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Phytochemical and Biological Studies of Aerial Parts of *Forsskaolea tenacissima* Linn. (Urticaceae)

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Abstract

Fractionation and purification of the methanolic extract of the aerial parts of *Forsskaolea tenacissima* L. (Urticaceae), afforded nine compounds identified as: lupeol (1), stearic acid (2), palmitic acid (3), arachidic acid (4), 1-hexadecanol (5), β -sitosterol/stigmasterol (6), maslinic acid (7), 2-hydroxy imino 3-phenyl propionic acid (8) which was first isolated from natural source in addition to β -sitosterol-3- β -D-glucopyranoside (9). Identification of these compounds had been established by physical, chemical and spectral methods. Compound 8 showed weak cytotoxic activity against tested normal cell line (Vero) and cancer cell lines (MCF-7, Caco-2 and HepG-2) exhibited IC₅₀ value 216.74 μ g/ml, 305.83 μ g/ml, 412.84 μ g/ml and 251.61 μ g/ml respectively, in comparison with the doxorubicin as potent chemotherapeutic agent exhibited IC₅₀ value 2.52 μ g/ml, 2.75 μ g/ml, 3.39 μ g/ml and 1.75 μ g/ml respectively. Total extract and methanol fraction showed high antiviral activity of 55.60% and 53.70%, respectively against Herpes Simplex Virus Type-1 (HSV-1). Ethyl acetate fraction showed significant antimicrobial activity against both Gram negative bacteria as *Escherichia coli* and *Pseudomonas aeruginosa* and Gram positive bacteria as *Staphylococcus aureus* and *Bacillus subtilis* while, both the total extract and different fractions did not show antifungal activity against *Aspergillus niger* and *Candida albicans*.

Keywords: *Forsskaolea tenacissima*, Urticaceae, fatty acids, triterpenes, sterols, antiviral, cytotoxic and antimicrobial.

1. Introduction

Family Urticaceae comprises 54 genera and more than 2000 species of herbs, shrubs, small trees, and a few vines, distributed primarily in tropical regions [1-3]. *Forsskaolea* is a small genus of six species distributed from Canary Islands and south eastward Spain to Pakistan and India [4-6]. *Forsskaolea tenacissima* L. is a member of the non-stinging nettles genus *Forsskaolea* and is in the same family as the stinging kind Urticaceae. Its synonyms: *Caidbeja adhaerens* Forssk and *Forsskaolea cossoniana* Webb [7, 8]. *Forsskaolea tenacissima* L. used in folk medicine in Pakistan as anti-inflammatory, antispasmodic, anti-diabetic and antipyretic [9]. The present study is dealing with the isolation and identification of the chemical constituents in addition to determination of the biological activities of the aerial parts of this plant.

2. Materials and Methods

2.1. Plant materials

The aerial parts of *Forsskaolea tenacissima* L. were collected in the period from April to June 2013. It is collected from El-kawthar city, Sohag, Egypt. The plant identified and authenticated by Prof. Dr. Salah M. El-Naggar Professor of Botany and Plant Taxonomy, Faculty of Science, Assuit University, Assuit, Egypt. A voucher sample (No. 0/7) was kept in the Herbarium of Faculty of Pharmacy, Minia University, Minia, Egypt.

2.2. Materials, equipments and cell lines

Melting points were measured on Electrothermal 9100 Digital Melting Point Instrument (England). ¹H and ¹³C-NMR spectra were recorded on a Bruker 400 MHz spectrometer (Germany). EI-MS data were estimated on JEOL JMS-600 mass spectrometer (Japan). Column chromatography was carried on silica gel (70-230, mesh, E-Merck) and Sephadex LH-20 (20-100 μ m, Sigma-Aldrich chemicals). TLC was carried on Precoated silica gel plates G60 F254 and Reversed phase silica RP-18 F254 (Aluminum sheets, E-Merck, Germany). The plates were examined under UV light (at 365 and 254 nm) and visualized by spraying with 10% v/v H₂SO₄ in MeOH, allowed to dry at room temperature followed by heating at 110-140 °C for 1-2 min.

Optical density data were acquired using ELISA reader (start fax-2100/ USA), Thermostar ELISA incubator (USA) and Jouan CO₂ incubator (France). Dimethyl sulfoxide (DMSO) (SDS / France). Minimum essential media (MEM) (Caisson / USA). Trypsin-EDTA 1:250 U (Molekulu / UK). MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (Biobasic / Canada). Doxorubicin as anticancer standard (Sigma-Aldrich Chemicals Co, Germany). Ampicillin as antibacterial agent standard and Amphotericin-B as antifungal agent standard (Sigma-Aldrich Chemicals Co, Germany). VERO cell line (African green monkey kidney cells) and Herpes Simplex Virus Type-1 (HSV-1). Caco-2 cell line (colon carcinoma cells), MCF-7 cell line (breast cancer cells) and HepG-2 cell line (human hepatocarcinoma cells).

2.3. Extraction and isolation

The air-dried powdered aerial parts of *Forsskaolea tenacissima* L. (4.5 kg) were exhaustively extracted by maceration in methanol/H₂O (70%). The methanolic extract was concentrated under reduced pressure to give dark green syrupy residue. The solvent-free residue (390 g) was subjected to fractionation on VLC using *n*-hexane, dichloromethane (DCM), ethyl acetate, methanol and 10% aqueous methanol, respectively till complete exhaustion in each case to give (38 g), (14 g), (13 g), (262 g) and (6 g) for *n*-hexane, DCM, ethyl acetate, methanol and 10% aqueous methanol, respectively.

The DCM fraction (14 g) was slurried with silica dried powdered and transferred to a column [5 (ID) X 120 (L) cm] packed with silica gel (420 g) slurried in *n*-hexane. The column was eluted initially with *n*-hexane followed by *n*-hexane-ethyl acetate gradients. Fractions of 100 ml were collected and the similar fractions were combined together and concentrated under reduced pressure to give 6 sub-fraction labeled FTD-1 to FTD-6. Fraction FTD-1 (2.2 g) was rechromatographed on silica gel CC using *n*-hexane-EtOAc gradient which afforded compound 1 (30 mg). Fraction FTD-2 (3 g) afforded compound 2 (15 mg), compound 3 (25 mg), compound 4 (10 mg) and compound 5 (8 mg) after repeated silica gel CC using *n*-hexane-EtOAc gradients and isochratic elution. Fraction FTD-3 (2.5 g) was also subjected to CC repeatedly using silica gel and *n*-hexane-EtOAc gradients as eluting systems, large amounts of crystals present as sediment in the tube, after repeated crystallization with methanol afforded compound 6 (800 mg).

The ethyl acetate fraction (13 g) was slurried with silica dried powdered and transferred to a column [5 (ID) X 120 (L) cm] packed with silica gel (390 g) slurried in DCM. The column was eluted initially with DCM followed by DCM-MeOH gradients. Fractions of 100 ml were collected and the similar fractions were combined together and concentrated under reduced pressure to give 5 sub-fractions labeled FTE-1 to FTE-5. Fraction FTE-2 (3.2 g) was subjected to repeated column chromatography on silica gel DCM-MeOH system gradients and sephadex LH 20 CC, afforded compound 7 (10 mg) and compound 8 (6 mg). Fraction FTE-3 (2.6 g) was rechromatographed on silica gel columns using DCM-MeOH with gradients elution that afforded compounds 9 (250 mg).

Compound 1: was isolated as white needle crystals, m.p. 215-216 °C. ¹H-NMR (400 MHz, CDCl₃): δ 4.65 (1H, br.s, H-29a),

4.54 (1H, br.s, H-29b), 3.15 (1H, m, H-3), 2.35 (1H, m, H-19), 1.96 (3H, s, H-30), 1.65 (3H, s, H-26), 1.02 (3H, s, H-23), 0.96 (3H, s, H-27), 0.94 (3H, s, H-25), 0.84 (3H, s, H-28) and 0.77 (3H, s, H-24). ¹³C-NMR (100 MHz, CDCl₃): δ 38.7 (t, C-1), 25.1 (t, C-2), 78.7 (d, C-3), 38.8 (s, C-4), 55.2 (d, C-5), 18.3 (t, C-6), 34.2 (t, C-7), 40.8 (s, C-8), 50.4 (d, C-9), 37.1 (s, C-10), 20.9 (t, C-11), 27.4 (t, C-12), 38.0 (d, C-13), 42.9 (s, C-14), 27.4 (t, C-15), 35.5 (t, C-16), 42.9 (s, C-17), 48.2 (d, C-18), 47.9 (d, C-19), 150.9 (s, C-20), 29.8 (t, C-21), 39.9 (t, C-22), 27.9 (q, C-23), 15.4 (q, C-24), 16.1 (q, C-25), 15.9 (q, C-26), 14.5 (q, C-27), 18.2 (q, C-28), 109.3 (t, C-29) and 19.3 (q, C-30).

Compound 2: was isolated as white waxy residue, m.p. 71-73 °C. ¹H-NMR (400 MHz, CDCl₃): δ 2.28 (2H, t, *J* = 7.5 Hz, H-2), 1.56 (2H, m, H-3), 1.19 (28H, m, H-4-H-17) and 0.80 (3H, t, *J* = 6.5 Hz, H-18). ¹³C-NMR (100 MHz, CDCl₃): δ 179.9 (s, C-1), 33.9 (t, C-2), 24.7 (t, C-3), 29.7-29.1 (t, C-4-C-15), 31.9 (t, C-16), 22.7 (t, C-17) and 14.1 (q, C-18).

Compound 3: was isolated as white crystalline powder, m.p. 63-64 °C. ¹H-NMR (400 MHz, CDCl₃): δ 2.28 (2H, t, *J* = 7.6 Hz, H-2), 1.56 (2H, m, H-3), 1.21 (24H, m, H-4-H-15) and 0.81 (3H, t, *J* = 7.2 Hz, H-16). ¹³C-NMR (100 MHz, CDCl₃): δ 179.97 (s, C-1), 34.04 (t, C-2), 24.68 (t, C-3), 29.68-29.07 (t, C-4-C-13), 31.93 (t, C-14), 22.69 (t, C-15) and 14.11 (q, C-16).

Compound 4: was isolated as white amorphous powder, m.p. 75-77 °C. ¹H-NMR (400 MHz, CDCl₃): δ 2.28 (2H, t, *J* = 7.4 Hz, H-2), 1.56 (2H, m, H-3), 1.19 (32H, m, H-4-H-19) and 0.81 (3H, t, *J* = 7 Hz, H-20). ¹³C-NMR (100 MHz, CDCl₃): δ 178.98 (s, C-1), 33.86 (t, C-2), 24.70 (t, C-3), 29.68-29.07 (t, C-4-C-17), 31.93 (t, C-18), 22.70 (t, C-19) and 14.12 (q, C-20).

Compound 5: was isolated as white amorphous powder, m.p. 49-51 °C. ¹H-NMR (400 MHz, CDCl₃): δ 3.57 (2H, br.s, H-1), 1.49 (2H, m, H-2), 1.18 (26H, m, H-3-H-15) and 0.81 (3H, t, *J* = 7.2 Hz, H-16). ¹³C-NMR (100 MHz, CDCl₃): δ 58.39 (t, C-1), 28.08 (t, C-2), 27.18 (t, C-3), 24.95-24.69 (t, C-4-C-13), 20.99 (t, C-14), 17.95 (t, C-15) and 9.37 (q, C-16).

Compound 6: was isolated as white crystalline needles, m.p. 134-136 °C. ¹H-NMR (CDCl₃, 400MHz): δ 5.32 (1H, m, H-6), 5.30 (1H, m, H-22b), 4.98 (1H, m, H-23b), 3.47 (1H, m, H-3), 2.24 (2H, m, H-4), 0.99 (3H, s, H-19), 0.66 (3H, s, H-18) and other methyl groups at δ 0.80-0.92 (m). ¹³C-NMR (CDCl₃, 100MHz): δ 37.2 (t, C-1), 31.8 (t, C-2), 71.7 (d, C-3), 42.3 (t, C-4), 140.7 (s, C-5), 121.6 (d, C-6), 31.9 (t, C-7), 31.6 (d, C-8), 50.1 (d, C-9), 36.5 (s, C-10), 21.1 (t, C-11), 39.7/39.6b (t, C-12), 42.1 (s, C-13), 56.7/56.8b (d, C-14), 24.3/24.3b (t, C-15), 28.2 (t, C-16), 56.0/55.9b (d, C-17), 11.8 (q, C-18), 19.3 (q, C-19), 36.1/40.5b (d, C-20), 18.8/21.1b (q, C-21), 33.9 (t, C-22)/138.3b (d, C-22), 26.1 (t, C-23)/129.3b (d, C-23), 45.8/51.2b (d, C-24), 29.2 (d, C-25)/31.9b (t, C-25), 19.8/18.9b (q, C-26), 19.0/21.2b (q, C-27), 23.0/25.4b (t, C-28) and 12.2 (q, C-29).

Compound 7: was isolated as white crystalline powder, m.p. 249-250 °C. ¹HNMR (DMSO-*d*₆, 400 MHz): δ 11.97 (1H, br.s, H-28), 5.18 (1H, br.s, H-12), 4.38 (1H, br.s, H-2), 4.28 (1H, br.s, H-3), 2.75 (1H, d, *J* = 8, H-18), 1.18 (3H, s, H-27), 1.15 (3H, s, H-23), 1.10 (3H, s, H-25), 0.91 (3H, s, H-30), 0.88 (3H, s, H-29), 0.75 (3H, s, H-24) and 0.71 (3H, s, H-26). ¹³C-NMR (DMSO-*d*₆, 100 MHz): δ 47.2 (t, C-1), 67.8 (d, C-2), 82.6 (d, C-3), 39.7(s, C-4), 55.2 (d, C-5), 18.5 (t, C-6), 32.5 (t, C-7), 40.1 (s, C-8), 47.5 (d, C-9), 38.1 (s, C-10), 23.7 (t, C-11), 121.5 (d, C-12), 144.7 (s, C-13), 41.7 (s, C-14), 27.6 (t, C-15), 23.4 (t, C-16), 46.1 (s, C-17), 41.8 (d, C-18), 45.9 (t, C-19), 30.7 (s, C-20), 33.8 (d, C-21), 32.8 (t, C-22), 29.3 (q, C-23), 16.8 (q, C-24), 17.3 (q, C-25), 17.5 (q, C-26), 26.1 (q, C-27), 179.9 (s, C-28), 33.2 (q, C-29) and 23.0 (q, C-30).

Compound 8: was isolated as yellowish white residue, m.p. 264-266 °C. ¹HNMR (DMSO-*d*₆, 400 MHz): δ 11.81 (1H, br.s, H-1), 7.99 (1H, br.s, N-OH), 3.52 (2H, br.s, H-3) and protons of benzene ring at δ 7.13-8.02 (5H, m, H-2'-H-6'). ¹³C-NMR (DMSO-*d*₆, 100 MHz): δ 166.49 (s, C-1), 136.88 (s, C-2), 132.67 (s, C-1'), 122.56 (d, C-2'-C-6'), 121.39 (d, C-3'-C-5'), 121.04 (d, C-4') and 29.47 (t, C-3).

Compound 9: was isolated as white amorphous powder, m.p. 276 – 278 °C. ¹HNMR (DMSO-*d*₆, 400 MHz): δ 5.34 (1H, m, H-6), 4.22 (1H, d, *J* = 8 Hz, H-1'), 3.33 (1H, m, H-3), 1.25 (3H, d, *J* = 6 Hz, H-21), 1.05 (3H, s, H-19), 0.90 (3H, s, H-26), 0.90 (3H, s, H-27), 0.83 (3H, t, *J* = 7 Hz, H-29), 0.66 (3H, s, H-18) and sugar protons 3.65-4.87 (5H, m, H-2'-H-6'). ¹³C-NMR (DMSO-*d*₆, 100 MHz): δ 36.69 (t, C-1), 29.65 (t, C-2), 77.37 (d, C-3), 38.00 (t, C-4), 140.92 (s, C-5), 121.69 (d, C-6), 31.90 (t, C-7), 33.72 (d, C-8), 50.07 (d, C-9), 35.95 (s, C-10), 21.00 (t, C-11), 40.41 (t, C-12), 42.33 (s, C-13), 56.64 (d, C-14), 24.25 (t, C-15), 28.25 (t, C-16), 55.40 (d, C-17), 12.15 (q, C-18), 19.09 (q, C-19), 35.95 (d, C-20), 19.57 (q, C-21), 33.70 (t, C-22), 24.25 (t, C-23), 45.60 (d, C-24), 29.66 (d, C-25), 19.41 (q, C-26), 20.19 (q, C-27), 23.00 (t, C-28), 12.26 (q, C-29), 101.25 (d, C-1'), 73.94 (d, C-2'), 77.37 (d, C-3'), 70.58 (d, C-4'), 77.24 (d, C-5') and 61.57 (t, C-6').

2.4. Methods for biological study

2.4.1. Antiviral activity

2.4.1.1. Cytotoxic assay ^[10, 11]: The lyophilized dried total extract and different fractions (100 mg each) of the aerial parts of *Forsskaolea tenacissima* L. were 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 days. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. 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. Measured maximum non-toxic concentrations of total extract and different fractions of *Forsskaolea tenacissima* L. aerial parts were used in antiviral assay (Table 1).

2.4.1.2. Antiviral assay

MTT cell viability test ^[12]: Plate 10,000 cells in 200 µl 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 (1:1 v/v) of non-lethal dilutions of tested samples (total extract and different fractions) and the virus (HSV-1) suspension for one hr. Add 100µl from viral/ sample suspension, place on a shaking table, 150 rpm for 5 minutes. Incubate (37 °C, 5 % CO₂) for 1- days to allow the virus to take effect. Make 2 ml of MTT solution at 5 mg/ml in phosphate buffer saline. Add 20 µl 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 % CO₂) for 1-5 hrs 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 200 µL DMSO. Place on a shaking table, 150 rpm for 5 minutes, to thoroughly mix the formazan into the solvent. Read optical density at 570 nm, optical density should be directly correlated with cell quantity. The antiviral activity for a given sample can be calculated from the following equation ^[13].

$$\% \text{ Antiviral activity} = [(A-B) / (C-B)] \times 100$$

Where, A: the mean optical density of treated cells, B: the mean optical density of virus control, C: the mean optical density of cell control (Table 2).

2.4.2. Antimicrobial activity

Agar cup diffusion method ^[14] was used to detect inhibition zones caused by the different extracts of *Forsskaolea tenacissima* L. aerial parts as follows: concentration of 100 mg/ml of *n*-hexane, dichloromethane, ethyl acetate, methanol and total extract were prepared in dimethyl sulphoxide (DMSO), using a sterile cork porer and under aseptic condition, cups were made in the medium in which fixed volumes from the tested fractions were dispensed to fill the cups, using sterile micropipette. Plates were placed carefully in the incubators at 37 °C for 48 hrs for bacteria and for 72 hrs for fungi. After incubation the diameter of the clear zone of inhibition surrounding the samples was measured. The results were interpreted according to National Committee for Clinical Laboratories Standards (NCCLS) ^[15] (Table 3).

2.4.3. Cytotoxic activity of compound 8 (2-hydroxy imino 3-phenyl propionic acid)

MTT cell viability test ^[16-18]: Isolated compound 8 and doxorubicin (positive control) were dissolved in dimethyl sulfoxide (DMSO) and diluted in the medium in final concentrations ranging from 1 to 1000 µg/mL in 96-well plates. Control cells were treated with vehicle alone. Plate 10,000 cells (Vero, Caco-2, Mcf-7, and HepG-2) in 200 µl media per well in a 96 well plate. Leave 8 wells empty for blank control. The other steps discussed in antiviral activity.

The experiment was performed triplicate, the cell viability (%) was calculated as the follows: (Table 4)

$$\text{Cell viability \%} = \frac{\text{Optical density (OD) of sample treated cells}}{\text{Optical density (OD) of untreated control cells}} \times 100$$

3. Results and discussion

3.1. Results and discussion for isolated compounds

Compound 1: It showed positive results with Liebermann-Burchard's test and Salkowski's test indicating its steroidal and/or triterpenoidal nature [21]. Careful investigation of ¹³C-NMR spectrum of compound **1** indicated the presence of 30 carbon atoms which confirmed its triterpenoidal nature. ¹H NMR spectra approved the presence of seven tertiary methyls at δ_{H} 0.77, 0.84, 0.94, 0.96, 1.02, 1.65 and 1.96 each (3H,s) corresponding to H-24, H-28, H-25, H-27, H-23, H-26 and H-30, which confirmed from ¹³C-NMR signals at δ_{C} C-24 (15.4), C-28 (18.2), C-25 (16.1), C-27 (14.5), C-23 (27.9), C-26 (15.9) and C-30 (19.3). Moreover ¹³C-NMR spectra showed characteristic signals at δ_{C} 150.9 (s, C-20) and δ_{C} 109.3 (t, C-29) which were approved from ¹HNMR at δ_{H} 4.65, 4.54 (each 1H, br.s) indicated the presence of terminal methylene moiety [19, 20]. Hydroxylated carbon C-3 was observed at δ_{C} 78.9 which was calculated from ¹HNMR signals at (δ_{H} 3.18 (1H, m) assigned to H-3 [22-24]. From the above mentioned data and by comparison with reported data [25], compound **1** was identified as lupeol, which was reported in this plant, this was confirmed by direct authentication (m.p. and co-chromatography).

Compound 2: Positive mode EI-MS showed [M]⁺ at m/z 284, corresponding to molecular formula C₁₈H₃₆O₂. Examination of the ¹³C-NMR spectra revealed signals at δ_{C} 179.3 and δ_{C} 14.10 assigned to carbonyl group of carboxylic moiety and terminal methyl group. In addition to group of signals at δ_{C} 22.7 to 33.9 assigned to CH₂ cluster, which diagnosed the existence of long chain fatty acid moiety. This was further approved from ¹HNMR signal at δ_{H} 0.80 (3H, t, $J = 6.5$ Hz), δ_{H} 1.19-1.56 (30H, br.s) and δ_{H} 2.28 (2H, t, $J = 7.5$ Hz) indicated to terminal methyl group, cluster of CH₂ and downfield shifted CH₂ group neighbor to carboxylic moiety [26-29]. It could be calculated that compound **2** was identified as stearic acid, which is first reported in the genus *Forsskaolea*.

Compound 3: EI-MS showed [M]⁺ at m/z 256, corresponding to molecular formula C₁₆H₃₂O₂. Examination of the ¹³C-NMR spectra revealed signals at δ_{C} 179.97 and δ_{C} 14.11 assigned to carbonyl group of carboxylic moiety and terminal methyl group. In addition to group of signals at δ_{C} 22.69 to 34.04 assigned to CH₂ cluster, which diagnosed the existence of long chain fatty acid moiety. This was further approved from ¹HNMR signal at δ_{H} 0.81 (3H, t, $J = 7.2$ Hz), δ_{H} 1.21-1.56 (26H, br.s) and δ_{H} 2.28 (2H, t, $J = 7.6$ Hz) indicated to terminal methyl group, cluster of CH₂ and downfield shifted CH₂ group neighbor to carboxylic moiety [26-29]. It could be calculated that compound **3** was identified as palmitic acid, which is first reported in the genus *Forsskaolea*.

Compound 4: EI-MS showed [M]⁺ at m/z 312, corresponding to molecular formula C₂₀H₄₀O₂. Examination of the ¹³C-NMR spectra revealed signals at δ_{C} 178.98 and δ_{C} 14.12 assigned to

carbonyl group of carboxylic moiety and terminal methyl group. In addition to group of signals at δ_{C} 22.70 to 33.86 assigned to CH₂ cluster, which diagnosed the existence of long chain fatty acid moiety. This was further approved from ¹HNMR signal at δ_{H} 0.81 (3H, t, $J = 7$ Hz), δ_{H} 1.19-1.56 (34H, br.s) and δ_{H} 2.28 (2H, t, $J = 7.4$ Hz) indicated to terminal methyl group, cluster of CH₂ and downfield shifted CH₂ group neighbor to carboxylic moiety [26-29]. It could be calculated that compound **4** was identified as arachidic acid, which is first reported in the genus *Forsskaolea*.

Compound 5: GC-MS showed [M⁺-H₂O] at m/z 224 suggested the presence of free hydroxyl moiety [30] corresponding to molecular formula C₁₆H₃₄O. Examination of the ¹³C-NMR spectra revealed signals at δ_{C} 58.39 and δ_{C} 9.37 assigned to carbon with hydroxyl group and terminal methyl group. In addition to group of signals at δ_{C} 17.95 to 28.08 assigned to CH₂ cluster, which diagnosed the existence of long chain hydrocarbon. This was further approved from ¹HNMR signal at δ_{H} 0.81 (3H, t, $J = 7.2$ Hz), δ_{H} 1.18-1.49 (28H, br.s) and δ_{H} 3.57 (2H, br.s) indicated to terminal methyl group, cluster of CH₂ and downfield shifted CH₂ group neighbor to hydroxyl moiety [30]. It could be calculated that compound **5** was identified as 1-hexadecanol, which is first reported in the genus *Forsskaolea*.

Compound 6: It showed positive results with Liebermann-Burchard's test and Salkowski's test indicating its steroidal and/or triterpenoidal nature [21]. Investigation of ¹³C-NMR spectrum of compound **6** indicated the presence of 29 carbon atoms which confirmed its steroidal skeleton [21], which were accounted for six methyl, nine methylene, eleven methane and three quaternary carbons. ¹³C-NMR has shown at δ_{C} 140.7 and δ_{C} 121.6 with δ_{H} 5.32 (1H, m) indicated the presence of double bond also, shown at δ_{C} 138.3 and δ_{C} 129.3 with δ_{H} 5.30 (1H, m) and δ_{H} 4.98 (1H, m) indicated the presence of other double bond [31-33]. In addition, δ_{C} 71.7 (d, C-3) with δ_{H} 3.47 (1H, m) indicated the presence of hydroxyl group at C-3 [22]. From the above mentioned physical, chemical, spectral data and by comparison with reported data [34], compound **6** was identified as a mixture of β -sitosterol (a) and stigmasterol (b), which was reported in this plant, this was confirmed by direct authentication (m.p. and co-chromatography).

Compound 7: It showed positive results with Liebermann-Burchard's test and Salkowski's test indicating its steroidal and/or triterpenoidal nature [21]. Careful investigation of ¹³C-NMR spectrum of compound **7** indicated the presence of 30 carbon atoms which confirmed its triterpenoidal nature, which were accounted for 7 methyls, 8 methylenes, 6 methines and 8 quaternary carbons. ¹HNMR spectra approved the presence of seven tertiary methyls at δ_{H} 0.71, 0.75, 0.88, 0.91, 1.10, 1.15 and 1.18 each (3H,s) corresponding to H-26, H-24, H-29, H-30, H-25, H-23 and H-27, which confirmed from ¹³C-NMR signals at δ_{C} C-26 (17.5), C-24 (16.8), C-29 (33.2), C-30 (23.4), C-25 (17.3), C-23 (29.26) and C-27 (26.1). In addition, the ¹HNMR spectrum indicated the existence of one chelated hydroxyl group at δ_{H} 11.97, one olefinic proton at δ_{H} 5.18 (br s, H-12) connected to C-12 (δ_{C} 121.5) and two oxymethine protons at δ_{H} 4.38 (br s, H-2) and 4.28 (br s, H-3) attached to

C-2 (δ_C 67.8) and C-3 (δ_C 82.6). These data suggested and the presence of double bond in position 12 indicated that compound 7 was an oleanane-type triperpenoid [19] showed an ortho-dihydroxy system at C-2 (δ_C 67.8) and C-3 (δ_C 82.6). The α and β orientation of C-2 and C-3 (OH) was approved from up field shift of C-2 from δ_C 71.5 to δ_C 67.8 and the down field shift of C-3 from δ_C 78.6 to δ_C 82.6 [19, 35]. From the above discussed data, compound 7 was identified as maslinic acid, which is first reported in the genus *Forsskaolea*.

Compound 8: GC-MS showed $[M]^+$ at m/z 179, corresponding to molecular formula $C_9H_9NO_3$. Examination of the 1H NMR spectral data of compound 8 revealed the presence of signals at δ_H 7.13-8.02 (5H, m) indicated mono-substituted benzene moiety [36] with methylene group H-2 at δ_H 3.52 (2H, br.s), δ_H 11.81 (1H, br.s) assigned to proton of carboxylic group (H-1) and δ_H 7.99 (1H, br.s) assigned to proton of hydroxy imino group which appear overlapped with aromatic protons. Moreover ^{13}C -NMR, further signal at δ_C 166.49 assigned to carboxylic group (C-1) and δ_C 136.88 indicate carbon attached to hydroxy imino group (C-2). From the above discussed data, compound 8 was identified as 2-hydroxy imino 3-phenyl propionic acid, which is first isolated from natural source.

Compound 9: It showed positive results with Liebermann-Burchard's test and Salkowski's test indicating its steroidal and/or triterpenoidal nature [21]. Investigation of ^{13}C -NMR spectrum of compound 9 indicated the presence of 35 carbon atoms among which 6 signals were attributed to sugar moiety and this was clear from 1H NMR signal at δ_H 4.22 (1H, d, $J = 8$ Hz) assigned to β -configured anomeric proton of sugar moiety [37, 38] and this was further indicated from ^{13}C -NMR signal at δ_C 101.25 of anomeric carbon. The chemical shifts suggest the presence of steroidal skeleton (29 carbon atoms), which were accounted for six methyl, nine methylene, eleven methane and three quaternary carbons. The 1H NMR spectrum showed six methyl groups at δ_H 0.66 (3H, s), 0.83 (3H, t, $J = 7$ Hz), 0.90 (6H, s), 1.05 (3H, s) and 1.25 (3H, d, $J = 6$ Hz) could be assigned to H-18, H-29, H-26 & H-27, H-19 and H-21 respectively. Moreover ^{13}C -NMR has shown at δ_C 140.92 and δ_C 121.69 with δ_H 5.34 (1H, m) indicated the presence of double bond. From the above mentioned data and by comparison with reported data [39], compound 9 was identified as β -sitosterol-3-O- β -glucopyranoside, which was reported in this plant, this was confirmed by direct authentication (m.p. and co-chromatography).

3.2. Results and discussion for biological activities

3.2.1. Antiviral activity

The total extract showed the highest antiviral activity followed by methanol fraction, ethyl acetate fraction, dichloromethane fraction and finally *n*-hexane fraction with lowest antiviral activity (Tables 1, 2 and Figure 2). The higher activity of total extract may be due to the existence of synergistic variety of compounds including phenolics, tannins, triterpenes, and sterols which produce greater antiviral activity more than the purified single compounds [13].

3.2.2. Antimicrobial activity

Ethyl acetate fraction showed remarkable antibacterial activity against both Gram negative bacteria as *Escherichia coli* and *Pseudomonas aeruginosa* and Gram positive bacteria as *Staphylococcus aureus* and *Bacillus subtilis* (Table 3) which may be attributed to the presence of triterpenes, sterols and phenolic compounds in this fraction [40]. The total extract and other fractions did not show any antibacterial or antifungal activities which could be attributed to low concentration and/or the absence of bioactive compounds responsible for the previously observed antimicrobial activity of ethyl acetate fraction.

3.2.3. Cytotoxic activity of compound 8

Cytotoxic activity of compound 8 (Figures 3, 4, 5, 6 and Table 4) on Vero, MCF-7, Caco-2 and HepG-2 cell lines at concentrations range 1 – 1000 μ g/ml expressed as a viability percentage are shown in table 44 and figures 113, 114, 115 and 116. IC_{50} value of compound 8 showed 216.74 μ g/ml, 305.83 μ g/ml, 412.84 μ g/ml and 251.61 μ g/ml against Vero, MCF-7, Caco-2 and HepG-2 cell lines respectively, While IC_{50} value of doxorubicin exhibited 2.52 μ g/ml, 2.75 μ g/ml, 3.39 μ g/ml and 1.75 μ g/ml against Vero, MCF-7, Caco-2 and HepG-2 cell lines respectively. From previous results, compound 8 showed weak cytotoxic activity against tested normal (Vero) and cancer cells (MCF-7, Caco-2 and HepG-2) in comparison with the doxorubicin as potent chemotherapeutic agent.

4. Conclusion

Aerial parts of *Forsskaolea tenacissima* L. family: Urticaceae, afforded nine compounds identified as: lupeol (1), stearic acid (2), palmitic acid (3), arachidic acid (4), 1-hexadecanol (5), β -sitosterol/stigmasterol (6), maslinic acid (7), 2-hydroxy imino 3-phenyl propionic acid (8) and β -sitosterol-3- β -D-glucopyranoside (9). Compound 8 was first isolated from natural source and showed weak cytotoxic activity against (Vero, MCF-7, Caco-2 and HepG-2) cell lines, in comparison with the doxorubicin as potent chemotherapeutic agent. Total extract and methanol fraction showed high antiviral activity against (HSV-1). Ethyl acetate fraction showed antimicrobial activity against both Gram negative and Gram positive bacteria while, both the total extract and different fractions did not show antifungal activity.

5. Acknowledgment

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6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

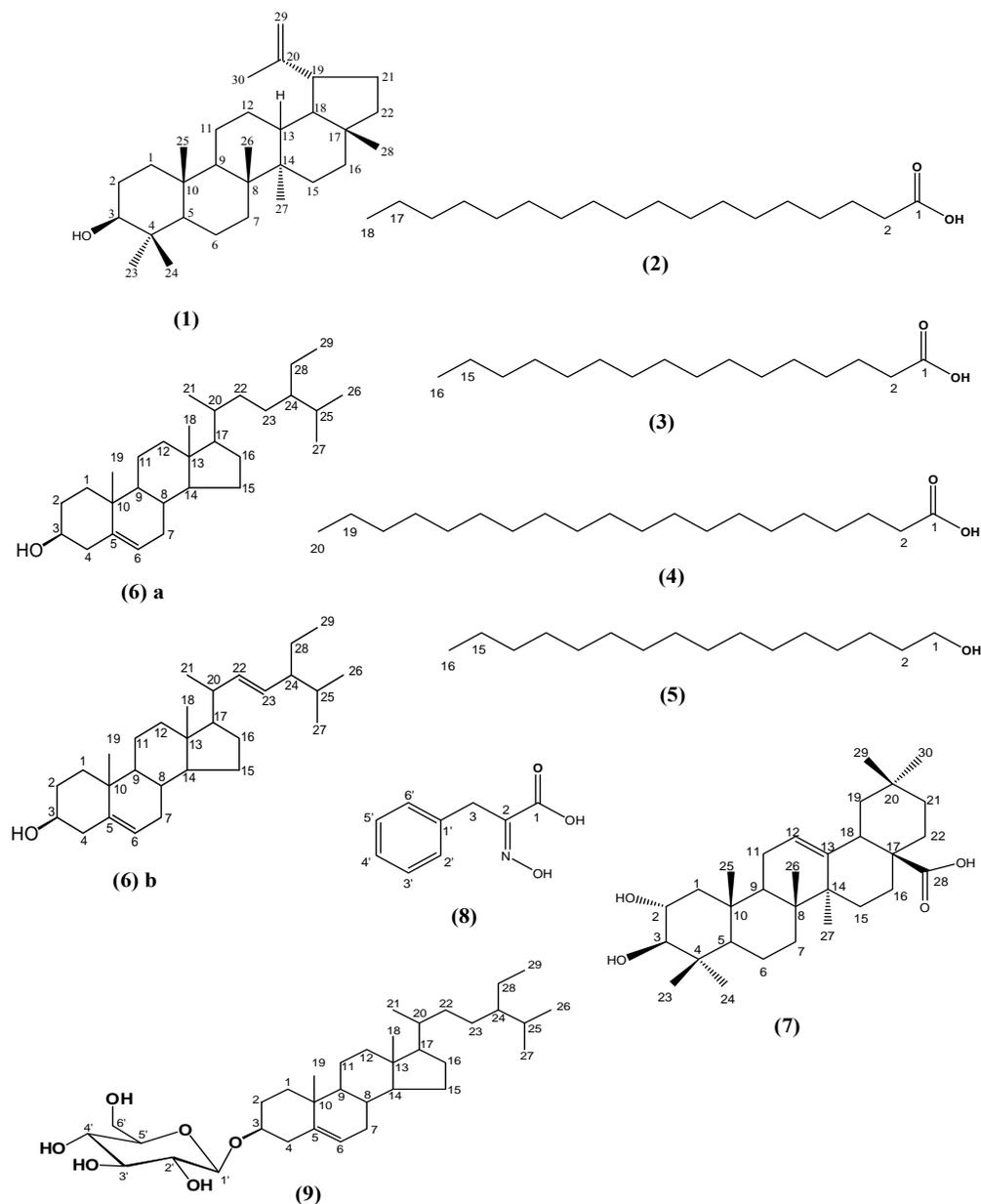


Fig 1: Structures of the isolated compounds 1- 9

Table 1: Maximum non-toxic concentrations (MNTC) of the total extract and different fractions of *Forsskaolea tenacissima* L. aerial parts.

Fractions	MNTC
Total extract	10 µg/ml
<i>n</i> -Hexane fraction	100 µg/ml
Dichloromethane fraction	20 µg/ml
Ethyl acetate fraction	20 µg/ml
Methanol fraction	100 µg/ml

Table 2: Antiviral activity (%) of total extract and different fractions of *Forsskaolea tenacissima* L. aerial parts

Fractions	Optical density	Mean O.D	% Antiviral activity
Total extract	0.252 / 0.260 / 0.283	0.265	55.60±0.70%
<i>n</i> -Hexane fraction	0.194 / 0.216 / 0.189	0.199	22.00±0.63%
Dichloromethane fr.	0.218 / 0.227 / 0.223	0.222	33.00±1.01%
Ethyl acetate fraction	0.230 / 0.238 / 0.219	0.229	37.33±1.22%
Methanol fraction	0.242/ 0.268 / 0.265	0.258	53.70±1.27%

Notes: Values of (%) antiviral activity are mean ± SE, O.D: optical density

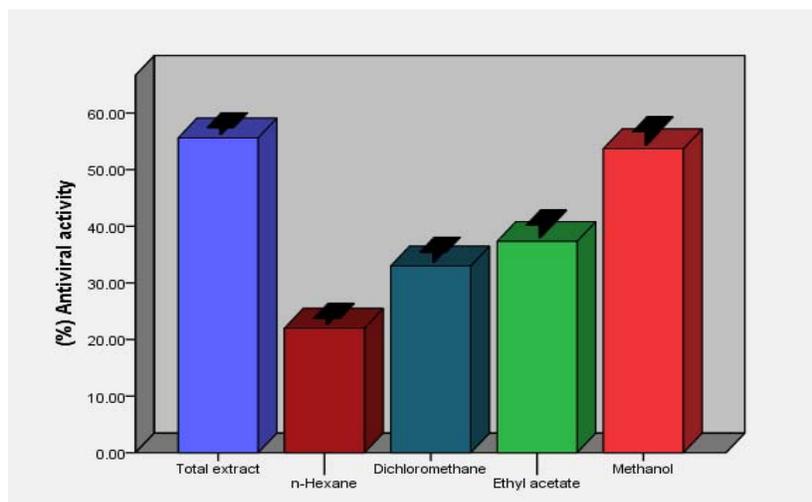


Fig 2: Antiviral activity (%) of total extract and different fractions of *Forsskaolea tenacissima* L. aerial parts on (HSV-1).

Table 3: Results of the antimicrobial activity of the different fractions and total extract of *Forsskaolea tenacissima* L. aerial parts.

Sample	Inhibition zone diameter (mm / mg sample)					
	<i>Escherichia coli</i> (G)	<i>Pseudomonas aeruginosa</i> (G)	<i>Staphylococcus aureus</i> (G ⁺)	<i>Bacillus subtilis</i> (G ⁺)	<i>Aspergillus niger</i> (Fungus)	<i>Candida albicans</i> (Fungus)
Control:DMSO	0.0	0.0	0.0	0.0	0.0	0.0
Ampicillin Antibacterial agent	22	17	18	20	—	—
Amphotericin B Antifungal agent	—	—	—	—	17	19
Total extract fr.	0.0	0.0	0.0	0.0	0.0	0.0
n-Hexane fr.	0.0	0.0	0.0	0.0	0.0	0.0
Dichloromethane fr.	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl acetate fr.	10	10	11	10	0.0	0.0
Methanol fr.	0.0	0.0	0.0	0.0	0.0	0.0

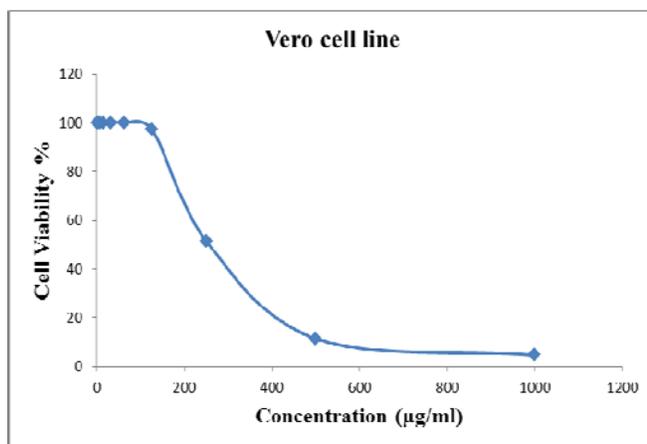


Fig 3: Dose-cytotoxicity curve of compound 8 isolated from *Forsskaolea tenacissima* L. aerial parts on Vero cell lines.

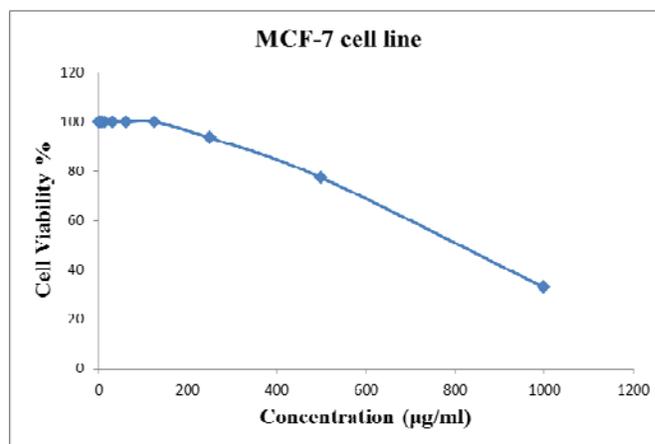


Fig 4: Dose-cytotoxicity curve of compound 8 isolated from *Forsskaolea tenacissima* L. aerial parts on MCF-7 cell lines.

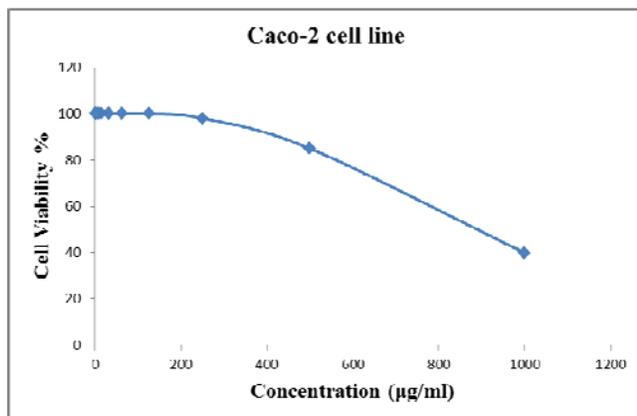


Fig 5: Dose-cytotoxicity curve of compound 8 isolated from *Forsskaolea tenacissima* L. aerial parts on Caco-2 cell lines.

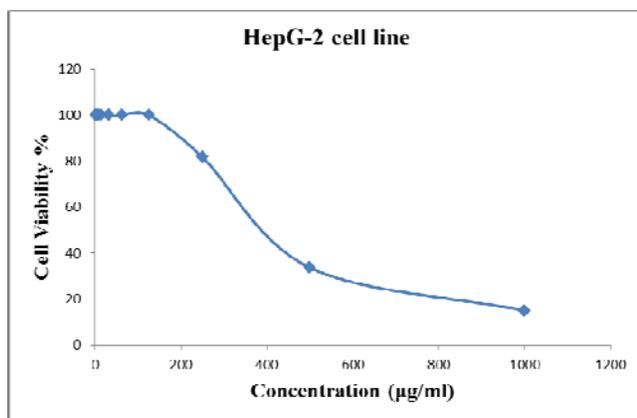


Fig 6: Dose-cytotoxicity curve of compound 8 isolated from *Forsskaolea tenacissima* L. aerial parts on HepG-2 cell lines.

Table 4: Cytotoxic activity of compound 8 isolated from *Forsskaolea tenacissima* L. aerial parts on Vero, MCF-7, Caco-2 and HepG-2 cell lines.

Sample	IC ₅₀ (µg/mL)			
	Vero	MCF-7	Caco-2	HepG-2
Compound 8 (2-hydroxy imino 3-phenyl propionic acid)	216.74 ± 5.56	305.83 ± 3.14	412.84 ± 4.36	251.61 ± 6.71
Doxorubicin (Positive control)	2.52 ± 0.24	2.75 ± 0.11	3.39 ± 0.75	1.75 ± 0.34

Values are mean of 3 experiment ± standard error (S.E.).

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