic, corosolic, alphitolic, arjunolic and asiatic acids [17, 28-31].

The related inhibitory activity can be explained by aptitude or capacity of extract and isolated compounds to inhibit the receptor complement, leading to the inhibition of a cell membrane associated enzyme called NADPH oxidase. Phagocytes activation is enclenched when zymosan A bind to type 3 complement receptor in the cell membrane, a process different to that of PMA, which activates O_2^- through the NADPH oxidase, directly acting on intercellular mediators [32].

5. Conclusion

C. aculeatum is used in traditional medicine for the treatment of various diseases such as blennorrhoea, helminthiasis, leprosy, rheumatic pains and other inflammatory diseases. The methanolic extract of the leaves has been found in this study to demonstrate potent inhibitory effect on ROS production. Compounds 3, 4, 5 and 9 were found to possess immunomodulatory activities in both luminal and lucigenin enhanced chemiluminescence assay compare to ibuprofen used as reference. These current results are deeply in agreement with the enthnobotanical uses of the plant and some previous reports on pharmacological activities such as anti-inflammatory, antioxidant, anticancer effects of some isolates and related previously isolated and identified from several plants. In general, pentacyclic triterpenes are very good anti-inflammatory and anticancer agents. This study demonstrated the anti-inflammatory potential of the methanolic extract of the leaves of *C. aculeatum* and some isolated compounds which may be lead compounds for further anti-inflammatory drug discovery process. However, further studies to unrevealed exact underlying molecular mechanism, as well as detailed *in vivo* studies and clinical trials are needed to evaluate the effects of this compound in reducing inflammation.

Ethics approval and consent to participate

For the donation of human blood samples, all process and procedure of collecting blood were accepted and approved by the independent ethics committee of ICCBS, University of Karachi, through the clearance N°: ICCBS/IEC-008-BC-2015/Protocol/1.0. The blood donors were provided information before their approval for the use of their blood for the purposes of this study. Animal facility was provided by ICCBS, University of Karachi and experiments were executed under the ethical guidelines of the International Association for the Study of Pain in Conscious Animals and guidelines set by for the scientific advisory committee, animal care, use and standards, ICCBS were followed (Protocol No. 1209004).

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

7. References

1. Maydell V. Trees and shrubs of the Sahel. Eschborn, 1986, 233-241.
2. Beentje HJ. Kenya trees, shrubs and lianas. National Museums of Kenya, 1994.
3. Burkill HM. Useful plants of West Tropical Africa. Royal Botanical Gardens, Kew 2. Families E-I, 1994.
4. Fratkin E. Traditional medicine and concepts of healing among Samburu pastoralists of Kenya. J of ethnobiology. 1996; 16:63-97.
5. Ahmed HA, Awatif AE, Waleed SK, Elbadri EO, Mahmoud MD. *In vitro* anticercarial activities of some Sudanese medicinal plants of the family Combretaceae. JFPI. 2014; 3:93-99.
6. Dawe A, Saotoing P, Tsala DE, Habtemariam S. Phytochemical constituents of Combretum Loefl. (Combretaceae). Pharmaceutical crops. 2013; 4:38-59.
7. Cepleanu F, Hamburger MO, Sordat B, Msonth JD, Gupta MP, Saadou M, Hostettmann K. Screening of tropical medicinal plants for molluscicidal, larvicidal, fungicidal and cytotoxic activities and brine shrimp toxicity. Int J Pharmacog. 1994; 32:294-307.
8. Sudipta R, Dilip G, Rabinarayan A, Rajiv R. Combretum (combretaceae): biological activity and phytochemistry. IAJPR. 2014; 4:5266-5299.
9. Elsheikh SH, Bashir AK, Suliman SM, Wassila ME. Toxicity of certain Sudanese plant extracts on cercariae and miracidia of Schistosoma mansoni. Int J Crude Drug Res. 1990; 28:241-245.
10. Morais Lima GR, Sales IRP, Caldas Filho MRD, Taveira de Jesus NZ, Sousa Falcão H, Barbosa-Filho JM *et al*. Bioactivities of the genus Combretum (Combretaceae). Molecules. 2012; 17:9142-9206.
11. Bartlett RR, Dimitrijevic M, Mattar T, Zielinski T, Germann T. A novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. Agents Actions. 1991; 32:10-21.
12. Loftus CG, Egan LJ, Sandborn WJ. Cyclosporine, tacrolimus and mycophenolate mofetil in the treatment of inflammatory bowel disease. Gastroenterol Clin North Am. 2004; 33:141-169.
13. Jantan I, Harun NH, Septama AW, Murad S, Mesaik MA. Inhibition of chemiluminescence and chemotactic activity of phagocytes *in vitro* by the extracts of selected medicinal plants. J Nat Med. 2011; 65:400-405.
14. Linn T, Germann H, Bretzel RG, Helmke K, Bicker U. Different effect of two immunomodulating agents, cyclosporin A and ciamexon on the insulin metabolism of cultured mouse islets. J Diabetes Res. 1987; 4:121-123.
15. Mesaik MA, Jabeen A, Halim SA, Begum A, Khalid AS. In silico and *in vitro* immunomodulatory studies on compounds of Lindelofia stylosa. Chem Biol Drug Des. 2012; 79:290-299.
16. El Ghazali GEB, El Tohami MS, El Egami AAB. Medicinal plants of the Sudan part III, Medicinal plants of the White Nile province. National centre for research, Medicinal and aromatic plants research institute, Khartoum, Sudan, 1994.
17. Aguirre MC, Delporte C, Backhouse N, Erazo S, Letelier ME, Cassels BK *et al*. Topical anti-inflammatory activity of 2α-hydroxy pentacyclictriterpene acids from the leaves of Ugni molinae. Bioor Med Chem. 2006; 14:5673-5677.
18. Goyal M, Nagori BP, Sasmal D. Review on ethnomedicinal uses, pharmacological activity and phytochemical constituents of Ziziphus mauritiana (Z. jujuba Lam., non Mill). Spatula DD. 2012; 2:107-116.
19. Li YB, Wei YL, Chang FC, Jing RW. Anti-tumor activities of triterpenes from Syzygium kusukusense. Nat Prod Commun. 2014; 9:1557-1558.
20. Marzouk AM. Hepatoprotective Triterpenes from Hairy Root Cultures of Ocimum basilicum. Z Naturforsch. 2009; 64:201-209.
21. Kumar UP, Sreedhar S, Purushothaman E. Secondary Metabolites from the Heart Wood of Combretum albidum G Don. IJPPR. 2015; 7:319-324.
22. Zimmerman M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983; 16:109-110.
23. Mesaik MA, Ul-Haq Z, Murad S, Ismail Z, Abdullah NR, Gill HK *et al*. Biological and molecular docking studies on coagulin-H: Human IL-2 novel natural inhibitor. Mol Immunol. 2006; 43:1855-1163.
24. Mahomoodally MF, Gurib-Fakim A, Subratty AH. Effect of exogenous ATP on Momordica charantia Linn. (Cucurbitaceae) induced inhibition of D-glucose, L-tyrosine and fluid transport across rat everted intestinal sacs *in vitro*. J Ethnopharmacol. 2007; 110:257-263.
25. Pacheco AG, Alcântara AFC, Abreu VGC, Corrêa GM. Relationships between chemical structure and activity of triterpenes against Gram-positive and Gram-negative bacteria in: Biochemistry, Genetics and Molecular Biology: A search for Antibacterial Agent, Varaprasad Bobbarala, 2012.
26. Babalola IT, Shode FO. Ubiquitous Ursolic Acid: A potential pentacyclic triterpene natural product. J Pharmacogn Phytochem, 2013; 2:214-222.
27. Wozniak L, Skapska S, Marszałek K. Ursolic ccid, a pentacyclic triterpenoid with a wide spectrum of pharmacological activities. Molecules. 2015; 20:20614-20641.
28. Reyes-Zurita FJ, Rufino-Palomares EE, Lupiáñez JA, Cascante M. Maslinic acid, a natural triterpene from Olea europaea L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. Cancer Lett. 2009; 273:44-54.
29. Reyes-Zurita FJ, Pachón-Peña G, Lizárraga D, Rufino-Palomares EE, Cascante M, Lupiáñez JA. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. BMC Cancer. 2011; 11:6-13.
30. Thakor P, Wenzhe S, Ramalingam BS, Thakkar VR, Vesey DA, Gobe GC. Maslinic acid inhibits proliferation of renal cell carcinoma cell lines and suppresses angiogenesis of endothelial cells. Journal of Kidney Cancer and VHL. 2017; 4:16-24.
31. Woo KW, Han JY, Choi SU, Kim KH, Lee KR. Triterpenes from Perilla frutescens var. acuta and their cytotoxic activity. Nat Prod Sci. 2014; 20:71-75.
32. Allen R. Bioluminescence and chemiluminescence, Academic Press, New York. 1986; 133:449-493.