NEW DOVYALICIN-TYPE SPERMIDINE ALKALOID FROM *DOVYALIS CAFFRA* (WARB.); FAMILY: SALICACEAE, CULTIVATED IN EGYPT

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Abstract

Phytochemical investigations of *Dovyalis caffra* (leaves and twigs) revealed a new dovyalicin-type spermidine alkaloid, named Dovyalicin G (1); which was identified as (*E*)-N-(4-(1,5-diazocan-1-yl)butyl)-N-methyltetradec-2-enamide, along with previously isolated *β*-sitosterol (2) and Hentriacontan-1-ol (3). The structures were established using ESI/MS, EI/MS, ^{1}H NMR, APT NMR, and two-dimensional NMR experiments. In addition to the biological studies of the different plant extracts including cytotoxicity, topoisomerase II inhibition, antimicrobial, and *in-vitro* anti-inflammatory activities. Screening of 5-lipoxygenase (5-LOX) and Cyclooxygenase-1 (COX-1) and -2 (COX-2) inhibition to evaluate anti-inflammatory activity were performed. The alkaloid fraction showed good antimicrobial activity against studied microorganisms and remarkable cytotoxic activities against studied cell lines (Besides, total methanolic and petroleum ether extracts). The total methanolic extract showed strong COX- inhibition activity and selectivity toward COX-2, comparing with celecoxib.

Keywords: *Dovyalis caffra*, anti-inflammatory, alkaloid, cyclooxygenase-1, 5-lipoxygenase, dovyalicin G

Introduction

Dovyalis caffra (Salicaceae) is an erect evergreen shrub or small dioceous tree. *D. caffra* is native to southern Africa and it is widely cultivated in Egypt as a hedge plant. Formerly, it was classified as a member of a defunct family; Flacourtiaceae, but now it belongs to family Salicaceae under the tribe of Flacourtieae (Chase et al. 2002; Cheek and Ngolan 2006; Chase et al. 2016; Lemke 1988; Steyn, Van Wyk, and Smith 2005). The roots of *Dovyalis caffra* and other *Dovyalis* spp. are used in African traditional medicine to treat amenorrhea and chest pain. Also, they are used by the Zulu to treat pain in rheumatic fever and rheumatism (Stanstrup et al. 2010). Recently, the total methanolic extract of the plant (leaves and twigs) was studied for its cytotoxic activity which was promising (Moustafa et al. 2014). Dovyalicin-type alkaloids are a class of amide alkaloids having a spermidine nucleus (NH_2 $(CH_2)_3$ NH $(CH_2)_4NH_2$), with at least one nitrogen atom involved in amide group. Till now, this type of alkaloids is exclusively isolated from the genus *Dovyalis* (*D. abyssinica, D. macrocalyx*, and *D.*

hebecarpa) and six members were identified and named as Dovyalicin A, B, C, D, E, and F (Rasmussen et al. 2006; Stærk et al. 2003). The previous phytochemical studies of *D. caffra* (Warb.) resulted in the isolation of polyamine alkaloids; aberiamine; and aberiamide, Flavonoids; Luteolin; apigenin; and apigenin-7-O-*β*-D-glucopyranoside, triterpenes; *α*- and *β*- amyrins, sterols; *β*-sitosterol; and *β*-sitosterol-O-*β*-D-glucopyranoside (Sayed et al. 2000), and phenolic glycosides; itoside A; and 4-hydorxytremulacin (Stanstrup et al. 2010). Biological investigations were performed on the total extract and showed antimicrobial activities (Zaki 1975), Cytotoxic Activity (Moustafa et al. 2014) and inhibition of proliferation of Hep G2 cells via apoptosis (El-Menshawi et al. 2010).

This study aimed to investigate different biological activities of the extracts – with different polarities- of *D. caffra* (Warb.), including cytotoxicity, topoisomerase II inhibition assay, antimicrobial, and *in vitro*- anti-inflammatory, along with phytochemical investigation of alkaloid and the less polar fractions.

Material and methods:

Experimental:

General experimental procedures: ESI/MS spectra were obtained using LC/MS positive and negative modes. NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H),100 (APT) MHz, and 2D (HSQC & HMBC) in $CDCl_3$ solution and chemical shifts were expressed in δ (ppm) with reference to TMS, and coupling constant (*J*) in Hertz. Column chromatographic separations was performed on silica gel 60 (Si gel 60, Merck), sephadex LH-20 (Pharmacia) and SPE-C-18 (Phenomenex) columns. TLC was performed on precoated TLC plates with silica gel 60 F254 (0.2mm, Merck). Developed chromatograms were visualized by spraying with Vanillin/ H_2SO_4 (before and after heating at 100 ^{0}C for 5 minutes) and Dragendorff's spraying reagents.

Plant material:

Dovyalis caffra leaves and young stems were collected from Al-Orman Botanic Garden, Giza, Egypt in November 2015. A voucher herbarium specimen had been deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Nasr city, Cairo, Egypt.

Extraction and isolation:

The air-dried; finely powdered leaves and young stems of *D. caffra* (2.5Kg.) was exhaustively extracted with 70% methanol and evaporated under reduced pressure to yield 320gm of dark green semisolid residue (total methanolic extract).

Separation of Purified Crude alkaloids and Isolation of Compound (1):

About 100gm of the total extract was suspended in 1.5L of 1% aqueous H_2SO_4, filtered, concentrated to 500ml (under reduced pressure), and then partitioned with petroleum ether (500ml x3). The aqueous layer was separated, alkalinized with 25% Na_2CO_3 solution till pH 11.5, and extracted with dichloromethane-DCM (500ml x4). The DCM-layer was evaporated under reduced pressure to give 21.5gm of crude alkaloid extract. The crude alkaloid extract was dissolved in 500ml DCM and extracted with 10% aqueous acetic acid (500ml x3). The acetic acid layer was concentrated (under reduced pressure), alkalinized with Na_2CO_3 powder (till no more effervescence), and extracted again with DCM (500ml x4). The DCM-fraction was evaporated under reduced pressure to give 15gm of purified crude alkaloid extract (Alkaloid Fraction) (Yubin et al. 2014). The alkaloid fraction (12gm) was fractionated on silica gel vacuum liquid chromatography (VLC) using DCM-Methanol-Ammonia (100:0:0 – 50:50:1%) to give eleven sub fractions; A-K. Sub fraction F was further chromatographed on silica gel column chromatography (CC) using DCM-Methanol-Ammonia (100:0:0 – 90:10:1%) to afford compound 1 (eluted with 97:3:1%), which was purified by prep. TLC using DCM-Methanol-Ammonia (95:5:1%) to give 8mg of yellowish white residue (Comp.1). About 10gm of the total extract was suspended in 500ml dist. water and partitioned successively with petroleum ether, DCM, ethyl acetate, and n-butanol to give pet. ether fraction (2.4gm), DCM-fraction (0.9gm), EtOAc fraction (0.5gm), and BuOH fraction (1.7gm)for biological studies and GC/MS experiments.

Isolation of Compounds (2) and (3):

The methanolic extract (about 210gm) was suspended in distilled water and fractionated with DCM, EtOAc, and n-BuOH, respectively. The DCM fraction (50gm) was fractionated on silica gel vacuum liquid chromatography (VLC) using n-hexane-EtOAc to give fourteen sub fractions; A-N. Sub fraction D was further chromatographed on silica gel column chromatography (CC) using n-hexane-EtOAc to afford 12mg of compound (3) and 40mg of compound (2) respectively.

Saponification of Pet. Ether Fraction:

Pet. Ether Fr. (1gm) was refluxed with 50ml of 10% alcoholic KOH for 8 hours (till complete saponification). The alcohol was evaporated under reduced pressure, then the residue was suspended in 100ml distilled water, and extracted with DCM (100ml x3).The DCM layer was concentrated to 100ml, washed with dist. water several times to remove any alkalinity, dried with anhydrous Na_2SO_4, then evaporated under reduced pressure to give the unsaponifiable matter (UNSAP). The aqueous alkaline layer was acidified with dil. HCl (20ml), and extracted with DCM. The DCM extract was washed with distilled water, dried with anhydous Na_2SO_4, and then evaporated under reduced pressure to give saponifiable matter (SAP), which was dissolved in 50ml absolute MeOH, 5ml Conc. H_2SO_4 was added, and then refluxed for 4 hours. After cooling and evaporation, 50ml distilled water was added and shaked well, then extracted with DCM, washed with distilled water, dried with anhydous Na_2SO_4, and then evaporated under reduced pressure to give methylated SAP fraction (El-Kashef et al. 2014).

Method of GC-MS analysis For SAP

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The initial column temperature was kept at 70 °C for 2 min. (isothermal) and programmed to 120 °C at a rate of 4 °C/min, and kept constant at 120 °C for 2 min. (isothermal) then programmed to 240 °C at a rate of 4 °C/min., and kept constant at 240 °C for 13 min. (isothermal) . Injector temperature was 250 °C. Helium carrier gas flow rate was 1.24 ml/min. All the mass spectra were recorded applying the following condition: filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 230°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 20).

Method of GC-MS analysis for UNSAP:

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The initial column temperature was kept at 50 °C for 3 min. (isothermal) and programmed to 300 °C at a rate of 5 °C/min., and kept constant at 300 °C for 10 min. (isothermal). Injector temperature was 280 °C. Helium carrier gas flow rate was 1.37 ml/min. All the mass spectra were recorded applying the following condition: filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 220°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15).

Compound 1:

Yellowish-white powder, ^{1}H-NMR (400 MHz, *CDCl$_3$*), δ 2.90 (4H, m, H-2, H-8), 1.24 (4H, m, H-3, H-7), 2.18 (4H, m, H-4, H-6), 1.5 (NH-5), 3.07 (2H, m, H-1’), 1.55 (2H, m, H-2’), 1.41 (2H, m, H-3’), 3.45(2H, m, H-4’), 3.05 (3H, s, N-CH$_3$), 6.19 (1H, d, *J*=14.5 Hz, H-2’’), 6.85 (1H, dt, *J*=14.5,7.1 Hz, H-3’’), 2.18 (2H, m, H-4’’), 1.23 -1.39 (18H, m, H-5’’ to H-13’’), 0.88 (3H, t, *J*=7.1, H-14’’); APT-NMR (100 MHz, *CDCl$_3$*) δ 53.74 (C-2, C-8), 32.03 (C-3, C-7), 45.14 (C-4, C-6), 53.81 (C-1’), 24.37 (C-2’), 28.45 (C-3’), 53.36 (C-4’), 167.51 (C-1’’), 119.81 (C-2’’), 147.80 (C-3’’), 32.69 (C-4’’), 29.36 -29.80 (C-5’’ to C-11’’), 31.01 (C-12’’), 22.79 (C-13’’), 14.22 (C-14’’), 35.56 (N-CH$_3$); ESI/MS *m/z* 408 [M$^+$+H] (calc. for $C_{25}H_{49}N_3O$).

Compound 2:

White crystals, ^{1}H-NMR (400 MHz, *CDCl$_3$*), δ 3.53 (1H, m, H-3), 5.35 (1H, m, H-6), 0.68 (3H, s, H-18), 1.01 (3H, s, H-19), 0.92 (3H,d, *J*= 6.5Hz, H-21), 0.81 (3H, d, *J*=6.5Hz, H-26), 0.83 (3H, d, *J*=6.5Hz, H-27), 0.84 (3H, t, *J*=7Hz, H-29); APT-NMR (100 MHz, *CDCl$_3$*) δ 37.38 (C-1), 31.75 (C-2), 71.96 (C-3), 42.41 (C-4), 140.88 (C-5), 121.88 (C-6), 32.05 (C-7), 32.02 (C-8), 50.24 (C-9), 36.63 (C-10), 21.21 (C-11), 39.90 (C-12), 42.45 (C-13), 56.89 (C-14), 24.44 (C-15), 28.39 (C-16), 56.17 (C-17), 12.00 (C-18), 18.91 (C-19), 36.28 (C-20), 19.16 (C-21), 34.06 (C-22), 26.15 (C-23), 45.95 (C-24), 29.25 (C-25), 19.97 (C-26), 19.54 (C-27), 23.18 (C-28), 12.12 (C-29); EI/MS *m/z* 414 [M]$^+$ (calc. for $C_{29}H_{50}O$).

Compound 3:

White amorphous powder, ^{1}H-NMR (400 MHz, *$CDCl_3$*), 3.67 (2H, t, *J*=6.6, H-1), 2.04 (1H, brs, OH), 1.66 - 1.45 (6H, m, H-2 to H-4), 1.34 – 1.15 (52H, m, H-5 to H-30), 0.87 (3H, t, J=7.1, H-31); APT-NMR (100 MHz, *$CDCl_3$*) δ 63.14 (C-1), 32.83 (C-2), 25.75 (C-3), 29.69 (enormous peak; C-4 to C-28), 31.95 (C-29), 22.72 (C-30), 14.11 (C-31); EI/MS *m/z* 452 $[M]^+$ (calc. for $C_{31}H_{64}O$).

Evaluation of cytotoxic activity:

Cytotoxic assay:

The cytotoxic activity was measured *in vitro* on human cancer cell line (MCF-7, HCT-116 and HEP-G2) using Sulforhodamine B stain (SRB) assay applying the method of Skehan, et al. In this study the cytotoxic activities (IC_{50}) of various extracts (70% methanol, petroleum ether, ethyl acetate, n-butanol and alkaloid fraction) from the leaves and young stems of *Dovyalis caffra* (Warb.) were measured against three different human cancer cell lines; breast adenocarcinoma (MCF-7); human colon carcinoma (HCT-116) and; hepatocellular carcinoma (HEP-G2). Cytotoxicity Assay by SRB method (Skehan et al. 1990) and the results were shown in table (3).

Evaluation of Topoisomerase II Inhibition Activity:

Alkaloid fraction and petroleum ether extract of *Dovyalis caffra* (Warb.) leaves and young stems were selected to be evaluated against topoisomerase II (MBS#942146) using human DNA topoisomerase 2-beta (TOP2B) ELISA kit according to manufacturer's instructions. Prepare all reagents, working standards, and samples. Add 100 µl of standard and sample per well and incubate for 2 hours at 37 ^{0}C. Remove the liquid of each well, don't wash. Add 100 µl of biotin-antibody to each well and incubate for 1 hour at 37 ^{0}C. Aspirate each well and wash three times. Add 100 µl of horseradish peroxidase (HRP-avidin) to each well and incubate for 1 hour at 37 ^{0}C. Repeat the aspiration/wash process for five times. Add 90 µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate to each well and incubate for 15-30 minutes at 37 ^{0}C, protect from light. Add 50 µl of stop solution to each well and determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. The values of % activity versus a series of extracts concentrations ranging from 0.01 µM to 100 µM (with semi-log decrease in concentration) were then plotted using non-linear regression analysis of sigmoidal dose response curve. The IC_{50} values for alkaloid fraction and petroleum ether extract against Topo II were determined by the concentration causing a half maximal percent activity and the data were compared with staurosporine as standard Topo II inhibitors (Abdelhaleem et al. 2018; Hassanin et al. 2018; Kassab and Gedawy 2018) and the results were shown in table (4).

Evaluation of antimicrobial activity:

The antimicrobial activity of different *Dovyalis caffra* extracts were tested against a range of micro-organisms including two Gram-positive bacteria (*Staphylococcus aureus* 'ATCC 25923' and *Bacillus subtilis* 'ATCC 6633'), two Gram-negative bacteria (*Salmonella typhi* 'ATCC 700931' and *Escherichia coli* 'ATCC

25922'**)** and one fungal species (*Aspergillus niger* 'ATCC 6275') using the agar well diffusion assay method (Liang et al. 2012) and the results were shown in (Table 5). All extracts were tested in concentration 50mg/ml and the inhibition zone was measure around each well after 24h.

Anti-inflammatory Activities:

1. Evaluation of Lipoxygenase Inhibition Activity (5-LOX):

The inhibitory activity of the total methanolic and ethyl acetate extracts of *D. caffra* (Warb.) against zileuton of 5-LOX was evaluated by using 5- lipoxygenase assay kit (Catalog # K980-100, Biovision incorporated). IC_{50} values of the tested extracts were carried out according to procedures and instructions given with the assay kit and in accordance to previously reported methods. Briefly, Test extract preparation: Dissolve the test compound in appropriate solvent. Prepare at such concentration so volume of test compound solution added to a well is no more than 2 µl in the final 100 µl reaction volume per well. Add 2µl test extract to each well of the 96 well white plate. For "Solvent Control", add 2 µl of the solvent used to prepare test compound solution at its final concentration in test wells, and for "Inhibitor Control" add 2 µl of Zileuton, the provided LOX inhibitor. Bring up the volume to 40 µl in each well by adding 38 µl of LOX Assay Buffer. For the "Enzyme Control" well, add 40 µl LOX Assay Buffer to a well. Reaction Mix: Mix enough reagents for the number of assays to be performed. For each well, prepare 40 µl Mix containing: 36 µl LOX Assay Buffer, 2 µl LOX Probe, 2 µl 5-LOX Enzyme; Mix well and add Reaction Mix to wells containing the Enzyme Control, Inhibitor Control, "Solvent Control" and Test Extracts. Incubate plate at RT for 10 minutes before adding substrate. There should be no bubbles in the wells. LOX Substrate: Dilute the provided LOX substrate (12500 X) in LOX Assay Buffer using 1:25 dilution factor to obtain a 500 X solution. Depending on the number of reactions to be performed, dilute the 500 X solution in LOX Assay Buffer at 1:100 to get 5X solution. 20 µl of 5X solution will be needed per reaction. Make up enough substrate depending on the number of reactions. Measurement: Start recording fluorescence at Ex/Em 500/536 nm on the second minute after the addition of the substrate at 30 second intervals for 10 - 20 minutes (Huang et al. 2019).

2. Evaluation of Cyclooxygenase- 1 & 2 Inhibition Activity (COX-1 & COX-2):

The COX-1 (human recombinant) and COX-2 (human recombinant) inhibitory assay was carried out for total methanolic and ethyl acetate extracts of *D. caffra* (Warb.) using a COX Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's protocol. Briefly, heme and COX enzymes, COX-1 and COX-2, respectively, were added to test tubes containing COX reaction buffer (0.1 M Tris–HCl, pH 8.0, containing 5 mM EDTA and 2 mM phenol). The mixture was vortexed and exposed to either reaction buffer or serum sample for 10 minutes at 37 °C. Celecoxib, the selective COX-2 inhibitor, was used at a concentration corresponding to its reported IC_{50} concentration (Reddy et al. 2000)(165 nM for COX-1

and 65.61 nM for COX-2) as a positive control. Subsequently, arachidonic acid solution was added to start the cyclooxygenase reaction. After incubation for 2 minutes and 37 °C 1 M hydrochloric acid was added to terminate the enzyme catalytic reaction followed by chemical reduction of prostaglandin (PG) PGH_2 to $PGF_{2\alpha}$ with saturated stannous chloride solution for 5 minutes at room temperature. The reaction products were stable for 1 week at 0–4 °C. The COX activity was measured based on the amount of $PGF_{2\alpha}$ generated in the reaction tube and detected by the enzyme immunoassay kit using a standard curve. The inhibitory activity before adding tested extracts was compared to the COX inhibition induced by the extracts supplementation. The difference was expressed as increase in COX inhibitory activity.

Statistical analysis (Wilcoxon matched pairs signed rank test) was performed using the Graph Pad prism software (Graph Pad Software Inc., San Diego, CA, USA). Significance was defined as $P < 0.05$ (Schäfer et al. 2006).

Results and discussion:

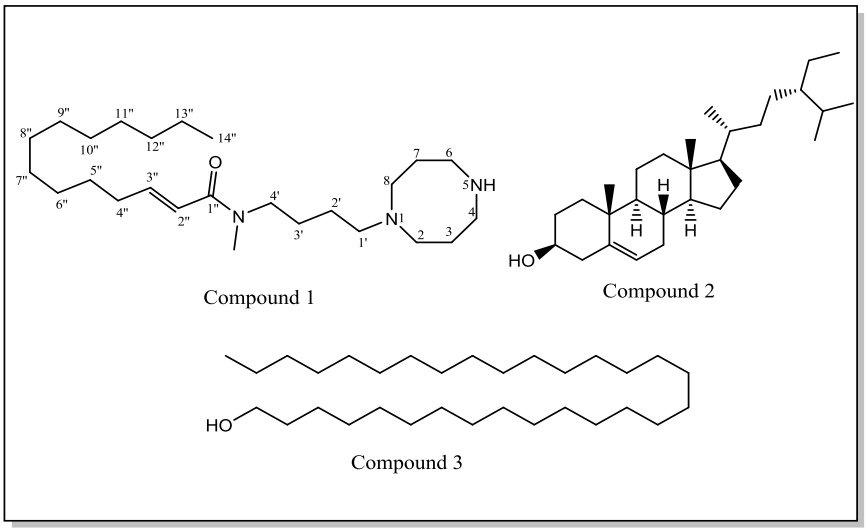

Figure 1: Compounds 1-3

Dovyalicin G; (*E*)-N-(4-(1,5-diazocan-1-yl)butyl)-N-methyltetradec-2-enamide (1): R_f = 0.4, TLC solvent systems (DCM-MeOH-NH_3 95:5:0.1), gave positive result with Dragendorff's spraying reagent, obtained as yellowish-white powder displayed a molecular ion peak at *m/z* 408 [M^++H]. Other fragment ions at *m/z* 336 ((M- $(CH_2)_4CH_3$) (base peak)) and *m/z* 265 (M- $(CH_2)_9CH_3$); suggesting a cleavage between C-4'' and C-5'' to give resonance stabilized carbocation (allylic carbocation) (Hiserodt et al. 2004), in addition to *m/z* 113 =$C_6H_{13}N_2$; suggesting 1,5- diazocane ring attached to the side chain at the nitrogen of position number 1).

The ^{1}H NMR signals at δ 2.90 (4H, m, H-2, H-8), 1.24 (4H, m, H-3, H-7), 2.18 (4H, m, H-4, H-6), 1.5 (1H, brs, NH-5) and APT- NMR signals at δ 53.74 (C-2, C-8), 32.03 (C-3, C-7), 45.14 (C-4, C-6) suggesting the presence of N-$(CH_2$-CH_2-$CH_2)_2$-NH moiety (Pretsch et al. 2009). In addition to ^{1}H NMR signals at δ 3.07 (2H, m, H-1'), 1.55 (2H, m, H-2'), 1.41

(2H, m, H-3'), and 3.45(2H, m, H-4'), & APT- NMR signals at δ 53.81 (C-1'), 24.37 (C-2'), 28.45 (C-3'), and 53.36 (C-4'), which suggest the presence of -N-$(CH_2)_4$-N- moiety. The presence of three APT- NMR signals at about 53ppm; two of them was equivalent (δ 53.73) and one at δ 53.81 indicating the presence of tertiary amine (in which the nitrogen atom attached to two equivalent carbons) (Pretsch et al. 2009). The previous data could be comply with 1,5-diazocane ring attached to butyl moiety at N-1, which is the nucleus of Dovyalicin –type spermidine alkaloids isolated from the genus Dovyalis (Rasmussen et al. 2006; Stærk et al. 2003).

The ^{1}H NMR spectrum showed, also two olefinic protons; δ 6.19 (1H, d, *J*=14.5Hz, H-2'') and 6.85 (1H, dt, *J*=14.5, 7.1Hz, H-3''), which indicating two protons on a double bond with trans- coupling as displayed by *J*-value of 14.5Hz. The more down fielded proton at δ 6.85 is adjacent to CH_2 (C-4'') of the straight chain of the tetradec-2-enamide moiety as displayed by triplet of *J*=7.1Hz, and signals at δ 2.18 (2H, m, H-4''), 1.23 -1.39 (18H, m, H-5'' to H-13''), 0.88 (3H, t, *J*=7.1, H-14'') also confirm this straight side chain. The APT-NMR spectrum exhibited two olefinic carbon signals at δ 119.8 (C-2''), and 147.8 (C-3''); suggesting a double bond adjacent to carbonyl group (*αβ*- unsaturated carbonyl moiety). The signal at δ 167ppm is typical for amide carbonyl group. Besides carbon signals of straight chain fatty acid residue at δ 32.7 (C-4''), 29.4 -29.8 (C-5'' to C-11''), 31.7 (C-12''), 22.8 (C-13''), 14.2 (C-14''). The presence of singlet at δ 3.05 in ^{1}H NMR spectrum and one methyl signal at δ 35.56 in APT-NMR spectrum indicating the presence of N-CH_3. The structure of compound (1) was confirmed by 2D-NMR experiment (HMBC) as illustrated in figure 2.

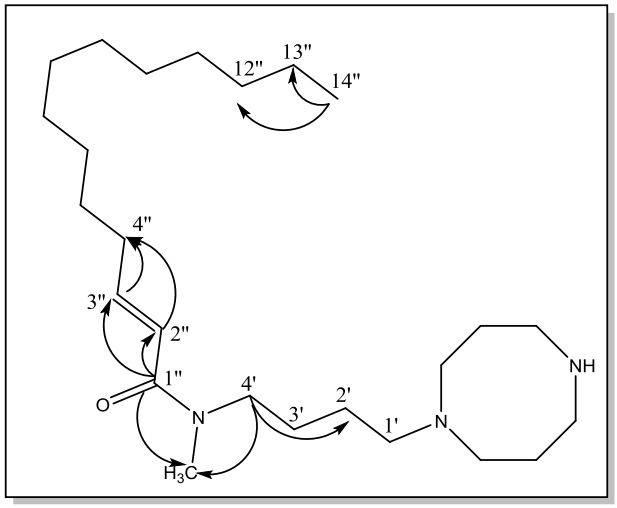

Figure 2: HMBC-correlations of Compound (1)

The HMBC (figure 2) showed the following correlations: C-1'' correlates with both H-2'' and H-3'' (confirming that the double bond is conjugated with the carbonyl group); the methyl protons of N-CH3 correlates with C-1''; H-4' correlates with the carbon of N-CH3 (confirming the position of N-CH3) and C-2'; H-4'' correlates with C-3'' and C-2''; and finally methyl protons of C-14'' correlates with C-13'' and C-14''.

Compound (2): By comparing the spectral data with that of the published literature (Aliba, Ndukwe, and Ibrahim 2018), the compound was identified as *β*-sitosterol which is previously isolated from the same plant (Sayed et al. 2000).

Compound (3): The spectral data of compound (3) was compared with that of published literature (Wang et al. 2006) and identified as Hentriacontan-1-ol. It is worth mentioning that this long chain fatty alcohol (Hentriacontan-1-ol) is first reported from the genus Dovyalis.

GC/MS of Petroleum Ether Fraction (Lipoid Matter) of *Dovyalis caffra*:

The results of GC/MS analysis of the saponifiable matter of *D. caffra* leaves and young stems shown in table (1) revealed the presence of 12 compounds from which four were identified as methyl esters of saturated fatty acids (63.77%), three were identified as methyl esters of unsaturated fatty acids (19.94%), one was identified as a saturated dicarboxylic acid methyl ester (9,12-Octadecadienoic acid methyl ester; 13.54%) and one methoxylated fatty acid (1.16%) whereas three compounds (1.57%) couldn't be identified.

Palmitic acid (Hexadecanoic acid) methyl ester was the major identified fatty acid methyl ester (53.27%) followed by linolenelaidic acid (9,12,15-(*Z*,*Z*,*Z*)-Octadecatrienoic acid) methyl ester (16.68%), linoleic acid (9,12-(*Z*,*Z*)-Octadecadienoic acid) methyl ester (13.54%), stearic acid methyl ester (7.12%), tetradecanoic acid methyl ester (2.74%) and 2-(*E*)- Hexadecenoic acid methyl ester (1.85%) while the other identified fatty acids methyl esters were in minor amounts.

Identification of the fatty acid methyl esters was done by comparison of their retention times and by matching their mass spectra with the national institute of standards and technology (NIST-11) library.

Table 1: GC/MS of saponifiable matter of *D. caffra* leaves and young stems

Peak No.	Retention Time (min.)	Name	Area (%)	Base Peak (m/z)
1	30.037	Tetradecanoic acid methyl ester	2.74	74.05
2	35.450	Unknown Compound	0.29	43.00
3	35.569	Hexadecanoic acid methyl ester	53.27	74.05
4	36.875	2-(*E*)- Hexadecenoic acid methyl ester	1.85	87.05
5	38.085	Heptadecanoic acid methyl ester	0.64	74.05
6	39.740	9,12-Octadecadienoic acid methyl ester	13.54	67.05
7	39.912	9,12,15-(*Z,Z,Z*)- Octadecatrienoic acid methyl ester	16.68	79.05
8	40.025	Unknown Compound	0.62	74.05
9	40.479	Methyl stearate	7.12	74.05
10	43.040	2-Methoxyoctanoic acid methyl ester	1.16	129.15
11	43.142	9,12,15-(*Z,Z,Z*)-Octadecatrienoic acid methyl ester	1.41	87.10
12	43.540	Mixture of more than one compound	0.66	69.10

The results of the GC/MS analysis of the unsaponifiable matter of *D. caffra* leaves and young stems shown in table (2) revealed the presence of 5 compounds from which 3 (66.87%) were identified whereas two compounds (33.13%) couldn't be identified. The identified compounds were classified as fatty alcohols (mono- or polyhydric) (54.49%), and steroids (45.51). *β*-sitosterol was the major identified compound from the unsaponifiable matter (45.51%) followed by different mono- and polyhydric fatty alcohols (the exact structures were not confirmed for two of them (33.13%) and the third was identified to be 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (16.32%)) and phytol (5.04%). Phytol, which is an acyclic diterpene alcohol functions as a precursor for vitamin E and K1 and it has antioxidant and anticancer activities (El-Kashef et al. 2014).

Identification of the compounds was carried out by matching their retention times and by matching their mass spectra with the national institute of standards and technology (NIST-05) library.

Table 2: GC/MS of unsaponifiable matter of *D. caffra* leaves and young stems

Peak No.	Retention Time (min.)	Name	Area (%)	Base Peak (m/z)
1	34.004	Phytol	5.04	71.05
2	37.271	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	16.32	43.10
3	52.539	Polyhydric fatty alcohol (the exact structure is not confirmed)	17.83	57.10
4	55.342	Polyhydric fatty alcohol with isoprenoid moiety (the exact structure is not confirmed)	15.30	57.05
5	56.432	*β*- Sitosterol	45.51	43.05

Cytotoxic activity:

All of Dovyalis caffra extracts showed promising activities against three cell lines (Table 3). Total extract, pet. ether fr. and alkaloid fr. were the most active against HEP-G2 and MCF-7 cell lines. Total extract, pet. ether fr. and BuOH fr. were the most active against HCT-116 cell line (Table 3). The promising activities pet. ether fr. and alkaloid fr. encourage us to explore mechanism of action of cytotoxicity by testing inhibition of activity of Topoisomerase II.

Table 3: IC_{50} in µg/ml of different *D. caffra* extracts against the specified cell lines:

	MCF-7	HCT-116	HEP-G2
Total Extract	4.01 ± 0.37	2.24 ± 0.24	2.18 ± 0.2
Alkaloid Fraction	3.87 ± 0.3	5.43 ± 0.5	3.25 ± 0.31
Pet. Ether Fraction	3.87 ± 0.31	3.18 ± 0.3	2.25 ± 0.21
EtOAc Fraction	11.13 ± 1.12	7.32 ± 0.76	5.41 ± 0.47
BuOH Fraction	4.21 ± 0.46	4.13 ± 0.48	3.74 ± 0.28

Topoisomerase II Inhibition Activity:

Topoisomerases are crucial enzymes that control the higher-order structural state of DNA. By selective cleaving, the problems of DNA are resolved by temporarily cleaving both strands of a DNA duplex to form a cleavage complex through which another DNA segment can be transported (Schoeffler and Berger 2008). Topoisomerases can be classified in two general categories, termed type I or type II, depending on whether one or both DNA strands of a single duplex are cleaved during a catalytic cycle, respectively (Deweese and Osheroff 2008). A variety of small-molecule agents capable of inducing such effects are widely prescribed as anti-cancer drugs which in turn increase the population of topoisomerase II (TOP2) breaking complexes, which leads to TOP2-mediated chromosome DNA cleavage and ultimately death of cancer cells (Pommier et al. 2010).

Alkaloid and pet. ether fractions were selected (based on cytotoxic activities) to study their inhibition potential for topoisomerase II; TOP2B (Table 4). Both tested fractions showed inhibitory activities at the sub-micromolar level (IC_{50} = 37 and 110 nM, for alkaloid and petroleum ether fractions respectively). It is worth mentioning that the IC_{50} of alkaloid fraction was close to that of the standard (Staurosporine; IC_{50} = 10 nM), suggesting that inhibition is one of the involved mechanisms of cytotoxicity.

Table 4: IC_{50} of Topoisomerase II Inhibition for Alkaloid and Pet. Ether Fractions of *D. caffra*:

	(TOP2B)
	IC_{50} conc. (nM)
Petroleum Ether Fraction	110
Alkaloid Fraction	37
Staurosporine (Reference Control)	10

Antimicrobial activities of the different extracts and purified alkaloid fraction of *Dovyalis caffra*:

In the worldwide as well as in the developing countries, the most human died due to infectious bacterial diseases (Nathan 2004). The bacterial organisms including Gram-positive and Gram-negative bacteria like different species of Bacillus, Staphylococcus, Salmonella and *E. coli* are the main source to cause severe infections in human because these organisms have the ability to survive in harsh condition due to their multiple environmental habitats (Ahameethunisa and Hopper 2010).

Herein we demonstrate the antimicrobial activity of the total extract, alkaloid, petroleum ether, DCM, ethyl acetate, and n-butanol fractions of *D. caffra*. The different extracts of *D. caffra* were tested against a range of microorganisms including two Gram-positive, two Gram-negative bacetria and one fungal species using the agar well diffusion assay method and the results were shown in Table (5).

The alkaloid fraction was the most active against all tested microorganisms, BuOH fraction was active against gram-positive bacteria (*Staph. aureus* and *B. subtilis*) and the tested fungal species (*A. niger*), while it showed no activity against tested gram-negative bacteria. The total extract showed activity against both *Staph. aureus* and *A.niger* and DCM-fraction showed activity against *A. niger* only. On the other hand, pet. ether and EtOAc fractions showed no antimicrobial activity against any of the tested microorganisms.

Table 5: Antimicrobial activity of *Dovyalis caffra* extracts and purified alkaloid fraction (zone of inhibition in mm)

		Staphylococcus aureus (ATCC 25923)	*Bacillus subtilis* (ATCC 6633)	*Salmonella typhi* (ATCC 700931)	*Escherichia coli* (ATCC 25922)	*Aspergillus niger* (ATCC 6275)
TEST SAMPLES (50mg/ml)	**Total Extract**	22	NA	NA	NA	29
	Alkaloid Fraction	24	17	15	14	30
	Pet. Ether Fraction	NA	NA	NA	NA	NA
	DCM Fraction	NA	NA	NA	NA	18
	EtOAc Fraction	NA	NA	NA	NA	NA
	BuOH Fraction	16	14	NA	NA	18
Positive control For Bacteria	**Gentamicin 10µg/ml**	20	21	19	24	
Positive control for fungi	**Fluconazole 50µg/ml**					25
Negative control	**DMSO**	NA	NA	NA	NA	NA

Anti-inflammatory Activities:

Inflammation is caused by release of chemicals from tissues and migrating cells. Most strongly implicated are the prostaglandins (PGs), leukotrienes (LTs), histamine, bradykinin, in addition to more recent; platelet-activating factor (PAF) and interleukin-1. Evidence for their involvement comes from studies with competitive antagonists for their receptors and inhibitors of their synthesis. H_1 histamine antagonists are effective for hay fever and some skin allergies such as urticaria, which indicates the importance of histamine in these conditions. Symptoms of rheumatoid arthritis are alleviated by the aspirin-like anti-inflammatory drugs, which inhibit the cyclooxygenase enzyme and reduce synthesis of prostanoids. (Vane and Botting 1987).

The *in vitro*- anti-inflammatory activities of total methanolic and ethyl acetate extracts of *D. caffra* leaves and young stems were investigated via determination of cyclooxygenase-1 and -2 (COX-1and COX-2) and 5-Lipoxygenase (5-LOX) inhibition activities. The selection of these two extracts for the study was based on folk medicine use of *D. caffra* and TLC profiling of different extracts of the plant.

Cyclooxygenase-1 and -2 (COX-1 and -2) inhibition activities:

Cyclooxygenases (COX, also known as Prostaglandin H Synthase or PGHS) are responsible for the biosynthesis of PGs under acute inflammatory conditions (Blobaum and Marnett 2007; Xie et al. 1991). The induced COX-2 is believed to be the target enzyme for the anti-inflammatory activity of non- steroidal anti-inflammatory drugs (Blobaum and Marnett 2007).

The total methanolic and ethyl acetate extracts of *D. caffra* leaves and young stems COX-1 and 2 inhibition activity results are shown in table (6). Both tested extracts showed remarkable inhibitory activities for both enzymes. It is worth mentioning that the total methanolic extract of *D. caffra* is more selective toward COX-2 as is expressed by selectivity ratio; which is the ratio between IC_{50} of COX-1 and COX-2, expressing the affinity toward both enzymes (IC_{50} for COX-1 / IC_{50} for COX-2; 1.91) (Botting 2006); comparing with the ratio for the standard, celecoxib (selectivity ratio = 2.51).

Table 6: IC_{50} of COX-1 and 2 inhibition activities for total extract and EtOAc fraction of *D. caffra*:

	COX-1 IC_{50} (nM)	**COX-2 IC_{50} (nM)**	**Selectivity ratio**
Total Extract	151.38	79.28	1.91
EtOAc Fraction	220.06	250.48	0.88
Celecoxib	165	65.61	2.51

1. 5-Lipoxygenase (5-LOX) inhibition activities:

5-Lipoxygenase (5-LOX) is a non-heme iron-containing dioxygenase that converts unsaturated fatty acids to epoxides; for example the synthesis of leukotrienes from arachidonic acid. It is involved in processes like cell proliferation, differentiation and inflammation and has been implicated in inflammation and hyper proliferation mediated diseases like asthma, rheumatoid arthritis and cancer(Choi et al. 2008). The total methanolic and ethyl acetate extracts of *D. caffra* (Warb.) leaves and young stems were assayed for the 5-LOX inhibitory activity to complete the *in vitro* anti-inflammatory investigation, while the results showed weak activity comparing with the specified standard, zileuton (table 7).

Table 7: IC_{50} of 5-LOX inhibition activity for total extract and EtOAc fraction of *D. caffra*:

	5-LOX IC_{50} (µM)
Total Extract	4.96 ± 0.22
EtOAc Fraction	3.31 ± 0.14
Zileuton	0.04

REFERENCES:

Abdelhaleem, Eman F, Mohammed K Abdelhameid, Asmaa E Kassab, and Manal M Kandeel. 2018. 'Design and synthesis of thienopyrimidine urea derivatives with potential cytotoxic and pro-apoptotic activity against breast cancer cell line MCF-7', *European journal of medicinal chemistry*, 143: 1807-25.

Ahameethunisa, Abdul R, and Waheeta Hopper. 2010. 'Antibacterial activity of *Artemisia nilagirica* leaf extracts against clinical and phytopathogenic bacteria', *BMC complementary and alternative medicine*, 10: 6.

Aliba, MO, IG Ndukwe, and H Ibrahim. 2018. 'Isolation and characterization of B-sitosterol from methanol extracts of the stem bark of large-leaved rock fig (*Ficus abutilifolia* Miq)', *Journal of Applied Sciences and Environmental Management*, 22: 1639-42.

Blobaum, Anna L, and Lawrence J Marnett. 2007. 'Structural and functional basis of cyclooxygenase inhibition', *Journal of medicinal chemistry*, 50: 1425-41.

Botting, RM. 2006. 'Inhibitors of cyclooxygenases: mechanisms, selectivity and uses', *Journal of physiology and pharmacology*, 57: 113.

Chase, M. W., M. J. M. Christenhusz, M. F. Fay, J. W. Byng, W. S. Judd, D. E. Soltis, D. J. Mabberley, A. N. Sennikov, P. S. Soltis, and P. F. Stevens. 2016. 'An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV', *Botanical Journal of the Linnean Society*, 181: 1-20.

Chase, Mark W, Sue Zmarzty, M Dolores Lledo, Kenneth J Wurdack, Susan M Swensen, and Michael F Fay. 2002. 'When in doubt, put it in Flacourtiaceae: a molecular phylogenetic analysis based on plastid rbcL DNA sequences', *Kew Bulletin*, 57: 141-81.

Cheek, Martin, and Rita Ngolan. 2006. 'A reassessment of the *Dovyalis spinosissima* (Flacourtiaceae-Salicaceae) complex in Africa, with a new species from Cameroon', *Kew Bulletin*, 61: 595-600.

Choi, Jongkeun, Jae Kyung Chon, Sangsoo Kim, and Whanchul Shin. 2008. 'Conformational flexibility in mammalian 15S-lipoxygenase: reinterpretation of the crystallographic data', *Proteins: Structure, Function, and Bioinformatics*, 70: 1023-32.

Deweese, Joseph E, and Neil Osheroff. 2008. 'The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing', *Nucleic acids research*, 37: 738-48.

El-Kashef, Dina, Ashraf Hamed, Hany Khalil, and Mohamed Kamel. 2014. Investigation of the Unsaponifiable and Saponifiable Matters of *Pachypodium*

lamerei Drake Leaves and Stems by GC/MS (Journal of Pharmacognosy and Phytochemistry, 3(1), 128-132.

El-Menshawi, Bassem S, Walid Fayad, Khaled Mahmoud, Salwa M El-Hallouty, and May El-Manawaty. 2010. 'Screening of natural products for therapeutic activity against solid tumors'.

Hassanin, Hany M, Rabah AT Serya, Wafaa R Abd Elmoneam, and Mai A Mostafa. 2018. 'Synthesis and molecular docking studies of some novel Schiff bases incorporating 6-butylquinolinedione moiety as potential topoisomerase II*β* inhibitors', *Royal Society open science*, 5, 1-13.

Hiserodt, Richard D, Barry M Pope, Michael Cossette, and Mark L Dewis. 2004. 'Proposed mechanisms for the fragmentation of doubly allylic alkenamides (tingle compounds) by low energy collisional activation in a triple quadrupole mass spectrometer', *Journal of the American Society for Mass Spectrometry*, 15: 1462-70.

Huang, Yuanzheng, Bin Zhang, Jiaming Li, Huicai Liu, Yanchun Zhang, Zhang Yang, and Wandong Liu. 2019. 'Design, synthesis, biological evaluation and docking study of novel indole-2-amide as anti-inflammatory agents with dual inhibition of COX and 5-LOX', *European Journal of Medicinal Chemistry*, 181: 41 - 50.

Kassab, Asmaa E, and Ehab M Gedawy. 2018. 'Novel ciprofloxacin hybrids using biology oriented drug synthesis (BIODS) approach: anticancer activity, effects on cell cycle profile, caspase-3 mediated apoptosis, topoisomerase II inhibition, and antibacterial activity', *European journal of medicinal chemistry*, 150: 403-18.

Lemke, David E. 1988. 'A synopsis of Flacourtiaceae', *Aliso: A Journal of Systematic and Evolutionary Botany*, 12: 29-43.

Liang, Hanqiao, Yongmei Xing, Juan Chen, Dawei Zhang, Shunxing Guo, and Chunlan Wang. 2012. 'Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicus* (Liliaceae)', *BMC complementary and alternative medicine*, 12: 238 - 43.

Moustafa, Seham MA, Bassem M Menshawi, Gamila M Wassel, K Mahmoud, and MM Mounier. 2014. 'Screening of some plants in Egypt for their cytotoxicity against four human cancer cell lines', *Int J Pharm Tech Res*, 6: 1074-84.

Nathan, Carl. 2004. 'Antibiotics at the crossroads', *Nature*, 431: 899.

Pommier, Yves, Elisabetta Leo, HongLiang Zhang, and Christophe Marchand. 2010. 'DNA topoisomerases and their poisoning by anticancer and antibacterial drugs', *Chemistry & biology*, 17: 421-33.

Pretsch, Ernö, Phillippe Buehlmann, Christian Affolter, Ernho Pretsch, P Bhuhlmann, and C Affolter. 2009. *Structure determination of organic compounds* (Springer).

Rasmussen, Bonnie, Aimee-Justine Nkurunziza, Matthias Witt, Hellen A Oketch-Rabah, Jerzy W Jaroszewski, and Dan Stærk. 2006. 'Dovyalicin-type spermidine alkaloids from Dovyalis species', *Journal of Natural Products*, 69: 1300-04.

Reddy, C Madhava, Vadiraja B Bhat, G Kiranmai, M Narsa Reddy, P Reddanna, and KM Madyastha. 2000. 'Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from Spirulina platensis', *Biochemical and biophysical research communications*, 277: 599-603.

Sayed, HM, DW Bishay, SA Yousef, MS Kamel, and RM Abdel-Salam. 2000. 'Alkaloids from the leaves of Aberia caffra'.

Schäfer, Angelika, Zuzana Chovanová, Jana Muchová, Katarína Sumegová, Anna Liptáková, Zdeňka Ďuračková, and Petra Högger. 2006. 'Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol)', *Biomedicine & Pharmacotherapy*, 60: 5-9.

Schoeffler, Allyn J, and James M Berger. 2008. 'DNA topoisomerases: harnessing and constraining energy to govern chromosome topology', *Quarterly reviews of biophysics*, 41: 41-101.

Skehan, Philip, Ritsa Storeng, Dominic Scudiero, Anne Monks, James McMahon, David Vistica, Jonathan T Warren, Heidi Bokesch, Susan Kenney, and Michael R Boyd. 1990. 'New colorimetric cytotoxicity assay for anticancer-drug screening', *Journal of the National Cancer Institute*, 82: 1107-12.

Stærk, Dan, Matthias Witt, Hellen A Oketch-Rabah, and Jerzy W Jaroszewski. 2003. 'A new class of spermidine-derived alkaloids', *Organic Letters*, 5: 2793-96.

Stanstrup, Jan, Anne-Mette Rusch, Sara Agnolet, Hasse B Rasmussen, Per Mølgaard, Johannes Van Staden, Gary I Stafford, and Dan Staerk. 2010. 'Itoside A and 4-hydroxytremulacin from Dovyalis caffra and Dovyalis zeyheri', *Biochemical Systematics and Ecology*, 38: 346 - 48.

Steyn, EMA, AE Van Wyk, and GF Smith. 2005. 'Ovule-to-seed development in *Dovyalis caffra* (Salicaceae: Flacourtieae) with notes on the taxonomic significance of the extranucellar embryo sac', *Bothalia*, 35: 101-08.

Vane, JOHN, and Regina Botting. 1987. 'Inflammation and the mechanism of action of anti-inflammatory drugs', *The FASEB Journal*, 1: 89-96.

Wang, J. D., M. L. Dong, W. Zhang, X. Shen, and Yue-Wei Guo. 2006. 'Chemical constituents of mangrove plant *Aegiceras corniculatum*', *Chinese Journal of Natural Medicines*, 4: 275-77.

Xie, WL, Jeffrey G Chipman, Donald L Robertson, RL Erikson, and Daniel L Simmons. 1991. 'Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing', *Proceedings of the National Academy of Sciences*, 88: 2692-96.

Yubin, JI, Yu Miao, Wang Bing, and Zhang Yao. 2014. 'The extraction, separation and purification of alkaloids in the natural medicine', *J Chem Pharm Res*, 6: 338-45.

Zaki, D. 1975. 'Biological investigation of *Dovyalis caffra*', *Planta medica*, 27: 330-32.

الملخص العربى

فصل قلويد جديد من نوع (دوفياليسين/ سبيرميدين) من نبات الدوفياليس كافرا المنتمى للعائلة الصفصافية والمزروع فى مصر

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أسفرت الدراسة الفيتوكيميائية –للأوراق والأغصان النامية- لنبات الدوفياليس كافرا عن فصل قلويد جديد من نوع (دوفياليسين/ سبيرميدين)، وتم التعرف عليه بواسطة الطرق الطيفية المختلفة وتسميته دوفياليسين جى (١). كما تم – أيضا- الفصل والتعرف على مركبين (سبق فصلهما والتعرف عليهما) وهما: بيتاسيتوسترول (٢)، والكحول الدهنى ١- هنترياكونتانول (٣). هذا بجانب بعض الدراسات الحيوية على الخلاصات المختلفة للنبات وهى: دراسة التأثير القاتل لبعض الخلايا السرطانية، والتأثير المثبط للتوبوأيزوميراز II، والفاعلية المضادة للميكروبات المختلفة، و-كذلك- الفاعلية المضادة للالتهاب (من خلال عمل مسح للتأثير المثبط لكل من: ٥-ليبوكسيجيناز والسيكلوكسيجيناز ١و٢). وتبين من الدراسة البيولوجية بأن الخلاصة الغنية بالقلويدات لها تأثير جيد مضاد للميكروبات بجانب تأثيرها الملحوظ بقتل الخلايا السرطانية و-كذلك- تأثيرها المثبط للتوبوأيزوميراز II بينما الخلاصة الكحولية لها تأثير جيد كمثبط للسيكلوكسيجيناز (وتحديدا سيكلواوكسيجيناز 2).