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Phytochemical and Biological Study of the Aerial Parts of *Chrozophora oblongifolia* (Delile) Spreng. (Euphorbiaceae)

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Abstract
Phytochemical investigation of the aerial parts of *Chrozophora oblongifolia* afforded four compounds isolated for first time from the species including: 1-octacosanol (1), lupeol (2), para-hydroxybenzoic acid (3) and methyl gallate (4), in addition to previously isolated compound, amentoflavone (5), were isolated from methylene chloride and ethyl acetate subfractions of methanolic extract of *C. oblongifolia* aerial parts. Their structures were elucidated on the basis of different physical, chemical and spectral techniques. The total extract and its subfractions showed variable in vitro antiviral activities against Herps Simplex Virus (HSV-1), where the methanol subfraction showed highest antiviral activity (68.1%) and *n*-hexane subfraction showed lowest antiviral activity (25.1%). Total extract and methanol subfraction showed significant wound healing activity on excision wound model in albino rats.

Keywords: Phytochemical; *Chrozophora oblongifolia*; Antiviral and Wound healing

1. Introduction
Euphorbiaceae, the spurge family, is a large family of flowering plants with 300 genera and around 7,500 species. Most spurges are herbs, but some, especially in the tropics, are shrubs or trees [(1)]. Genus *Chrozophora* is a large genus belonging to family Euphorbiaceae with different species which possess different biological activities as *C. oblongifolia* which possess antioxidant and antimicrobial activity [(2)], *C. hierosolymitana* which possess antifungal and antiyeast activities [(3)], *C. rottleri* is traditionally used by the tribes and native medical practitioners for the treatment of various diseases, in Sudan, powdered stems or whole plant are applied to wounds to improve healing, in Ethiopia, an infusion of the seeds and leaves is taken as a laxative. The plant is also used medicinally in Saudi Arabia, Pakistan and India (e.g. against jaundice and purifying blood), in Senegal, the plant is not browsed by most stock, except occasionally by sheep and goats, as it causes vomiting and diarrhea, where as in Kenya, camels graze it, in Nepal, juice of the fruit is given in cases of cough and colds, purifying agent (leaf) and laxative (seed), the leaves are very much beneficial in treating skin diseases and also used as a depurative agent [(4)], On reviewing the available current literatures a few report on phytochemical and biological investigation on the plant *C. oblongifolia* have been obtained, and this enhances the interest of the phytochemical and biological investigation of the plant.

2. Material and methods
2.1. Instruments and Chemicals
EI-MS was measured on JEOL JMS 600 Hz (Japan) and Shimadzu Qp-2010 plus (Japan). NMR analysis (^{1}H-NMR, ^{13}C-NMR and DEPT) were measured on Bruker Mercury-VX-400 MHz spectrometer (Germany), Varian Mercury VX-300 MHz spectrometer (USA) and JEOL TNM-LA-400 MHz spectrometer (Japan) using TMS as internal standard. Column chromatography was carried on silica gel (70-230, mesh, E-Merck, Germany), Sephadex LH-20 (Fluka, 25-100µm, Sigma-Aldrich chemicals, Switzarland), TLC was carried on precoated silica gel plates G_{60} F_{254} (E-Merck, Germany). The plates were examined under UV light at (365 and 254 nm). The spots are sprayed with 10% *v/v* H_2 SO_4 in MeOH and heated at 110-140 ^{0}C till maximum spot intensity. Authentic reference materials lupeol and 4-hydroxybenzoic acid were obtained from Pharmacognosy Departement, Fculty of Pharmacy,

Al-Azhar University, Assuit Branch, Assuit, Egypt. The following solvent systems were used for TLC:

1. Methylene chloride-methanol (95: 5 v/v)
2. *n*-hexane - ethyl acetate (80:20 v/v)
3. Methylene chloride-methanol (93:07 v/v)
4. Methylene chloride-methanol (90:10 v/v)

All solvent used are of analytical grade.

2.2 Materials for antiviral activity

2.2.1 Instruments

Optical density data were acquired using ELISA reader (start fax-2100/USA). ELISA incubator (Thermostar ELISA incubator/USA) and Jouan CO2 incubator/France).

2.2.2. Chemicals

Dimethyl sulfoxide (DMSO) (SDS/France), Minimum essential media (MEM) (Caisson/USA), Trypsin-EDTA 1:250 U (Molekulu/UK) and (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) (MTT) (Biobasic/Canada).

2.2.3. Cells and viruses

VERO cell line (African green monkey kidney cells) and Herpes simplex virus type-1 (HSV-1) are obtained from Al-Azhar University Center for Viral Research and Studies.

2.3 Materials for wound healing activity

2.3.1. Chemicals

Thiopental sodium injection (500mg) obtained from Egyptian International Pharmaceutical Industry Co (E.I.P.I.Co). NaCl (0.9%) obtained from (El-Nasr Pharmaceutical and Chemical Co., Egypt) (ADWIC). Gentamicin ointment (5%) obtained from Memphis Co for Pharmaceutical industries. Bees wax and white soft paraffin obtained from (El-Nasr Pharmaceutical and Chemical Co., Egypt) (ADWIC).

2.3.2. Animals

The healthy Wistar albino rats (20 rat) of either sex, weighing 150–200 g, were housed under standard environmental conditions of temperature and humidity (25±0.50 ◦C) and 12 h light/dark cycle) were utilized for the studies. The animals were fed with standard pellet diet and water. The rats were divided into four groups (5 rats in each).

2.3.3. Preparation of test ointments

Total extract (T1) and methanol fraction (T2) of C. oblongifolia aerial parts are incorporated into simple ointment base in concentrations of 5% w/w and simple ointment base served as control preparation in this study. Gentamicin ointment used as reference standard for comparison with tested ointments (total extract, methanol fraction and simple ointment base) (5).

2.4. Plant material

The aerial parts of *C. oblongifolia* were collected in March 2013 from Saint Catherin, South Siniai, Egypt. The plant identified and Authenticated by Prof. Dr. Salah El-Naggar, Professor of Botany and Plant Taxonomy, Faculty of Science Assuit University, Assuit-Egypt. A voucher specimen (COE-1) was kept in the Department of Pharmacognosy, Fculty of Pharmacy, Al-Azhar University, Assuit, Egypt.

3. Extraction and isolation

The air-dried powdered aerial parts (4 Kg) of *C. oblongifolia* were extracted by maceration in methanol (70%) till complete exhaustion [three times each 8 L, overnight]. The methanolic extracts were concentrated under reduced pressure till constant weight to give a dark brown syrupy residue (430g). A part of the methanolic extract (400 g) was subjected to successive solvent fractionation on VLC with *n*-hexane, methylene chloride (MC), ethyl acetate (EtOAC), methanol (MeOH) and finally with aqueous methanol till complete exhaustion in each case to give *n*-hexane (30 g), MC (5 g), EtOAC (30 g), MeOH (180 g) and aqueous methanol (70 g) sub fractions.

A part of the MC soluble fraction (4 g) was chromatographed over silica gel column chromatography (160 g), eluted with *n*-hexane (100%), *n*-hexane-MC gradient elution, MC (100%) and finally eluted with MeOH (100%). Fractions 100 ml, each were collected. Similar fractions were grouped and pooled together, concentrated under reduced pressure to give 7 subfractions labeled (MCF 1) to (MCF 7). Subfraction (MCF 2) was rechromatographed on silica gel column chromatography and eluted with *n*-hexane followed by *n*-hexane-EtOAC gradient elution from (99:1) to (80:20), the fractions eluted with *n*-hexane- EtOAC (98:2) afforded compound 1 (30 mg). Subfraction (MCF 5) was rechromatographed on silica gel chromatography and eluted with *n*-hexane-EtOAC gradient elution from (98:2) to (80:20), the fractions eluted with *n*-hexane-EtOAC (93:7) afforded compound 2 (40 mg).

A part of the EtOAC-soluble fraction (20 g) was chromatographed over silica gel column chromatography (800 g), eluted with *n*-hexane (100%), *n*-hexane-EtOAC gradient elution from (99:1) to (75:25), EtOAC (100%) and finally eluted with MeOH (100%). Fractions 100 ml, each were collected. Similar fractions were grouped and pooled together, concentrated under reduced pressure to give 10 subfractions labeled (EAF 1) to (EAF 10). Subfraction (EAF 5) was rechromatographed on silica gel column chromatography and eluted with MC- methanol gradient elution from (99:1) to (85:15), the fractions eluted with MC- MeOH (93:7) was collected to give (EAF 5') fraction (130 mg), which was further rechromatographed on sephadex LH-20 using MC-MeOH (1:1). Fractions 5 - 12 were collected and concentrated under reduced pressure, afforded compound 3 (20 mg). Subfraction (EAF 6) was rechromatographed on silica gel column chromatography and eluted with MC- MeOH gradients(98:2) to (80:20), the fractions eluted with MC-MeOH (92:8) was collected to give (EAF 6') fraction (85 mg), which was further rechromatographed on sephadex LH-20 using MC-MeOH (1:1). Fractions 4-7 were collected and concentrated under reduced pressure, afforded compound 4 (15 mg). Subfraction (EAF 7) was rechromatographed on silica gel column chromatography and eluted with MC-MeOH gradient elution from (98:2) to (85:15), the fractions eluted with MC- MeOH (90:10) afforded compound 5 (15 mg).

Compound (1): Obtained as white crystals from MeOH (30 mg), m.p 83 0C. Rf = 0.68 (system, I) EI MS showed peak at m/z 392 [M-H2O] +, other peaks at m/z 378 [M-H2O-CH2]+, 364 [M-H2O-C2H4]+, 336 [M-H2O-C4H8]+, 308 [M-H2O-C6H12]+, 168 [M-H2O-C16H32]+ and 42 [M-H2O-C25H50]+ were identified. 1H-NMR (CD Cl3, 400 MHz): δH 0.87 (3H, t, H-28), 1.22-1.29 (48H, br.s. H-3 to H-27), 1.56 (2H, m, H-2) and 3.63 (2H, t, H-1). 13C NMR (CD Cl3, 100

MHz): δc 14.08 (C-28), 22.65 (C-27), 25.68 (C-3), 29.32–29.66 (CH2 residue), 31.88 (C-26), 32.77 (C-2) and 63.08 (CH2-OH).

Compound (2): Obtained as white needle crystals from acetone (40 mg), m.p. 215-216 0C. Rf = 0.87 (system, II). 1H-NMR (CD Cl3, 400 MHz): δH 0.76 (3H, s, H-28), 0.80 (3H, s, H-27), 0.91 (3H, s, H-23), 0.94 (3H, s, H-25), 1.00 (3H, s, CH3-26), 1.1 to 1.6 (br.s. methylene & methane groups), 1.24 (3H, s, H-24), 1.67 (3H, s, H-30), 2.39 (1H, m, H-19), 3.18 (1H, m, H-3), 4.54 (1H, br. s, H-29b) and 4.66 (1H, br.s. H-29a). 13C NMR (CD Cl3, 100 MHz): δc 38.68 (C-1), 27.42 (C-2), 78.97 (C-3), 38.84 (C-4), 55.26 (C-5), 18.29 (C-6), 34.25 (C-7), 40.80 (C-8), 50.41 (C-9), 37.15 (C-10), 20.91 (C-11), 25.10 (C-12), 38.02 (C-13), 42.81 (C-14), 27.39 (C-15), 35.55 (C-16), 42.97 (C-17), 48.27 (C-18), 47.96 (C-19), 150.96 (C-20), 29.83 (C-21), 39.98 (C-22), 27.97 (C-23), 15.35 (C-24), 16.10 (C-25), 15.95 (C-26), 14.52 (C-27), 17.98 (C-28), 109.30 (C-29) and 19.28 (C-30).

Compound (3): Obtained as white crystals from MeOH (20 mg), m.p. 214–2150C. Rf = 0.58 (system, III). EI-MS showed the molecular ion peak at m/z 138. 1H-NMR (CD3 OD, 300 MHz): δH 6.82 (2H, d, J=6.9 Hz, CH2-3 and CH2-5) and 7.88 (2H, d, J=6.9 Hz, H-2 and H-6).

Compound (4): Obtained as white amorphous powder from MeOH (40 mg), m.p. 200–202 oC. Rf = 0.55 (system, IV). EI-MS showed the molecular ion peak at m/z 184 [M] +, other peaks at m/z 153 [M-OCH3]+ and 124.9408 [M-COO-CH3]+ . 1H-NMR (CD3 OD, 400 MHz): δH 3.71 (3H, s, H-8) and 6.94 (2H, s, H-2 and H-6). 13C NMR (CD3 OD, 100 MHz): δC 120.03 (C-1), 108.63 (C-2 and C-6), 145.10 (C-3 and C-5), 138.0 (C-4), 167.62 (C-7) and 50.85 (C-8).

Compound (5): Obtained as yellow amorphous powder from MeOH (30 mg) m.p 346-348 0C. Rf = 0.42 (system, IV). EI-MS (positive mode) showed the molecular ion peak at m/z 539. 1H-NMR (CD3 OD, 400 MHz): δH 6.18 (1H, d, J= 2 Hz, H-6), 6.37 (1H, s, H-6''), 6.42 (1H, d, J= 2 Hz, H-8), 6.58 (2H, d, J= 3.8 Hz, H-3''' and H-5'''), 6.71 (1H, s, H-3'''), 6.74 (1H, s, H-3), 7.10 (1H, d, J= 8.6 Hz, H-5'), 7.50 (2H, d, J= 8.8 Hz, H-2''' and H-6'''), 7.88 (1H, d, J= 2.3 Hz, H-2'), 7.94 (1H, d, J= 2.3 Hz, H-6'). 13C NMR (CD3 OD, 100 MHz): δC 164.77 (C-2), 102.61 (C-3), 182. 40 (C-4), 161.77 (C-5), 98.56 (C-6), 164.62 (C-7), 93.74 (C-8), 159.53 (C-9), 104.01 (C-10), 121.81 (C-1'), 127.53 (C-2'), 121.81 (C-3'), 161.10 (C-4'), 115.94 (C-5'), 131.38 (C-6'), 164.77 (C-2''), 101.98 (C-3''), 182.83 (C-4''), 161.14 (C-5''), 98.77 (C-6''), 164.54 (C-7''), 103.92 (C-8''), 155.06 (C-9''), 104.01 (C-10''), 120.14 (C-1'''), 127.89 (C-2'''), 115.43 (C-3'''), 162.08 (C-4'''), 115.43 (C-5''') and 127.89 (C-6''').

4. Biological study

4.1. Antiviral activity

Antiviral activities of total extract and subfractions of aerial parts of *C. oblongifolia* are determined through the following sequences.

Cytotoxicity assay

Cytopathogenic effect assay method: detection of any sign of cell toxicity such as (loss of monolayer sheet, granulation and vacuolization in the cytoplasm (6). The dried total extract in addition to different fractions (*n*-hexane, methylene chloride, ethyl acetate and methanol) (100mg of each) of the aerial parts of C. oblongifolia was dissolved in 1 ml DMSO. Growth medium was decanted from 96 well micro titer plates after confluent sheet of VERO cell was formed, cell monolayer was washed twice with wash media, then about 1 ml of wash media was added and the plates were incubated at room temperature for 5-10 minutes. Ten-fold serial dilutions of total extract and different fractions were made in MEM, starting from 100 (mg/ml) till 10-6 dilution. 0.2 ml of each dilution was tested in different wells leaving 6 wells as control, receiving only maintenance medium. Plate was incubated at 37 °C and examined frequently for up to 3 successive days. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding and shrinkage or cell granulation. The maximum non-toxic concentration [MNTC] of each extract was determined which can be defined as the dilution of extract at which, by microscopic examination, cells showed normal morphology and cell density when compared with control cells grown without extract.

Antiviral assay

This is the colourimetric assay (8). In this assay water soluble Yellow MTT [3-(4, 5- Dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide, a tetrazole] is reduced to insoluble purple formazan in the mitochondria of living cells. This reduction based on the mitochondria succinate dehydrogenase enzymes activity. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength by a spectrophotometer. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance (7). Plate 10,000 cells in 200µl media per well in a 96 well plate. Leave 8 wells empty for blank control. Incubate at (37 °C, 5% CO2) overnight to allow the cells to attach to the wells. Incubate equal volume (1:1v/v) of non-lethal dilutions of tested samples (total extract and different fractions) and the virus (HSV-1) suspension at (37 °C, 5% CO2) for 4 successive days until the cells in the virus control wells showed a complete virus-induced cytopathic effect as observed under the light microscope. Make 2ml or more of MTT solution at 5 mg/ml in phosphate buffer saline. Add 20µl MTT solution to each well. Place on a shaking table, 150 rpm for 5 minutes, to thoroughly mix the MTT into the media. Incubate at (37 °C, 5% CO_2) for 1-5 hours to allow the MTT to be metabolized. Dump off the media, wash two times with phosphate buffer saline and dry plate on paper towels to remove residue if necessary. Resuspend formazan (MTT metabolic product) in 200µl DMSO. Place on a shaking table, 150 rpm for 5 minutes, to thoroughly mix the formazan into the solvent. Read optical density at λmax 492 nm. Optical density should be directly correlated with cell quantity.

The antiviral activity for a given sample can be calculated from the following equation (8).

% antiviral activity = [(A-B) / (C-B)] x 100

Where A, B and C are the OD492 of treated infected, untreated infected, and untreated uninfected cells, respectively.

4.2. Wound healing activity

Excision wound model is the wound type used in this experiment in which the rats in each group were anesthetized by administering thiopental sodium (50 mg /kg, i.p) (9). A full thickness of the excision wound of circular area (approx. 200mm2) and 2mm depth (10) was inflicted on the shaved back of the rats 30 min after anesthesia, the administration of thiopental sodium injection. The wounding day was

considered as day 0. The wounds were treated with topical application of the ointment preparations mentioned above, one time daily till the wounds were completely healed. The wounds were monitored and the area of wound was measured on 3, 6, 9, 12 and 15 post-wounding days till the wounds were completely healed. The wounds were traced on 1- mm² graph paper and the wound healing rate was expressed as (%) of wound healing from the following equation

% of wound closure = (wound area on day 0-wound area on day n x 100)/(wound area on day 0)

Where n = number of days 3rd, 6th, 9th, 12th and 15th (11).

5. Statistical Analysis

Experimental results are expressed as mean ± standard error. Results were statistically analyzed using analysis of variance (one-way ANOVA) followed by Tukey's t test for comparison between different groups. SPSS 20 version was used for the statistical analysis.

6. Results and discussion

6.1. Phytochemical study

Compound (1)

The EI-MS gave peak at m/z 392 [M-H2O] + corresponding to molecular formula C28 H58 O – H2O. Other mass units differing by 14 mass units (-CH2) or its multipliers, this fragmentation patterns typical for a long-chain fatty alcohol (12). Examination of the 1H-NMR spectral data showed a triplet signal at δH 3.63 which assigned for protons of oxygenated carbon attached to aliphatic chain, broad singlet signal at δH 1.22-1.29 which assigned for long chain methylene clusters and triplet signal at δH 0.87 for prephral methyl group. This finding was confirmed from 13C NMR spectrum which showed signal at δc 14.08 which assigned for terminal methyl group, while other signals at δc (22.65 to 32.77) which assigned for methylene groups, in addition to downfield shifted methylene group at δc 63.08 to alcoholic methylene moiety.

From the previous mentioned physical, chromatographic and spectral data (EI-MS, 1H-NMR and 13C-NMR) it was concluded that compound (1) is *n*-octacosanol and this is the first report for its isolation from the *C. oblongifolia*.

Compound (2)

It gave a red colour with Salkowiski test and violet ring with Libermann-Burchard's test suggested its steroidal or triterpenoidal nature (13). Examination of 1H-NMR spectrum showed signals at δH (1.67, 1.24, 1.003, 0.94, 0.91, 0.80 and 0.76) assigned for seven tertiary methyls, signals for two olefinic protons at δH 4.66 and 4.54 (each 1H, br.s), indicative for terminal methylene group and signal for oxygenated proton at δH 3.18 (1H, m). In addition to methylene and methine groups at δH 1.1 to 1.6. The data obtained from 1H NMR suggested the compound (2) is pentacyclic triterpen, this suggestion was confirmed from 13C-NMR signals, which indicated the presence of 30 carbons, with the general features of lupeol triterpene. Also the spectra revealed one oxygenated carbon at δC 78.97 which assigned for (C-3) and carbon resonances at δC 150.96 and δC 109.30 ppm which assigned for (C-20 and C-29 respectively).

From previous mentioned physical, chemical, chromatographic and spectral data (1H-NMR and 13C-NMR) and by comparing with published data of lupeol (14), in addition to co-chromatography with an authentic sample, it was concluded that the compound (2) is lupeol and this is the first report for its isolation from the *C. oblongifolia.*

Compound (3)

It gave violet colour with FeCl3 (T.S.) indicating its phenolic nature (15). EI-MS showed the molecular ion peak at m/z 138 calculated for molecular formula to be C7 H6 O3. Examination of the 1H-NMR spectral data revealed downfield shifted double doublet aromatic signals at δH 6.82 (2 H, dd, J= 6.9, 2.1 Hz) assigned to H-3 and H-5 and at δH 7.88 (2H, dd, J= 6.9, 2.4 Hz) assigned to H-2 and H-6 which, indicated the presence of 1,4 disubstituted benzyl moiety(16).

From previous mentioned physical, chemical, chromatographic and spectral data (1H-NMR) and authentication with authentic reference material, it was concluded that the compound (3) is 4-hydroxybenzoic acid and this is the first report for its isolation from the C. oblongifolia.

Compound (4)

It gave bluish green colour with FeCl3 (T.S.) indicating its phenolic nature (17, 18). EI-MS showed the molecular ion peak at m/z 184 calculated for molecular formula to be C8H8O5. Examination of the 1H-NMR spectral data revealed downfield shifted aromatic signal at δH 6.94 (2H, s, H-2, H-6) indicated the presence of tetra-substituted benzyl moiety (19, 20), furthermore presence of upfield shifted aliphatic signal at δH 3.71 (3H, s) which suggested the presence of methyl group attached to carbonyl moeity (ester linkage). This suggestion was confirmed from 13C-NMR signals which displayed signals at δ_C C 120.03 (C-1), 108.63 (C-2, C-6), 145.10 (C-3, C-5) in addition to δ_C C 167.62 attributed to carbonyl moeity (C-7) and δ_C C 50.85 (C-8) attributed to aliphatic CH3 group (21).

From the previous mentioned physical, chemical, chromatographic and spectral data (EI-MS, 1H-NMR and 13C-NMR) it was concluded that compound (4) is methyl gallate and this is the first report for its isolation from the *C. oblongifolia.*

Compound (5)

It gave yellow colour with dilute solution of sodium hydroxide indicating its flavonoid nature (22) and gave a green colour with FeCl3 (T.S.) which indicated presence of free hydroxyl groups (23). EI-MS (positive mode) showed the molecular ion peak at m/z 539 calculated for molecular formula to be C30 H18 O10. 1H-NMR and 13C-NMR spectra displayed signals characterstic to biflavonoid moiety (24). Examination of the 1H-NMR spectral data showed a three singlet signals at δH 6.37, 6.71 and 6.74 each (1H) for H-6", H-3" and H-3 respectively. In addition to presence of four sets of meta-coupled aromatic protons at δH (6.18, d, J= 2), (6.42, d, J= 2), (7.88, d, J= 2.3), and (7.94, d, J= 2.3) assigned for H-6, H-8, H-2' and H-6' respectively. Furthermore, presence of three sets of ortho-coupled aromatic protons at δH (6.58, d, J= 3.8), (7.10, d, J= 8.6) and (7.50, d, J= 8.8), assigned for (H-3"', H-5"'), H-5' and (H-2"', H-6"') respectively. 13C-NMR spectrum showed the presence of six oxygenated carbons at δC 161.10, 161.14, 161.77, 162.08,164.54 and 164.62 which were assigned to C-4', C-5", C-5, C-4", C-7" and C-7 respectively, in addition to presence of two carbonyl moieties at δC 182.40 and 182.83 which were assigned to C-4 and C-4" respectively.

From previous mentioned physical, chemical, chromatographic and spectral data (1H-NMR and 13C-NMR) it was concluded that compound (5) is amentoflavone and the compound was previously reported in the species (25).

6.2. Biological study

6.2.1. Cytotoxicity assay

Cytopathogenic effect assay method: detection of any sign of cell toxicity such as (loss of monolayer sheet, granulation and vacuolization in the cytoplasm (6). The maximum non-toxic concentrations [MNTC] of total extract and different fractions of the aerial parts of C. oblongifolia are listed in table 1. The toxic effect of total extract and different fractions of C. oblongifolia aerial parts on Vero cell line in comparison with control Vero cell line are listed in figure 2.

6.2.2. Antiviral activity

The obtained results showed that the methanol fraction showed the highest antiviral activity followed by total extract, methylene chloride fraction, ethyl acetate fraction and finally *n*-hexane fraction with lowest antiviral activity. The activity of total extract and different fractions of C. oblongifolia aerial parts against (HSV-1) are listed in table 2 and represented in figure 3. Triterpenoids, flavonoids, tannins and sterols being constituents of many plants with biological activities which may be responsible for antiviral activity obtained, so the highest activity of methanol fraction (68.1%) against (HSV-1) was attributed to their higher contents of flavonoids, tannins and other polyphenolic compounds. Also the relatively high activity of methylene chloride fraction (42.8%) against (HSV-1) was attributed to their higher contents of triterpenoids. The activity of total extract (51.9%) against (HSV-1) was attributed to their total contents of flavonoids, triterpenoids, tannins and sterol (26).

6.3. Wound healing activity

The healing process begins with the clotting of blood and is completed with remodeling of the cellular layers of the skin. However, the wound healing process may be prevented by the presence of ROS (Reactive oxygen species) or microbial infection, since the type of cells to be first recruited to the site of injury is neutrophiles which is produced in response to cutaneous injury which has a role in antimicrobial defense (27).

The results of wound healing activity by excision wound model of (T1) and (T2) of *C. oblongifolia* aerial parts compared with standard group (gentamicin) and control group (simple ointment base) are represented in table 3 (wound area in mm2), table 4 (% of wound closure), figure 4 (graphical presentation of % wound closure in day 15) and figure 5 (progression of wound healing process).

The results showed marked increase in wound healing activity of T1 and T2 groups compared with control group. The wound healing activity of T1 is slightly higher than the standard group and the activity of T2 is slightly lower than the standard group. The higher wound healing activity of T1 was attributed to presence of different classes of active constituents which possess different activities in wound healing process as sterols (β-sitosterol) which is one of the active compounds which may be responsible for the epithelization activity (28), anti-inflammatory, anti-pyretic, antiarthritic and anti-ulcer activities(11), triterpens as lupeol shows activities as antiprotozoal, anti-inflammatory and antimicrobial which are also supporting the wound healing process, while polyphenolic compounds like tannins which reported to have good wound healing activity, tannins are known for their astringent activity which responsible for anti-bacterial activity (11).

Polyphenolics act as free radical scavengers which remove free radicals from site of injury which cause more tissue damage and delay wound healing process. The slightly lower activity of T2 group than standard group was attributed to lack of anti-inflammatory constituents as sterols and triterpens in the MeOH fraction and thus the wound healing activity of T2 was attributed to presence of polyphenolics as flavonoids which act as free radical scavenger and tannins which act as astringent.

Epithelialization period is varied from 15 day in case of T1 group, 17 day in case of standard group, 18 day in case of T2 group and finally 20 day in case of control group.

7. Conclusion

The phytochemical study of aerial parts of C. oblongifolia resulted in the isolation of five compounds, four of them are reported for first time in the *C. oblongifolia* aerial parts which was identified as *n*-octacosanol, lupeol from MC fraction, 4-hydroxybenzoic acid and methyl gallate from EtOAC fraction and one is previously reported in the same plant is amentoflavone from EtOAC fraction. The total extract and fractions of the aerial parts of *C. oblonfgifolia* showed variable activities against HSV-1 at [MNTC] varied from 68.1% for MeOH fraction to 42.8% for MC fraction. Total extract and MeOH fraction of the aerial parts of C. oblongifolia showed variable wound healing activity on exicison wound model in albino rat in comparison with gentamicin.

Table 1: The maximum non-toxic concentrations [MNTC] of total extract and different fractions of *C. oblongifolia* aerial parts

	Fraction	MNTC
1	Total extract	(100µg/ml)
2	*n*-hexane Fr.	(100µg/ml)
3	Methylene chloride Fr.	(50µg/ml)
4	Ethyl acetate Fr.	(100µg/ml)
5	Methanol Fr.	(50µg/ml)

Table 2: The optical density, mean optical density and % antiviral activity of total extract and different fractions of *C. oblongifolia* aerial parts.

	Test	O.D	Mean O.D	%antiviral activity
1	Control VERO cell line	0.154/0.155/0.156	0.155	---
2	Virus control(HSV-1&VERO)	0.054/ 0.051 / 0.050	0.051	---
3	Total extract	0.101/ 0.106/ 0.109	0.105	51.9±2.6%
4	*n*-hexane Fr.	0.078/ 0.075/ 0.080	0.075	25.1±1.6%
5	Methylene chloride Fr.	0.092/ 0.096/ 0.1	0.096	42.8±2.7%
6	Ethyl acetate Fr.	0.087 /0.092/ 0.090	0.084	36.7±1.9%
7	Methanol Fr.	0.125 / 0.119 / 0.122	0.121	68.1±1.6%

Values of (%) antiviral activity are mean ± SE M; OD = Optical density; Fr = fraction

Table 3: Effect of total extract and methanol fraction ointments (T1 and T2) on the wound area in mm2.

	Control group	Standard group	T_1 group	T_2 group
Day (0)	197.9±1.4	197.6±1.4	197.9±2	197±1
Day (3)	192.2±1.3	183.9±1.6	174.7±2	189±0.9
Day (6)	172.2±0.84	157.2±2.1	147.1±1.6	164±0.7
Day (9)	114.9±1.9	82.9±1.9	74±1.5	103.2±1.2
Day (12)	66.8±1.3	24.9±1.9	18.3±1.5	40.3±1.2
Day (15)	21.4±0.8	2.3±0.7	0	7±0.5

Values are mean ± SE.M; n = 5

Table 4: Effect of total extract and methanol fraction ointments of *C. oblongifolia* aerial parts (T1 and T2) on the (%) wound contraction.

	Control group	Standard group	T_1 group	T_2 group
Day (3)	2.9±0.06%	7.9±0.2%	11.7±0.2%	4.0±0.3%
Day (6)	12.7±0.2%	20.6±0.5%	25.7±0.1%	16.7±0.5%
Day (9)	42.0±0.3%	58.1±0.7%	62.6±0.4%	47.7±0.5%
Day (12)	66.3±0.4%	87.4±0.9%	90.8±0.7%	79.7±0.6%
Day (15)	89.2±0.4%	98.9±0.3%	100%	96.4±0.3%

Values are mean ± SE.M; n = 5

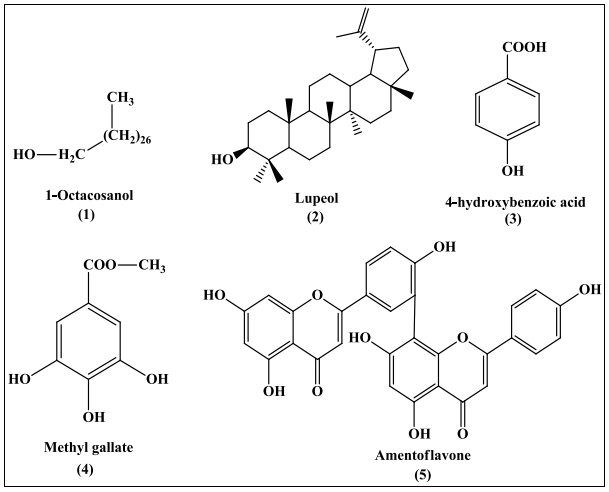


Fig 1: Isolated compounds (1-5):

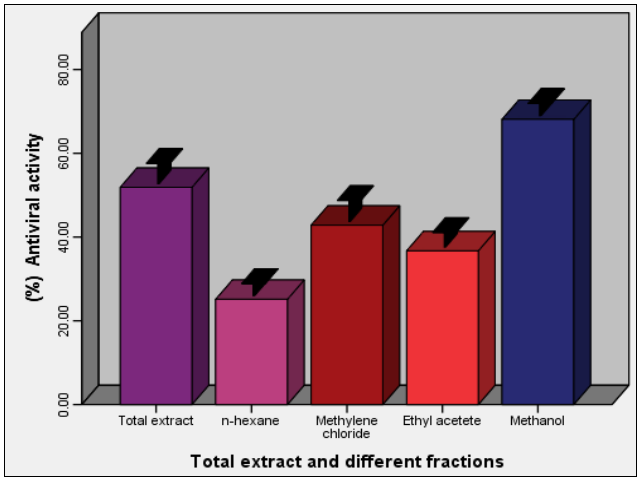
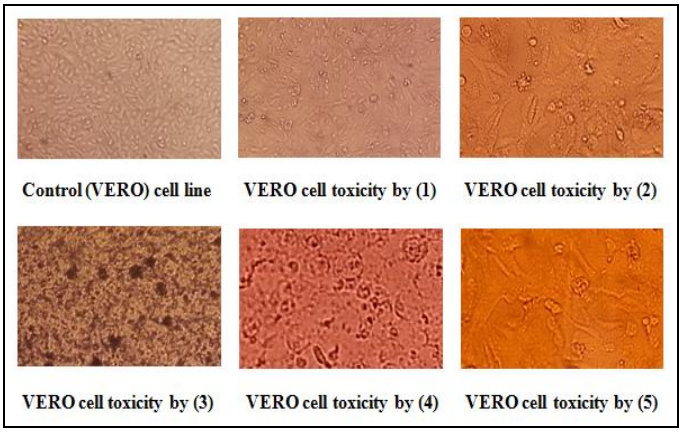
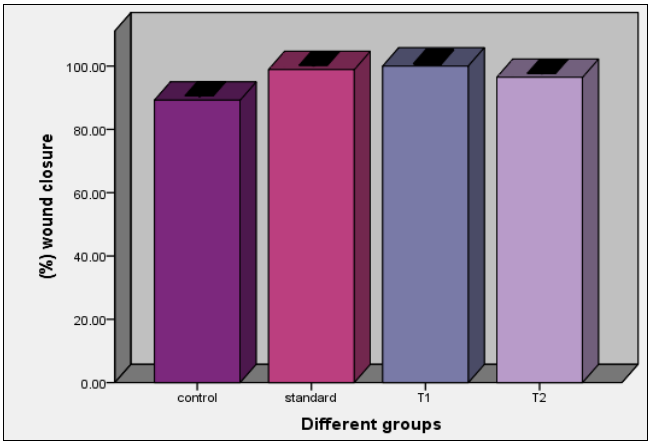


Figure 3: (%) antiviral activity of total extract and different fractions of *C. oblongifolia* aerial parts on (HSV-1).



Source (1) = Total extract, (2) = *n*-hexane fraction, (3) = MC fraction, (4) = EtOAC fraction and (5) = MeOH fraction

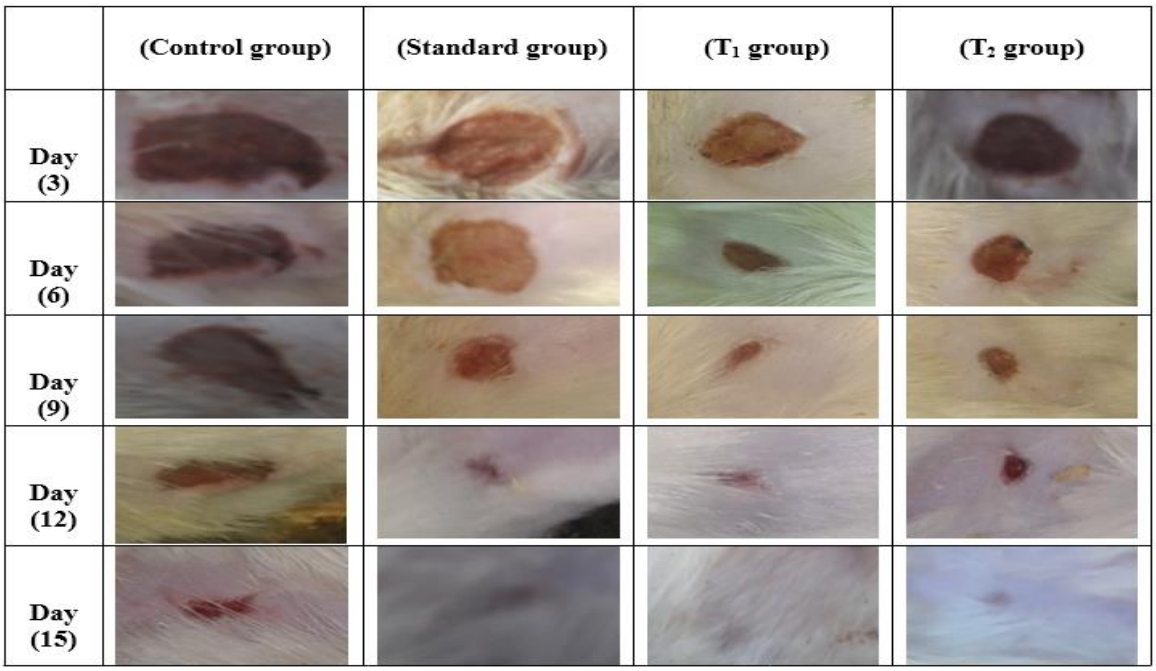
Fig 2: Toxic effect of total extract and different fractions of *C. oblongifolia* aerial parts on Vero cell line in comparison with control Vero cell line.



Source: Total extract and methanol fraction of *C. oblongifolia* aerial parts

Fig 4: (%) wound closure of treated groups in day 15.

Fig 5: Progression of wound healing of rats on day (3, 6, 9, 12 and 15) in T1 and T2 in comparison with standard and control groups.



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