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PHYTOCHEMICAL ANALYSIS AND WOUND HEALING ACTIVITY OF *CALOTROPIS PROCERA* (AIT.) R. BR. LEAVES

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ABSTRACT: *Calotropis procera* (Ait) R. Br. (Asclepiadaceae) is a small, hardy, pubescent, evergreen, erect and compact shrub that grows wild in South-Eastern Asia. It is prescribed to treat anasarca, asthma, ascites, bronchitis, cough, cutaneous diseases, intestinal worms, leprosy, and eczema. A methanolic extract of the leaves of *Calotropis procera* was subjected to chromatography over silica gel column to isolate as six phytoconstituents, and their structures were characterized as *n*-nonanyl octadec-9-enoate (*n*-nonanyl oleate, 1), lup-12, 20 (29)-dien-3β-olyl hexadecanoate (lupenyl palmitate, 2), 1-methyl-4-(2'-*n*-decanyl)-cyclohex-1-en-5-one (3), (Z)-eicos-9-enoic acid (gadoleic acid, 4), *n*-decanoyl-α-L-arabinopyranoside (*n*-capryl arabinoside, 5) and stigmast-5- en- 3β- ol- 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside-2″-hexadecanoate (β-sitosterol-diglucosyl palmitate, 6). The structures of all these natural products have been established on the basis of spectral data analyses for the screening of the wound healing activity of all 6 compounds, and we found Compound-1, 2, 4, and compound 6 having excellent results during wound healing in rats. Our results clearly authenticate the beneficial effects of Compound-1, 2, 4, and 6 for an accelerated wound healing activity.

INTRODUCTION: The relationship between human and traditional systems of medicine has been established in ancient civilizations, and the majority of the compounds isolated from medicinal plants having a different kind of active constituents are now in use worldwide. An average of 25% of the therapeutic drugs in developing countries are plant derivatives, and their medicinal use is well known in many countries [1].

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A survey about this problem indicates that Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day [2].

All over the world, there are a lot of reports indicating certain cases that are related to drug resistance against human pathogenic bacteria, and this problem has an issue due to the excessive and extensive use of antibiotics [3-4]. And this kind of antibiotics is effective in pathogenic bacterial infection; these sometimes initiated certain adverse effects such as hypersensitivity, immune-suppression, and allergic reactions [5, 6, 7]. Therefore, treating infectious diseases in a clinical setting has become a global challenge [8].

According to World Health organization (WHO), nearly 80% of the world's population depends on traditional medicine and therapies, which involves the use of active constituents from various plant extracts. Research on plants and identifying the wound healing agents is an emerging field that integrates microbiology and Ethnobotany [9].

In view of the increasing incidences of wound and ulcers, despite of the development of western medicine to treat these ailments, it is a matter of prime importance to scientifically evaluate the herb isolated compounds recommended as a wound healer. In the present study, the six isolates from *Calotropis procera* isolated by column chromatography. And the main observations were wound contraction, a period of epithelialization, of healed tissue in excision wound and tensile strength in incision wounds was found to have significant results which were related or important for wound healing as also emphasized in the introduction.

Wounds are known as physical injuries that result in an opening or break of skin. So the proper healing of the wound is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. Wound Healing is a complex and intricate process initiated in response to an injury that restores the function and integrity of damaged tissue [10]. Wound healing is a dynamic process involving biochemical and physiological phenomena that behave in a harmonious way in order to guarantee tissue restoration [11]. For Therapeutic concern, measurement of wound healing are aimed not only at accelerating the process but also at grafting the wound in order to minimize the local deformity and scar marks that are likely to arise after the healing process is complete. The mechanism of wound healing involves a number of inflammatory reactions, such as the complement system and growth factors. During the inflammatory process, the majority of macrophages are recruited from the bloodstream as monocytes during the inflammatory phase and differentiate into macrophages at the site of injury [12]. The complement system is an important effecter pathway of the non-specific humoral immune response. Proteolytic cleavages of the complement components lead to the generation of biologically active complement activation products that may increase local vascular permeability, attract leucocytes (chemotaxis), mediate immune adherence and modulate antibody production. The complement system is involved in inflammatory process and in immunological defence reactions [13]. Injury or infection can result in the activation of the plasmin and kinin cascade that produces vasoactive peptides, which act on vascular endothelium to increase vascular permeability. The enzymes of the kinin cascade also can activate the complement cascade. The plasmin cascade is important in the remodeling of the extra-cellular matrix that accompanies wound healing [14].

There are a lot of treatment options analgesic antibiotics. Non-steroidal anti-inflammatory drugs are available for wound management. Still, most of these therapies produce numerous unwanted side effects in recent years. Several studies have been carried out on herbal drugs to clarify their potential effect in wound healing management. These natural herbs proved their effectiveness as an alternative treatment to available synthetic drugs for the treatment of the wound.

Calotropis procera (Ait) R. Br. (Asclepiadaceae), known as Apple of Sodom, Milkweed or Swallow-wort, is a small, hardy, pubescent, evergreen, erect and compact shrub, up to 4.5 m high, covered with cottony tomentum. It exudates copious milky sap when given a cut. It grows wild in southeastern Asia, including India, Pakistan, and Afghanistan, tropical Africa, Indochina, Morocco, and Senegal, mainly in drier and warm regions up to 1,050 m altitude on course, sandy and alkaline soils. Its growth is luxuriant on rubbish heaps, waste or fallow lands, along roadsides, seashores and river banks [15]. The root is cylindrical, branched, curved, light, woody and grayish-white. It resembles the root of *Cephaelis ipecacuanha* (Broter) A. Richard (family Rubiaceae) in action and is substituted for it. The roots are alterative, anthelmintic, depurative, diaphoretic, emetic, expectorant, febrifuge, and purgative; prescribed to treat anasarca, asthma, ascites, bronchitis, cough, cutaneous diseases, intestinal worms, leprosy, and eczema [16-17]. The root powder promotes gastric secretion; the fresh root is used as a toothbrush to cure toothache [15]. A root paste mixed with the leaves of *Ocimum sanctum* is taken orally to relieve menorrhoea [18]. The phytoconstituents cardenolides [19-20], flavone glycoside [21], pentacyclic triterpenoids [22-28], sterols

[21-29], monoterpenic, diterpenic, and phenolic glycosides [30-32], fatty acids [19], and a norditerpenyl ester [26], have been reported from the plant. This manuscript describes the isolation and characterization of a variety of chemical constituents from the leaves of *C. procera* collected from Delhi, India.

MATERIALS AND METHODS:

General: Melting points were determined on a Perfit apparatus without correction. The IR spectra were measured in KBr pellet on a Bio-Red FT-IR spectrometer. Ultraviolet (UV) spectra were obtained in methanol with a Lambda Bio 20 spectrometer. The ^{1}H (400 MHz), ^{13}C (100 MHz) NMR spectra were recorded on Bruker spectrospin spectrometer. $CDCl_3$ and DMSO-d6 (Sigma-Aldrich, Bangalore, India) were used as solvents and TMS as an internal standard. ESI MS analyses were performed on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) Mass Spectrometer. Column chromatography separations were carried out on silica gel (Merck, 60–120 mesh, Mumbai, India). Precoated silica gel plates (Merck, Silica gel 60 F_{254}) were used for analytical thin layer chromatography and visualized by exposure to iodine vapors and UV radiations.

Plant Material: The leaves of *C. procera* were collected from the herbal garden of Jamia Hamdard, New Delhi, and identified by Dr. H.B. Singh, Scientist F and Head, Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. A voucher specimen of the leaves was deposited in the Raw Materials Herbarium and Museum, NISCAIR, New Delhi, with a reference number NISCAIR/RHMD/Consult /-2008-09/1169/201.

Assessment of Wound Healing Activity: Evaluation of Wound healing activity has been performed by two different kinds of models: the excision wound inducing model, and the second is the incision wound-induced model.

Excision Wound Induction: The animals were weighed individually, and the skin on the dorsolateral flank area cleaned, before wounding preparation with 70% alcohol, 1cm × 1cm area of skin was measured and cut on the right dorsolateral flank area. The skin wound's resulting area was measured using vernier calipers, which was previously sterilized using 80% alcohol solution. Homeostasis was secured by the application of compound 30 mg per kg bodyweight as interval until complete wound re-epithelialization has taken place. It varies for all treatments [33].

After complete healing, blood samples withdrawn for Biochemical estimations and skin samples of different treatments were stored in -80 °C deep.

Measurement of Wound Contraction Period/ Measurement of Period of Epithelialization in Excision Wound Model: Contraction, which mainly contributes to wound closure, was studied by vernier calipers. The wound is measured on 4^{th}-day 8^{th}-day 12^{th} day and till wounds were completely covered with epithelium [34]. Wound contraction was calculated as a percentage change in the initial wound size *i.e.*

WC (%) = Initial wound size – Specific day wound size / Initial wound size

The epithelialization period was monitored by noticing the number of days required for eschar to fall away, leaving no raw wound area behind [35].

Histopathological Studies: Rats were sacrificed under light ether anesthesia and liver and skin were removed and washed with normal saline. A specimen sample skin tissues from each of the treated groups of rats were taken out from the healed wounds of the animals in the above excision wound model and fixed in neutral buffered 10% formalin. Later paraffin section of 5 micron was made and stained with hematoxylin and Eosine (H & E) stain for histopathological examinations [36].

Incision Wound Induction and Measurement of Tensile Strength: Lightly anesthetize the animal with intraperitoneal injections with ketamine hydrochloride, and clip the hair from the entire trunk. All animals to be studied should first be shaved before proceeding with the wounding protocol. After induction of surgical anesthesia, quickly move each animal on to the operating board, which has been covered with a sterile surgical sheet. Position of animal prostrate and pointed in a caudal direction towards the operator.

Applying 75% alcohol for skin preparation Use the toothed forceps to elevate the starting point of the incision at the lower left posterior flank but 1 cm lateral to the spine. Use a sharp-blunt scissor to make a small cut through the whole thickness of the skin. Insert the blunt tip of the scissor through the cut in to the loose aereolar tissue. Reposition the forceps to hold the tissue at the incision site and "pushcut" the skin with the scissor along the peraspinous longitudinal line until a 4 cm incision is made. Position the animal's head to the left 0f the operator. Grasp both ends of the wound with forceps to create a wound pocket and apply test material if this to be done per the experimental plan.

With one end of the wound under tension, apply to the grasp wound, approximate the wound margin with three evenly spaced cotton bandages. Removal of the bandage after 5 days of wound applying the pure compound twice in a day [37].

TABLE 1: EXCISION AND INCISION WOUND GROUPING FOR EXPERIMENTS

Groups (n=6)	Treatment	Dose of drug
Group-1	Control (negative control)	Normal saline treatment
Group-2	Feramycetin treated group (Standard)	30 mg /Kg b.w.
Group-3	CP-1	30 mg /Kg b.w.
Group-4	CP-2	30 mg /Kg b.w.
Group -5	CP-3	30 mg /Kg b.w.
Group-6	CP-4	30 mg /Kg b.w.
Group-7	CP-5	30 mg /Kg b.w.
Group-8	CP-6	30 mg /Kg b.w.

The drug was applied topically once a day. On the 5^{th} day bandage will be removed, and applied drugs till the wound will cover completely.

Measurement of the Tensile Strength of the Wound: Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists breaks under tension and may indicate the quality of the repaired tissue. For this purpose, the newly repaired tissue, including scar, was measured to excise, to measure the tensile strength [38]. Tensile strength of wound was determined in all groups on day 20^{th} by a texture analyzer.

The rats were lightly anesthetized with light ether. The skin was removed with 1cm on each side of the wound. The tensile strength was measured using a texture analyzer (*TA XT2*), and the increase in tensile strength served as a measure of wound healing. Tensile strength was determined using the following equation [39].

Tensile strength = Breaking load (force) /Area

Area = Thickness × Width

Sample Collection and Tissue Preparations: At the end of complete epithelialization of wound or till wound were completely covered with epithelium, the rats were anesthetized and sacrificed to measure the zero time point parameters. Blood has been collected from the retro-orbital plexus of the rats using capillary tubes, and serum was separated by centrifugation by 5 min at 4000 rpm at 4 °C. Serum from each rat was aliquoted in 0.5 ml fractions and stored at -20 °C.

Extraction and Isolation: The air-dried leaves (2 kg) of *C. procera* were coarsely powdered and extracted exhaustively in a Soxhlet apparatus with methanol for 72 h. The methanolic extract was concentrated under reduced pressure to obtain dark brown viscous mass. A small portion of the extract was analyzed chemically to determine the presence of different chemical constituents. The viscous dark brown mass was adsorbed on silica gel (60-120 mesh) for column chromatography after being dissolved in little quality of methanol for preparation of the slurry. The slurry (200 g) was air-dried and subjected to chromatography over a silica gel column packed in petroleum ether. The column was eluted successively with petroleum ether, a mixture of petroleum ether and chloroform (9:1, 3:1, 1:1, and 1:3), chloroform, and the mixture of chloroform and methanol (99:1, 97:3, 95:5, 92:8, 9:1, 3:1, 1:1, 1:3). Various fractions were collected separately and matched by TLC to check homogeneity. Similar fractions having the same Rf values were combined and crystallized. The isolated compounds were recrystallized to get the pure compounds **Fig. 1**.

***n*-Nonanyl oleiate (1):** Elution of the column with petroleum ether-chloroform (1:1) furnished colourless flakes of 1, recrystallized from acetone-MeOH (1:1), 213 mg (0.49% yield), R_f: 0.51 (petroleum ether -chloroform, 1:1), m. p. 98-100 °C, UV λ_{max} (MeOH): 215 nm (log ε 3.8); IR ν_{max} (KBr): 2926, 2854,1735, 1645, 1463, 1379, 1245,

1167, 1025, 982, 721 cm^{-1}; ^1H NMR (DMSO-d6): δ 5.31 (1H, m, H-9), 5.29 (1H, m, H-10), 4.39 (2H, m, H2-1′), 2.28 (2H, t, J=7.2 Hz, H2- 2), 2.20 (2H, m, H2-8), 1.98 (2H, m, H2-11), 1.67 (2H, m, CH_2), 1.62 (2H, m, CH_2), 1.58 (2H, m, CH_2), 1.51 (2H, m, CH_2), 1.34 (2H, m,CH_2), 1.31 (26H, brs, 13 × CH_2), 0.83 (3H, t, J=6.2 Hz, Me-18), 0.81 (3H, t, J=6.0 Hz, Me-9′); +ve ESIMS *m/z* (rel. Int.): 408 $[M]^+$ (C27H52O2) (31.8).

Lupenyl palmitate (2): Elution of the column with chloroform gave colourless powder of 2, recrystallized from acetone, 312 mg (0.71% yield), Rf 0.61 (petroleum ether – chloroform, 1:1), m. p. 139-140 °C, UV λ_{max} (MeOH): 207 nm (log ε 3.8); IR λ_{max} (KBr): 2924, 2852, 1734, 1645, 1461, 1379, 1247, 1026, 981 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.10 (1H, d, J= 3.5 Hz, H-12), 4.99 (1H, brs, H2-29a), 4.65 (1H, brs, H2-29b), 4.15 (1H,dd, J=5.5, 9.3 Hz, H-3α), 1.76 (3H, brs, Me-30), 1.13 (3H, brs, Me-23), 1.01 (3H, brs, Me-24), 0.78 (3H, brs, Me-25), 0.63 (3H, brs, Me-26), 0.70 (3H, brs, Me-27), 0.57 (3H, brs, Me-28), 2.07 – 1.26 (26H, m, 11 × CH_2; 4 x CH), 2.26 (2H, t, J= 7.2 Hz, H_2-2′), 1.30 (16H, brs, 8 × CH_2), 1.22 (6H, brs, 3 × CH2), 0.81 (3H, t, J=6.0 Hz, Me-16′); ^{13}C NMR (CDCl3): δ 39.20 (C-1), 27.58 (C-2), 80.99 (C-3), 38.42 (C-4), 55.37 (C-5), 18.17 (C-6), 34.36 (C-7), 41.07 (C-8), 50.33 (C-9), 37.78 (C-10), 21.28 (C-11), 118.87 (C-12), 139.82 (C-13), 42.32 (C-14), 29.33 (C-15), 34.16 (C-16), 48.28 (C-17), 48.69 (C-18), 47.98 (C-19), 150.92 (C-20), 31.90 (C-21), 36.68 (C-22), 27.93 (C-23), 21.60 (C-24), 16.48 (C-25), 16.32 (C-26), 14.68 (C-27), 17.68 (C-28), 109.34 (C-29), 20.93 (C-30), 171.01 (C-1′), 42.16 (C-2′), 37.01 (C-3′), 35.56 (C-4′), 29.67 (C-5′), 29.67 (C-6′), 29.67 (C-7′), 29.67 (C-8′), 29.67 (C-9′), 29.65 (C-10′), 29.33 (C-11′), 29.30 (C-12′), 27.02 (C-13′), 23.68 (C-14′), 22.69 (C-15′), 14.08 (C-16′). +ve ESI MS *m/z* (rel.int.): 662 $[M]^+$ ($C_{46}H_{78}O_2$) (21.8).

1-Methyl-4 (2′-n-decanyl)-cyclohex-1-en-5-one: Elution of the column with chloroform-methanol (99:1) gave a colourless powder of 3, recrystallized from acetone, 145 mg (0.33% yield), Rf : 0.89 (chloroform-methanol, 99:1), m.p.: 98-100 °C, U V λ_{max} (MeOH): 238 nm (log ε 1.3); IR νmax (KBr): 2919, 2850, 1710, 1640, 1463, 1378, 1288, 720 cm^{-1}; ^1H NMR (DMSO-d6): δ 5.10 (1H, m, H-2), 2.48 (2H, brs, H2- 6), 2.25 (1H, m, H-4), 1.98 (2H, m, H2-3), 1.61 (1H, m, H-8), 1.55 (3H, brs, Me-7), 1.51 (2H,m, CH2), 1.22 (12 H, brs, 6 x CH2), 0.89 (3H, d, J=6.1 Hz, Me-9), 0.83 (3H, t, J=6.0 Hz, Me-17); ^{13}C NMR (DMSO-d6): δ 192.83 (C-5), 139.22 (C-1), 114.01 (C-2), 50.11 (C-4), 42.31 (C-6), 42.26 (C-3), 37.23 (C-8), 33.79 (C-8), 29.65 (6 × CH_2), 24.68 (Me-7), 22.65 (CH2), 14.06 (Me-9), 13.87 (C-17); +ve ESI MS *m/z* 250 $[M]^+$ ($C_{17}H_{30}O$) (2.5).

Gadoleic acid (4): Elution of the column with chloroform-methanol (19:1) gave a colourless powder of 4, recrystallized from chloroform-methanol (1:1), 132 mg (0.30% yield), R_f 0.40 (chloroform-methanol, 19:1), m.p. 78-80 °C, UV λ_{max} (MeOH), 213 nm (log ε 3.1); IR ν_{max}(KBr): 3240, 2918, 2849, 1709, 1630, 1463, 1377, 1294, 941, 720 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.33 (1H, m, H-9), 5.30 (1H, m, H-10), 2.48 (2H, t, J=7.2 Hz, H2-2), 2.18 (2H, m, H2- 8), 2.14 (2H, m, H2- 11), 1.96 (2H, m, CH_2), 1.52 (2H, m, CH_2), 1.47 (2H, m, CH_2), 1.23 (20H, brs, 10 × CH_2), 0.85 (3H, t, J=6.1 Hz, Me-20); ^{13}C NMR ($CDCl_3$): δ 178.70 (C-1), 130.08 (C-9), 129.77 (C-10), 33.88 (CH_2), 31.95 (CH_2), 29.70 (8 × CH_2), 29.35 (CH_2), 29.25 (CH_2), 29.12 (CH_2), 27.27 (CH_2), 24.77 (CH_2), 22.67 (CH_2), 14.01 (Me-20); +ve ESI MS *m/z* (rel. Int.): 310 $[M]^+$ ($C_{20}H_{38}O_2$) (3.5).

***n*-Capryl arabinopyranoside (5):** Elution of the compound with chloroform-methanol (93:7) gave pale yellow crystals of 5, recrystallized from chloroform-methanol (1:1), 138 mg (0.32 % yield), Rf: 0.91 (chloroform-methanol, 19:1), m.p. 120 °C, U V λ_{max} (MeOH) : 227, 249 nm (log ε 3.1, 3.3), IR ν_{max} (KBr): 3450, 3383, 2924, 2853, 1732, 1462, 1376, 1179, 1071, 908 cm^{-1}; ^1H NMR (DMSO-d6): δ 5.03 (1H, d, J= 6.5, H-1′), 4.67 (IH, m, H-4′), 3.99 (1H, m, H-2′), 3.54 (IH, m, H-3′), 3.20 (2H, d, J=8.5 Hz, H2-5′), 2.41 (2H, t, J= 7.5 Hz, H2-2), 1.90 (2H, m, CH_2), 1.71 (2H, m, CH_2), 1.53 (2H, m, CH_2), 1.29 (8 H, brs, 4 × CH_2), 0.87 (3H, t,J=6.1 Hz, Me-10); ^{13}C NMR ($CDCl_3$): δ 171.81 (C-1), 103.15 (C-1′), 70.11 (C-2′), 67.42 (C-3′), 65.77 (C-4′), 63.23 (C-5′), 34.01 (C-2), 31.88 (CH2), 30.18 (CH_2), 29.65 (CH2), 29.31 (CH2), 27.17 (CH2), 25.61 (CH2), 22.67 (CH2), 14.11 (Me-10); +ve ESI MS *m/z* (rel.int.): 304 $[M]^+$ ($C_{15}H_{28}0_6$) (2.5).

β-Sitosterol diglucosyl palmitate (6): Elution of the column with chloroform-methanol (9:1) yielded colourless powder of 6, recrystallized from

methanol, 123 mg (0.28% yield), R_f 0.88 (chloroform-methanol, 9:1), m.p. 160-161 °C, UV λ_{max} (MeOH): 214 nm (log ε 5.1); IR ν_{max} (KBr) : 3490, 3416, 3350, 3265, 2929, 2850, 1735, 1640, 1456, 1377, 1261, 1167, 1066, 1026, 720 cm^{-1}; ^1H NMR (DMSO-d6): δ 5.30 (1H, m, H-6), 5.05 (1H, d, J=7.8 Hz, H-1′), 4.96 (1H, d, J=7.2 Hz, H-1″), 4.41 (1H, m, H-5′), 4.33 (1H, m, H-5″), 4.27 (1H, m, H-2″), 4.21 (1H, m, H-2′), 3.79 (1H, m, H-3′), 3.70 (1H, m, H-3″), 3.65 (1H, brm, w1/2 = 16.5 Hz, H-3α), 3.36 (2H, m, H-4′, H-4″), 3.15 (1H, d, J=12.0 Hz, H2- 6′a), 3.11 (1H, d, J=12.0 Hz, H2-6′b), 3.08 (1H, d, J= 11.2 Hz, H2-6″a), 3.05 (1H, d, J=11.2 Hz, H2-8″b), 2.48 (2H, t, J=7.2 Hz, H2-2‴), 2.25 - 1.21 (24 H, brs, 12 × CH_2), 1.01 (3H, brs, Me-19), 0.93 (3H, d, J=6.1 Hz, Me-21), 0.89 (3H, d, J=6.0 Hz,

Me-26), 0.87 (3H, d, J=6.3 Hz, Me-27), 0.80 (3H, d, J=5.8 Hz, Me-29), 0.78 (3H, t, J=6.2 Hz, Me-16‴), 0.67 (3H, brs, Me-18); ^{13}C NMR (DMSO-d6): δ 36.52 (C-1), 31.90 (C-2), 71.86 (C-3), 41.90 (C-4), 141.96 (C-5), 116.41 (C-6), 31.66 (C-7), 33.94 (C-8), 51.24 (C-9), 37.26 (C-10), 21.07 (C-11), 39.76 (C-12), 42.30 (C-13), 56.87 (C-14), 24.17 (C-15), 28.67 (C-16), 55.96 (C-17), 11.99 (C-18), 19.41 (C-19), 36.68 (C-20), 18.79 (C-21), 34.03 (C-22), 27.08 (C-23), 45.83 (C-24), 27.28 (C-25), 19.83 (C-26), 18.99 (C-27), 23.11 (C-28), 11.87 (C-29), 103.62 (C-1′), 75.04 (C-2′), 73.25 (C-3′), 69.54 (C-4′), 75.51 (C-5′), 60.94 (C-6′), 94.81 (C-1″), 77..61 (C-2″), 73. 44 (C-3″), 68.26 (C-4″), 75.16 (C-5″), 60.37 (C-6″), 173.21 (C-1‴), 37.67 (C-2‴), 32.86 (C-3‴), 31.22 (C-4‴), 29.87 (C-5‴), 29.61 (C-6‴), 29.46 (C-7‴), 29.31 (C-8‴), 29.25 (C-9‴), 29.25 (C-10‴), 29.25 (C-11‴), 27.38 (C-12‴), 22.68 (C-13‴), 14.12 (C-14‴);; +ve ESI MS m/z (rel. Int.): 978 $[M]^+$ ($C_{57}H_{100}O_{12}$) (51.3), 563 (14.1), 413 (23.5), 325 (4.8), 255 (21.1), 239 (9.7), 163 (12.6).

Hydrolysis of Compound-6: Compound-6 (35 mg) was dissolved in ethanol (5 ml), dil HCL (3 ml) added and the reaction mixture refluxed for 1 hr. The solvent was evaporated under reduced pressure and the residue was dissolved in petroleum ether to separate palmitic acid, m.p. 61-62 °C, co-TLC comparable. The residue was redissolved in $CHCl_3$ to isolate β-sitosterol, m.p. 136-137 °C, co –TLC comparable. The residue was again dissolved in water and chromatographed on SiO_2 TLC using n-BuOH-AcOH-H_2O (4:1:5) as a developing solvent. The sugar was identified and D-glucose, R_f 0.12.

RESULTS AND DISCUSSION: Compound-1, named *n*-nonanyl oleiate, was obtained as colorless flakes from petroleum ether-chloroform (1:1) eluent. Its IR spectrum showed characteristic absorption bands for ester group (1735 cm^{-1}), unsaturation at (1645 cm^{-1}), and long aliphatic chain (721 cm^{-1}). The mass spectrum of Compound-1 exhibited a molecular ion peak at *m/z* 408 $[M]^+$ corresponding to a molecular formula of a fatty acid ester, $C_{27}H_{52}O_2$. The ^{1} H NMR spectrum of 1 displayed two one-proton multiplets at δ 5.31 and 5.29 assigned to vinylic H-9 and H-10 protons, respectively. A two-proton multiplet at δ 4.39 was ascribed to oxygenated methylene H2-1′ protons. A two-proton triplet at δ 2.28 (J=7.2 Hz) was attributed to methylene H2-2 protons adjacent to the ester group. Two-three- proton triplets at δ 0.83 (J=6.2 Hz) and 0.81 (J=6.0 Hz) were accounted to terminal C-18 and C-9′ primary methyl protons, respectively. The remaining methylene proton signals appeared between δ 2.20-1.31. Acid hydrolysis of Compound-1 yielded oleic acid. Based on this evidence, the structure of Compound-1 was established as *n*-nonanyl octadec-9-enoate, a new fatty acid ester.

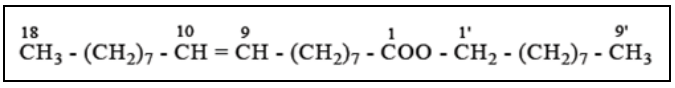

FIG. 1: COMPOUND-1 N-NONANYLOLEIATE 1

Compound-2, named lupenyl palmitate, was obtained as a colorless powder from chloroform (1:3) eluents. Its IR spectrum exhibited characteristic absorption bands for the ester group at 1734 cm^{-1} and unsaturation at 1645 cm^{-1}. Its mass spectrum displayed a molecular ion peak at m/z 662 $[M]^+$ consistent with the molecular formula of a triterpenic ester, $C_{46}H_{78}O_2$. The ^1H NMR spectrum of Compound-2 showed a one-proton doublet at δ 5.10 (J=3.5 Hz) assigned to vinylic H-12 proton. Two one-proton broad singlets at δ 4.99 and 4.65 were ascribed to unsaturated methylene H2-29 protons of a lupene -type molecule. A three- broad proton singlet at δ 1.76 was attributed to C-30 methyl protons attached to the vinylic C-20 carbon. Six three-protons broad singlets at δ 1.13, 1.01, 0.78, 0.63, 0.70, and 0.57

were associated with the tertiary C-23, C-24, C-25, C-26, C-27, and C-28 methyl protons, all attached to the saturated carbons. A three-proton triplet at δ 0.81 (J=6.0 Hz) was accounted to C-16′ primary methyl protons. A one-proton double doublet at δ 4.15 with coupling interactions of 5.5 and 9.3 Hz was due to oxygenated C-3 methine proton, and its shifting in the deshielded region supported the attachment of the ester function at C-3. The remaining methylene and methine protons resonated from δ 2.26 to 1.22. The ^{13}C NMR spectrum of Compound-2 displayed signals for ester carbon at δ 171.01 (C-1′), vinylic carbons at δ 118.87 (C-12), 139.82 (C-13), 150.92 (C-20) and 109.34 (C-29), oxygenated methine carbon at δ 80.99 (C-3), methyl carbons from δ 27.93 to 14.08 and remaining methine and methylene carbons from δ 55.37 to 21.28.

The ^{1}H and ^{13}C NMR values of the triterpenic unit of Compound-2 were compared with the related compounds [40-42]. Acid hydrolysis of Compound-2 yielded palmitic acid and lup-12, 20 (29)- dien-3β-ol, co-TLC comparable. On the basis of spectral data analysis and chemical reactions, the structure of Compound-2 was formulated as lup-12, 20 (29)-dien-3β-olyl hexadecanoate, a known triterpenic ester isolated from *C. procera* for the first time.

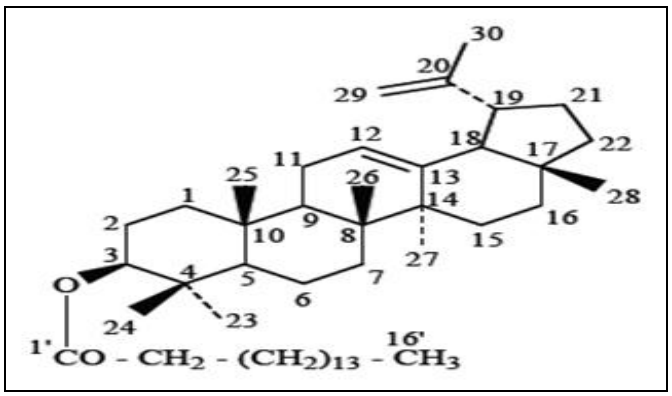

FIG. 2: COMPOUND-2 LUPENYL PALMITATE 2

Compound-3, a cyclohexenone derivative, was obtained as a colourless powder from chloroform-methanol (99:1) eluents. Its IR spectrum showed characteristics absorption bands for keto group (1710 cm^{-1}), unsaturation (1640 cm^{-1}) and aliphatic chain (720 cm^{-1}). On the basis of mass and ^{13}C NMR spectra the molecular weight of Compound-3 was established at *m/z* 250 $[M]^{+}$ consistent with a molecular formula of a cyclohexenone derivative, $C_{17}H_{30}O$.

It indicated three double bond equivalents; one each was adjusted in the cyclohexyl ring, venylic linkage, and carbonyl function. The ^{1}H NMR spectrum of Compound-3 showed a one- proton multiplet at δ 5.10 assigned to vinylic H-2 proton. A two-proton broad singlet at δ 2.48 and a one-proton multiplet at δ 2.25 were attributed to methylene H2-6 and methine H-4 protons, respectively, linked to C-5 carbonyl carbon. A three-proton broad singlet at δ 1.55 was ascribed to C-7 methyl protons attached to the C-1 vinylic carbon. A three-proton doublet at δ 0.89 (J=6.1 Hz) and a three- proton triplet at δ 0.83 (J=6.0 Hz) were associated with secondary C-9 and primary C-17 methyl protons, respectively. The remaining methine and methylene protons appeared from δ 1.98 to 1.22. The ^{13}C NMR spectrum exhibited signals for vinylic carbons at δ 139.22 (C-1) and 114.01 (C-2), carbonyl carbon at δ 192.83 (C-5), methyl carbons at δ 24.68 (Me-7), 14.06 (Me-9) and 13.87 (Me-17), methine carbons at δ 50.91 (C-4) and 37.23 (C-8) and methylene carbons from δ 42.31 to 22.65. On the basis of the spectral data analysis, the structure of Compound-3 has been established as 1-methyl-4 (2′-*n*-decanyl)-cyclohex-1-en-5-one. It is a new cyclohexenone derivative.

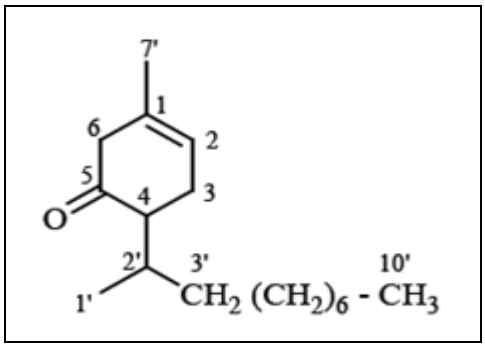

FIG. 3: COMPOUND-3 1-METHYL-4(2'-N-DECANYL)-CYCLOHEX-1-EN-5-ONE 3

Compound- 4, a known fatty acid characterized as gadoleic acid (eicos-9-enoic acid).

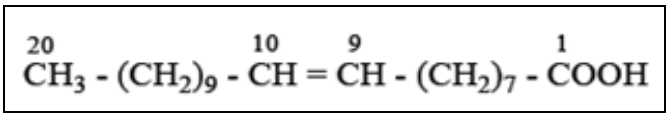

FIG. 4: COMPOUND-4 GADOLEIC ACID 4

Compound 5, named *n*-caprylarabinoside, was obtained as a pale yellow crystalline mass from chloroform-methanol (97: 3) eluents. It responded to glycoside tests positively. Its IR spectrum showed distinctive absorption bands for hydroxyl

groups (3450, 3383 cm^{-1}) and ester function (1732 cm^{-1}). On the basis of the mass spectrum, its molecular ion peak was established at m/z 304 $[M]^+$ corresponding to the molecular formula of a fatty acid glycoside, $C_{15}H_{28}O_6$. The ^1H NMR spectrum of 5 exhibited a one- proton doublet at 5.03 with coupling interaction of 6.5 Hz assigned to anomeric α- H-1′ proton. Three one- proton multiplets at δ 4.67, 3.99 and 3.54 and a two-proton doublet at δ 3.20 (J=8.5 Hz) were ascribed to oxygenated methine H-4′, H-2′ and H-3′, and methylene H2-5′ protons, respectively. A two-proton triplet at δ 2.41 (J=7.5 Hz) was attributed to methylene. H2-2 protons adjacent to the ester function. Three two-proton multiplets at δ 1.90, 1.71 and 1.53 and a broad singlet at δ 1.29 (8 H) were associated with the methylene protons of the fatty acid chain. A three- proton triplet at δ 0.87 (J=6.1 Hz) was accounted to terminal C-10 primary methyl protons. The ^{13}C NMR spectrum of Compound-5 showed signals for ester carbon at δ 171.81 (C-1), anomeric carbon at δ 103.15 (C-1′), other sugar carbons between δ 70.11 – 63.23, methyl carbons from δ 34.01 to 22.67 and methyl carbon at δ 14.11 (C-10). Acid hydrolysis of 5 yielded capric acid and α-L-arabinoside, Rf 0.18 [*n*- butanol – acetic acid-water (4:1:5)]. Based on this evidence, the structure of **5** has been determined as *n*-decanoyl-α-L-arabinopyranoside.

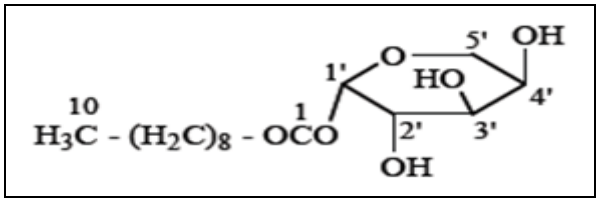

FIG. 5: COMPOUND-5 N-CAPRYLARABINOSIDE

Compound-6, named β-sitosterol diglucoside palmitate, was obtained as a colourless powder from chloroform-methanol (9:1) eluents. It gave positive tests for glycosides. Its IR spectrum displayed characteristics absorption bands for hydroxyl groups (3490, 3416, 3350, 3265 cm^{-1}), ester function (1735 cm^{-1}), unsaturation (1640 cm^{-1}) and a long aliphatic chain (720 cm^{-1}). On the basis of ESI mass spectrum, the molecular weight of Compound-6 was determined at m/z 977 $[M+H]^+$ corresponding to the molecular formula of a steroidal diglucosidic ester $C_{57}H_{101}O_{12}$. The ^1H NMR spectrum of Compound-6 showed a one-broad proton signal at δ 5.30 assigned to vinylic H-6 proton. Two one- proton doublets at δ 4.90 (J=7.8 Hz) and 4.85 (J=7.0 Hz) were ascribed to anomeric H-1' and H-1'' protons, respectively. A one- proton broad multiplet at δ 3.65 with half-with of 16.5 Hz was attributed to oxygenated α-oriented H-3 methine proton. Four one - proton doublets at δ 3.15 (J=12.0 Hz), 3.11 (J=3.11 Hz) and at 3.08 (J=11.2 Hz) and 3.05 (J=11.2 Hz) were due to hydroxyl methylene protons, H_2-6' and H_2-6'', respectively. The remaining sugar protons appeared between δ 4.41-3.36. Two three-proton broad signals at δ 1.01 and 0.67 and three doublets at δ 0.93 (J=6.1 Hz), 0.89 (J=6.0 Hz), and 0.87 (J=6.3 Hz) integrating for three-protons each were accounted to C-21, C-26, and C-27 secondary methyl protons, respectively. A three-proton doublet at δ 0.80 (J=5.8 Hz) and a three- proton triplet at δ 0.78 (J=6.2 Hz) were accommodated to primary C-29 and C-16''' methyl protons, respectively. A two- broad proton signals at δ 2.48 was due to C-2''' methylene protons adjacent to the ester function. The remaining methine and methylene protons resonated from δ 2.25 to 1.21.

The appearance of the sugar protons in the deshielded region at δ 4.27 (m, H-2'') and 4.21 (dd, J=7.8, 6.2 Hz H-2') indicated the attachment of the ester group at C-2'' and second sugar unit at C-2'. Acid hydrolysis of CP-6 yielded β-sitosterol, β-D-glucose and palmitic acid.

On the basis of spectral data analysis and chemical reactions, the structure of Compound-6 has been formulated as stigmast –5-en-3β-ol-3 β-D-glucopyranosyl(12)-β-D-glucopyranoside-2''-hexa-decanoate. It is new steroidal glucoside.

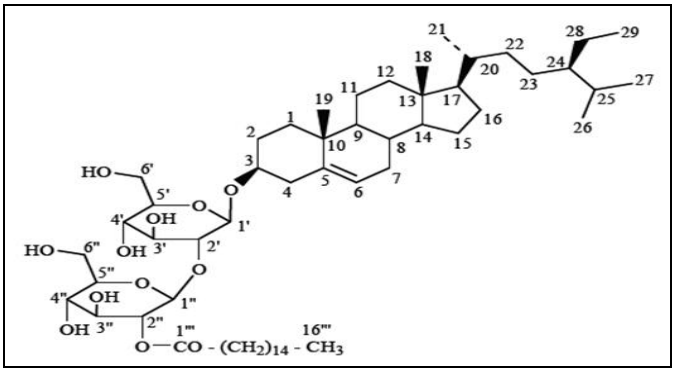

FIG. 6: COMPOUND-6 β-SITOSTEROL DIGLUCOSIDE PALMITATE

Table 1: Post wounding reduction in percentage and period of epithelization of six isolates**.**

Compound-1 showed a contraction of the wound, which was found to be much faster as compared to control, a period of epithelization was observed significantly reduced from eighteen to twelve days. Compound-2 treated group was observed good wound contraction as compared to control. The epithelization period significantly decreased from eighteen to thirteen-day; Compound-3 demonstrated a period of epithelization was seventeen days, Compound-4 showed average wound contraction, and the period of epithelization was observed to significantly reduce from eighteen days to seventeen days. Compound-5 treated group showed very slow wound contraction, a period of epithelization was found to eighteen to nineteen days similar to control group, and compound Compound-6 was found excellent healer, similar to Compound-2 and the epithelization period significantly decreased from eighteen to twelve days, and the period epithelization of the six isolates and control-treated groups was also observed as compared to the standard group.

TABLE 1: POST WOUNDING REDUCTION IN PERCENTAGE AND PERIOD OF EPITHELIZATION OF SIX ISOLATES

Treatments	Post wounding reduction in the percentage														
	0 days	4th day		8th day		12th day		14th day		16th day		18th day		Period of epithelization	
	Mean	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
Control	0.00	0.83	0.31	13.00	1.86	54.17	2.21	72.67	2.19	90.00	2.21	97.50	1.50	17.83	0.31
Standard	0.00	5.33	2.12	26.33	3.27	68.83	1.54	94.17	2.17	99.00	0.00	99.00	0.00	14.00	0.26
Compound-1	0.00	16.00	1.46	45.50	2.62	90.00	3.29	99.00	0.00	99.00	0.00	99.00	0.00	12.00	0.26
Compound-2	0.00	6.83	2.20	24.17	1.60	69.50	2.75	92.50	2.06	99.00	0.00	99.00	0.00	13.00	0.52
Compound-3	0.00	1.33	0.21	11.17	2.10	50.67	2.40	83.50	2.23	94.50	2.01	99.00	0.00	17.50	0.43
Compound-4	0.00	15.00	1.79	33.83	0.48	72.17	3.21	96.50	1.52	99.00	0.00	99.00	0.00	13.67	0.42
Compound-5	0.00	2.50	0.20	5.2	0.10	15.23	1.20	30.12	2.32	65.30	1.76	85.06	2.04	18.26	0.31
Compound-6	0.00	10.02	1.34	45.32	2.10	95.20	1.02	100	0.00	100	0.00	100	0.00	12.52	0.20

Values are expressed as mean ± S.E. for six animals. Compared with the corresponding control. CP-1, CP-2, CP-6 treated group more significant as compared to others. Data represented by similar letter are not significantly different at $p \leq 0.05$ according to DMRT.

Table 2: showed the Tensile strength of all six isolates. Tensile strength of control, Compound-1, Compound-2, and Compound-6 treated animals on the twentieth day healed tissue, results indicate a significant increase in healing of Compound-1, Compound-2, Compound-4, and Compound-6 treated groups as compared with control. A slight decrement was observed in Compound-3, and Compound-5 treated groups.

After complete epithelialization after twelfth, fourteenth, sixteenth eighteenth day. The activity of serum enzyme ALT and AST was measured, and the liver was found normal.

TABLE 2: TENSILE STRENGTH OF ALL SIX ISOLATES

Treatments	Mean (grams)	SE ±
Control	2585.02[f]	311.00
Standard	8654.24[b]	522.42
Compound-1	11104.35[a]	1554.78
Compound-2	8446.19[c]	467.87
Compound-3	5237.68[d]	605.09
Compound-4	8167.09[e]	506.11
Compound-5	2123.06[f]	212.01
Compound-6	11120.32[a]	1432.3

Values are expressed as mean ± S.E. for six animals. Compared with corresponding control. CP-1, CP-2, CP-6 treated group more significant as compared to others. Data represented by similar letter are not significantly different at $p \leq 0.05$ according to DMRT

CONCLUSION: Phytochemical investigation of a methanolic extract of the leaves of *Calotropis procera* resulted in the isolation of one each of fatty ester, lupenyl palmitate, alkyl cyclohexenone, fatty acid, capryl arabinoside, and steryl diglucosyl palmitate. All these compounds were isolated from the plant for the first time. This work has enhanced my understanding of the phytoconstituents of the plant. These compounds may be used as chromatographic markers for the standardization of the plant leaves. Further research work relating to bioactivities of the isolated phytoconstituents and leaf extracts should be carried out to justify the medicinal properties of the plant.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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