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Short communication

Stachaegyptin A-C: *Neo*-clerodane diterpenes from *Stachys aegyptiaca*

M-E.F. Hegazy[a,*], Ahmed R. Hamed[a,b], El-Sayeda A. El-Kashoury[c], Alaa M. Shaheen[a], Wafaa A. Tawfik[a], Paul W. Paré[d], Essam Abdel-Sattar[c]

[a] Phytochemistry Department, National Research Centre,33 El-Bohouth St., Dokki, Giza, 12622, Egypt
[b] Biology Unit, Central Laboratory for Pharmaceutical and Drug Industries Research Division, National Research Centre,33 El-Bohouth St., Dokki, Giza, 12622, Egypt
[c] Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[d] Department of Chemistry & Biochemistry Texas Tech University, Lubbock, TX 79409, USA

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ABSTRACT

Phytochemical investigation of *Stachys aegyptiaca* resulted in the characterization of three new diterpenes (**1-3**) together with eleven known compounds including four *neo*-clerodane diterpenes and seven flavonoid aglycones. Structure elucidation was performed by spectroscopic analysis by HRFABMS, 1D and 2D NMR and X-ray. Isolated compounds were screened for anti-inflammatory activity using a lipopolysaccharide-induced nitric oxide inhibition assay employing murine macrophage cells. Among the assayed compounds, **13** (calycopterin) showed a concentration-dependent inhibition of LPS-induced nitric oxide release with a IC_{50} of 62.5 μM.

1. Introduction

Stachys is one of the largest genera in the Lamiaceae family including *ca.* 300 species distributed in temperate and tropical regions except the continent of Australia (Tundis et al., 2014). *Stachys* species are reported to have anti-inflammatory, cytotoxic, antibacterial and antioxidant activities (Háznagy-Radnai et al., 2008). Previous phytochemical studies of *Stachys aegyptiaca,* locally named Qourtom, has resulted in the isolation of essential oils (Halim et al., 1991), diterpenes (Mohamed and Mohamed, 2014; ; Melek et al., 1991) and flavonoids (El-Desoky et al., 2007; Sharaf, 1998; El-Ansari et al., 1991, 1995). Herein, is reported the isolation and structures elucidation from the aerial parts of *S. aegyptiaca* seven *neo*-clerodane diterpenes including three new compounds (Fig. 1), as well as seven previously isolated flavonoids. Chemical characterization of the newly identified compounds was established by comprehensive spectroscopic analysis while known compounds were identified by comparison of NMR data with literature reports. Biological activity as anti-inflammatory agents is presented.

2. Results and discussion

A methylene chloride/methanol (1:1) extract of air dried, aerial parts of *S. aegyptiaca* was purified using normal and reversed phase chromatography to afford three new compounds (**1-3**), in addition to eleven known compounds (**4-14**) (Fig. 1).

Compound **1** was isolated as a colorless crystals (m.p. 123–126 °C) with an optical rotation of $[\alpha]_{25}^{D}$ −19.0 (*c* 0.01, MeOH). HR-FAB-MS analysis showed a molecular ion peak at *m/z* 325.2140 $[M+Na]^+$ (calcd. for $C_{20}H_{30}O_2Na^+$, 325.2144) corresponding to a molecular formula of $C_{20}H_{30}O_2$. The ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals including: four methyls, six methylenes (two olefinic), five methines (one oxygenated and two olefinic), and five quaternary carbons (one keto and two olefinic) (see Table 1). Based on *neo*-clerodane type diterpene structures commonly observed in this genus (Adinolfi et al., 1984), a characteristic oxygenated H-7 was identified at δ_H 4.07 (brd, J = 3.4). In turn, NMR signals at δ_H 1.51/2.17 and δ_H 1.62 were assigned to H_2-6 and H-8, respectively via DQF-COSY analysis. The HMBC correlations observed between oxygenated H-7 and δ_C 41.5 (C-6), δ_C 38.9 (C-8) and a methyl signal at δ_C 12.6 was assigned to C-17. Also from the HMBC spectrum, a correlation was observed between C-8 and methyl protons at δ_H 1.04 (s, 3H) which were assigned to H-20. H_3-20 correlated as well with δ_C 39.5 (C-9), δ_C 45.9 (C-10) and δ_C 37.8 (C-11). Correlation between C-11 and δ_H 1.97 allowed for the assignment of H_2-12 (Fig. 2). H_2-12 proton signal correlates with δ_H 1.41 (H_2-11) in the DQF-COSY spectrum and a down-field quaternary olefinic at δ_C 146.5 (C-13) in the HMBC spectrum. C-13 also correlates with δ_H 1.41 (H-12), δ_H 6.28 (dd, J = 11.0, 17.0 Hz, H-14), δ_H 5.00/5.14 (H-15) and $_H$ δ 4.93/4.95 (H-16) in the HMBC spectrum. The down fielded olefinic proton H-14 correlated with two olefinic methylene carbons at δ_C 113.2 (C-15) and 116.0 (C-16) characteristic for a vinyl group. The down-field proton signals indicated

* Corresponding author.
E-mail address: elamir77@live.com (M.-E.F. Hegazy).

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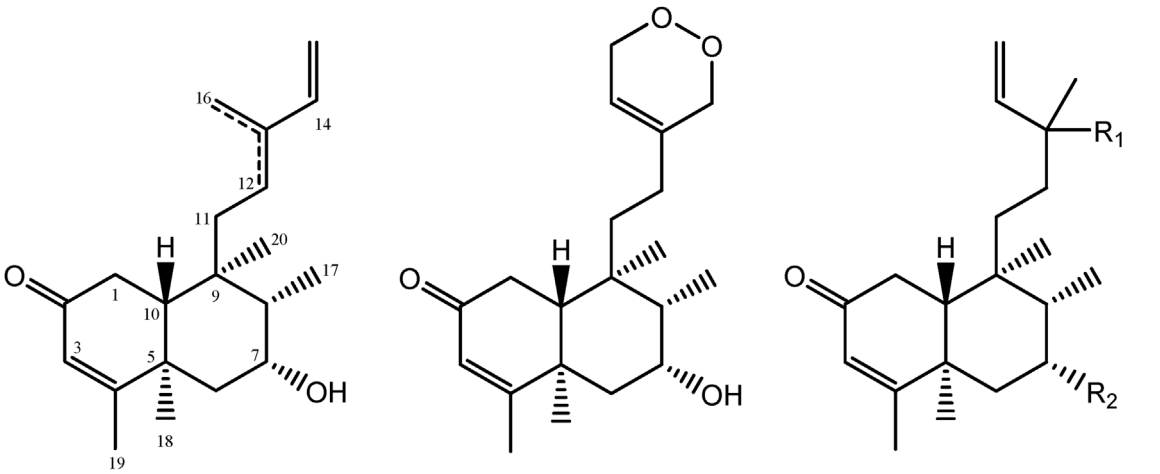
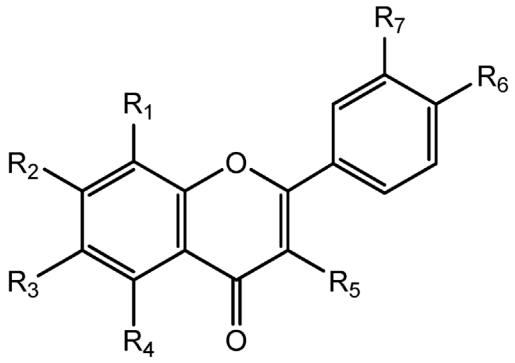


1 = $\Delta^{13,16}$
2 = Δ^{12}

3

4 R_1 = OH, R_2 = H
5 R_1 = OH, R_2 = OH
6 R_1 = OAc, R_2 = OH
7 R_1 = OAc, R_2 = OAc



8 R_1 = OMe, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = H, R_6 = OH, R_7 = H
9 R_1 = H, R_2 = OMe, R_3 =H, R_4 = OMe, R_5 = OMe, R_6 = OH, R_7 = OMe
10 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = H, R_6 = OMe, R_7 = OMe
11 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = OMe, R_6 = OMe, R_7 = H
12 R_1 = OH, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = OMe, R_6 = OMe, R_7 = H
13 R_1 = OMe, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = OMe, R_6 = OH, R_7 = H
14 R_1 = OMe, R_2 = OMe, R_3 = OMe, R_4 = OH, R_5 = OMe, R_6 = OMe, R_7 = H

Fig. 1. The isolated metabolites from *S. aegyptiaca.*

exocyclic and vinyl group double bonds at $\Delta_{13\text{-}16}$ and $\Delta_{14\text{-}15}$, respectively as being a part of the side chain system. Correlation between C-10 and methylene protons at δ_H 2.34 (dd, J = 3.4, 14.0) and 2.43 (dd, J = 14.0, 17.0), allowed the assignment of H-1. DQF-COSY showed a correlation between H-1 and δ_H 1.92 and allowed for the assignment of H-10. An HMBC correlation between H-1 and a keto group at δ_C 200.0 (C-2), as well as a broad singlet at δ_H 5.66 allowed for the assignment of H-3. Correlation between H-10 and methyl carbon at δ_C 20.2 allowed for the assignment of H-19 (δ_H 1.37, s) as well as correlation between H-19 and an olefinic carbon at δ_C 173.1 allowed for the assignment of C-4 (Fig. 2).

The presence of the olefinic unit between C3/C-4 is based on the NMR chemical shift and is consistent with an endocyclic double bond often present in neoclerodane type diterpene (Popa et al., 1972; Melek et al., 1992; Adinolfi et al., 1984).

The relative stereochemistry assignment of H-7 to a β-configuration was based on biogenetic precedent and was consistent with previously reported NMR chemical shift data for similar *neo*-clerodane type diterpenes (Popa et al., 1972; Melek et al., 1992; Adinolfi et al., 1984). The NOESY correlations between H-7 (δ_H 4.07) and H-8 (δ_H 1.62) indicated these protons are on the same β-side of the ring. H_3-17 (δ_H 1.01) showed a NOESY correlation with H_3-20 (δ_H 1.03) and H_3-19 (δ_H 1.37) indicating that these methyl groups are all on the same side in an α-configuration (Fig. 3). The structure assignments and relative stereochemistry was confirmed by x-ray crystallography (Fig. 4). Thus, **1** was assigned as 2-oxo-*neo*-cleroda-3,13(16),14-trien-7-ol and named as stachaegyptin A, a *neo*-clerodane-diterpene.

Compound **2** was obtained as a colorless oil with an optical rotation $[\alpha]_{25}^{D}$ −6.0 (*c* 0.01, MeOH). HR-FAB-MS analysis showed a molecular ion peak at *m/z* 325.2141 $[M+Na]^+$ (calcd. for $C_{20}H_{30}O_2Na^+$, 325.2144), corresponding to a molecular formula of $C_{20}H_{30}O_2$. Spectral data of **2** is shows similarity to **1** except for the position of one of the

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data for **1-3.**

No	1		2		3	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	2.34 dd (3.4, 17.0) 2.43 dd (14.0, 17.0)	35.0	2.38 dd (3.4, 17.0) 2.49 dd (14.0, 17.0)	35.1	2.31 dd (3.4, 17.0) 2.45 dd (13.7, 17.0)	34.8
2		200.4		200.3		200.1
3	5.66 brs	125.0	5.64 brs	125.0	5.68 brs	125.1
4		173.1		172.9		172.8
5		38.4		39.4		38.2
6	1.51 dd (3.4, 14.0) 2.17 dd (2.7, 14.0)	41.5	1.46[a] m 2.12* m	41.3	1.51 dd (3.4, 14.0) 2.19 dd (2.7, 14.0)	41.5
7	4.07 brd (3.4)	73.1	4.02 brd (3.4)	73.0	4.08 brd (3.4)	73.2
8	1.62 m	38.9	1.50[a] m	39.4	1.57 m	38.9
9	–	39.5	–	39.8	–	39.4
10	1.92 m[a]	45.9	2.14 dd (4.0, 14.0)	46.5	1.92 m[a]	45.9
11	1.41 m	37.8	1.89 dd (7.0, 16.5) 2.04 dd (7.5, 16.5)	37.2	1.75 m	25.5
12	1.97 m[a]	24.5	5.32 brt (7.5)	127.6	1.42 m[a]	36.3
13		146.5		136.3		135.4
14	6.28 dd (11.0, 17.0)	138.9	6.27 dd (11.0, 17.0)	141.6	5.62 brs	117.3
15	5.00 d (17.0) 5.14 d (11.0)	113.2	4.88 d (17.0) 5.03 d (11.0)	111.3	4.53 brs –	70.0
16	4.93 s 4.95 s	116.0	1.72 s	12.7	4.41 brs	72.5
17	1.01 d (7.0)	12.6	1.03 d (7.0)	12.7	1.02 d (7.0)	12.5
18	1.89 s	19.2	1.87 brs	19.3	1.91 s	19.1
19	1.37 s	20.2	1.37 s	20.3	1.39 s	20.2
20	1.03 s	19.6	1.08 s	19.1	1.08 s	19.6

[a] Overlapped signals.

double bonds making **2** a positional isomer of **1**. This hypothesis was confirmed by the presence of only one exo-oelfinic methylene group in **2** instead of two in **1** and one aliphatic methylene group in **1** was replaced by a methyl group in **2**, with the same number of degree of unsaturation. The position of double bond at $\Delta^{13,16}$ in **1** was located into $\Delta^{12,13}$ in **2**. In the DQF-COSY spectrum, the protons of H_2-11 methylene group are correlated with only one olefinic proton (5.32, H-12) in **2**, instead of two aliphatic protons in **1**. Furthermore, the downfield

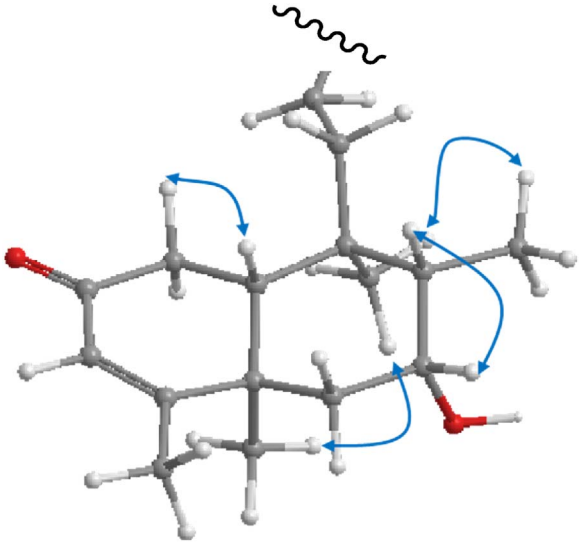


Fig. 3. Observed NOESY correlations for **1-3.**

shifted proton signal at δ_H 4.66 (brt, *J* = 7.5, H-12) is correlated with carbon signals at δ_C 37.2 (C-11), 136.3 (C-13) and 12.7 (C-16) in the HMBC spectrum, indicating that the exomethylene double bond (C-13/16) in **1** changed to C-12/C-13 in **2**, as well as, the appearance of an additional methyl group located on olefinic carbon (C-13). The similarity of stereochemistry of **2** to that of **1** was further confirmed by investigation of NOSEY spectrum which showed correlations between H-7 and H-8 and between H_3-17 and H_3-19 and H_3-20. From the aforementioned discussion and from intensive analysis of the DQF-COSY, HMQC, NOESY and HMBC spectral data, allowed for **2** to be assigned as 2-oxo-*neo*-cleroda-3,12,14-trien-7-ol and named stachaegyptin B, a new *neo*-clerodane diterpene.

Compound **3** was isolated as a colorless oil with an optical rotation of $[\alpha]_{25}^{D}$ −17.0 (*c* 0.001, MeOH). HRFABMS analysis showed a molecular ion peak at *m/z* 357.2033 $[M + Na]^+$ (calcd. for $C_{20}H_{30}O_4Na^+$, 357.2042), corresponding to a molecular formula of $C_{20}H_{30}O_4$. From ^{13}C NMR and DEPT spectra, **3** showed the presence of 20 carbon signals (Table 1): 4 methyls, 6 methylenes (2 oxygenated), 5 methines (1 oxygenated, 2 olefinic), and 5 quaternary carbons (1 keto and 2 olefinic), with six degrees of unsaturation. By comparison of its spectral spectral data (^1H and ^{13}C NMR) with **1** indicated that **3** is an *ent*-clerodane type diterpene skeleton (Adinolfi et al., 1984). The difference

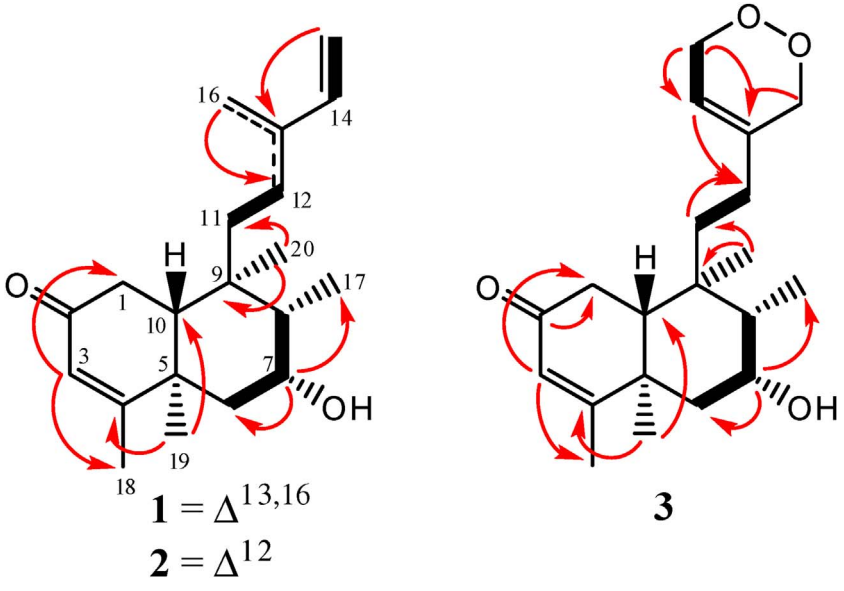


Fig. 2. Observed ^1H-^1H COSY and HMBC correlations for **1-3.**

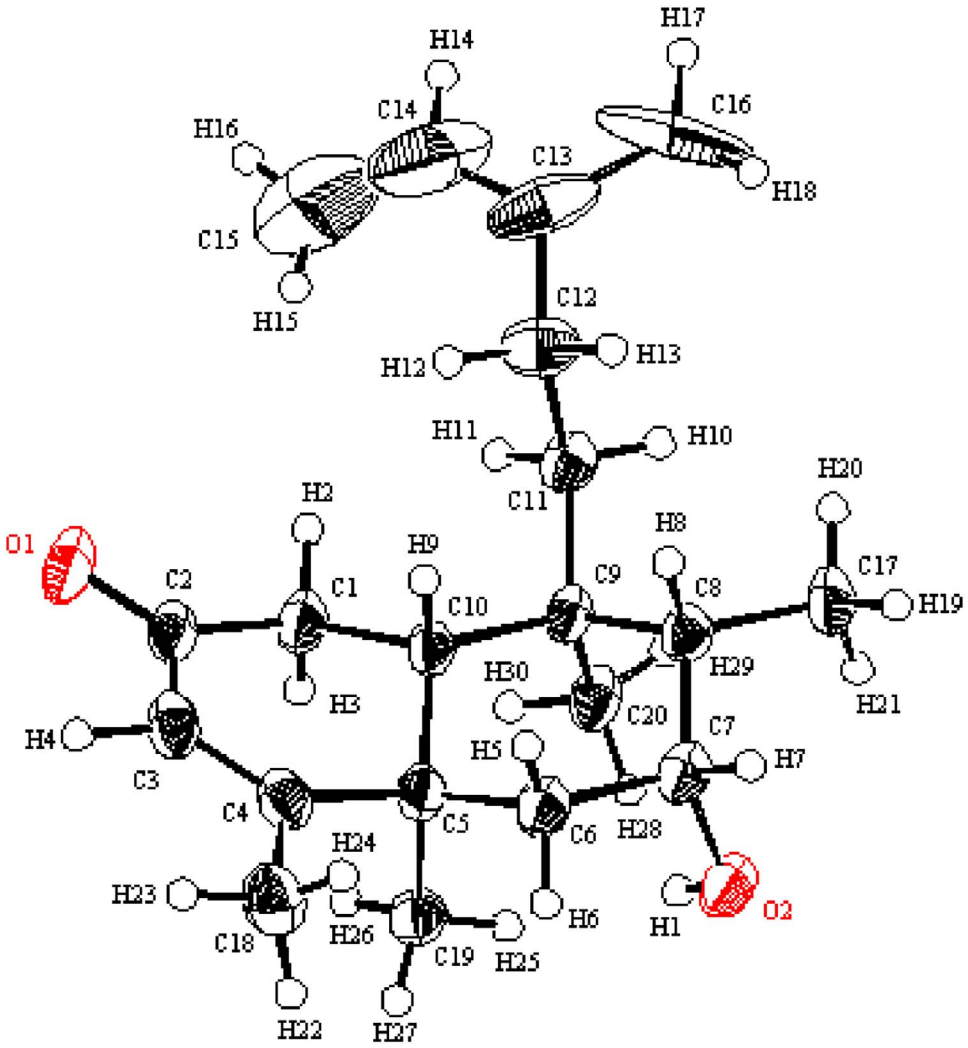


Fig. 4. X-ray of **1**.

between **3** and **1** is in the side chain at C-9 which lacks one double bond and instead showed two broad singlets in the ^{1}H NMR of two oxygenated methylene protons at δ_H 4.41 (2H, brs) and 4.53 (2H, brs). HMBC showed correlation between a characteristic methyl singlet at δ_H 1.08 (H-20), and a methylene carbon at δ_C 25.5 allowing for the assignment of C-11 and it proton signals at δ_H 1.75 (H-11). In addition, methylene protons at δ_H 1.42 (m) correlate with C-11, quaternary olefinic carbon at δ_C 135.4, and quaternary carbon at δ_C 39.4, allowing for the assignment of H-12, C-13, and C-9, respectively. The appearance of two broad singlets (^{1}HNMR) for two oxygenated methylene protons at δ_H 4.41 (2H, brs) and 4.53 (2H, brs) correlated with two olefinic carbons at δ_C 117.3 (d) and 135.4 (q) in HMBC spectrum, indicating a dioxane ring which was confirmed by HRFABMS. HMBC correlation for δ_H 4.53 (2H, brs), 4.41 (2H, brs) and 5.62 (1H, brs) with the same quaternary carbon C-13, allowed for the assignment of H-15, H-16 and H-14, respectively. All of above data combined with HRFABMS confirm the presence of a dioxane ring as a side chain at C-12.

The relative configuration was determined based on the NOESY data. H-7 was assigned to a β-configuration (Fig. 4), based on biogenetic precedent and was consistent with previously reported NMR chemical shift data for similar neoclerodane type diterpene (Popa et al., 1972; Melek et al., 1992; Adinolfi et al., 1984). Therefore, **3** was assigned as 2-oxo-*neo*-cleroda-3, 13-dien-7-ol- 15,16-endoperoxide, stachaegyptin C, a new clerodane-diterpene.

Eleven known compounds were also identified and include: 4′-hydroxy-3,5,7,3′- tetramethoxy flavones (**9**) (Likhitwitayawuid et al., 2006), eupatilin-7-methyl ether (**10**) (Balboul et al., 1997) and 5,8-dihydroxy-3,6,7,4′-tetramethoxy flavone (**12**) (Maldonado et al., 1992) have been reported for the first time from the genus *Stachys*; 5-hydroxy-3,6,7,4′-tetramethoxy flavone (**11**) (Meremeti et al., 2004; Rasool et al., 2009), having been reported for the first time from *Stachys aegyptiaca;* and other compounds identified as roseostachenone (**4**) (Pacheco et al., 2009), stachysolone (**5**) (Adinolfi et al., 1984; Popa et al., 1972), 13-monoacetyl-stachysolone (**6**) (Adinolefi et al., 1984), diacetyl stachysolone (**7**) (Adinolefi et al., 1984), xanthomicrol (**8**) (El-Ansari et al., 1991), calycopterin (**13**) (El-Ansari et al., 1991), 5-hydroxy-aurantin (**14**) (El-Ansari et al., 1991) having been reporetd before from *Stachys aegyptiaca.*

Murine macrophage cells were employed to assay compounds for LPS-induced NO release. In a preliminary screen (data not shown), all compounds were tested at single concentration of 100 μM. Among the tested compounds, **13** caused more than 50% inhibition of LPS-induced NO release in culture supernatant as revealed from the Griess assay. Therefore, this compound was subjected to further concentration − response testing. As shown in Fig. 5, co-treatment of RAW 264.7 cells with 100 ng/ml LPS and increasing serial concentrations of **13** (0–100 μM) resulted in a concentration dependent inhibition of LPS-induced NO release. The IC_{50} of this inhibition was calculated statistically as 62.5 μM, with this inhibition considered moderate.

3. Experimental

3.1. General procedures

Specific rotation was measured with Perkin–Elmer-341 MC digital

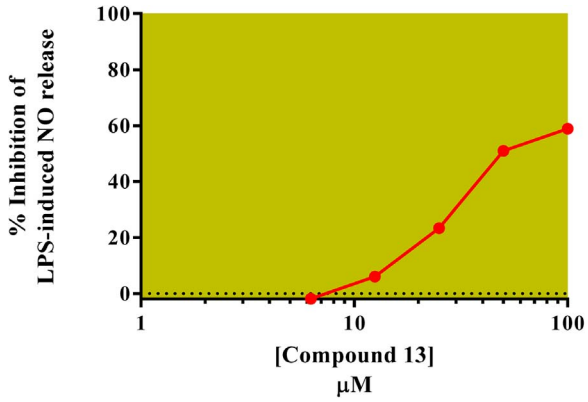


Fig. 5. Concentration-dependent inhibition of NO release by **13**.

polarimeter (Wellesley, MA, USA) and IR spectra were collected on a JASCO FT/IR-6300 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a JEOL ECA- 600 spectrometer (600 MHz= for ^{1}H and 150 MHz for ^{13}C) (JEOL Ltd., Tokyo, Japan). All chemical shifts (δ) are given in ppm units with reference to TMS as an internal standard and coupling constants (J) are reported in Hz. FAB-MS experiments were performed using a Thermo ISQ Single Quadrupole system and HR-FAB-MS experiments were performed on Fourier transform ion cyclotron mass spectrometer (Thermo Scientific, San Jose, CA, USA). High performance liquid chromatography (HPLC) was performed on an Agilent pump equipped with an Agilent-1200 with refractive index (RI) detector (Santa Clara, CA, US) and a semi-preparative reversed-phase column (Econosphere™, RP-C_{18}, 5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL, USA). Silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany) was used for column chromatography; reversed-phase silica gel for column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh. Pre-coated silica gel plates (Kieselgel 60 F_{254}, 0.25 mm, Merck, Darmstadt, Germany) were used for TLC analyses. Spots were visualized by heating after spraying with 10% H_2SO_4.

3.2. Plant material

Stachys aegyptiaca Pers plants were collected in June 2013, from South Sinai, Egypt and aerial parts air-dried. A voucher specimen has been deposited in the herbarium of St. Katherine protectorate, Egypt, as well as the herbarium of the National Research Centre (voucher ID 213), Cairo, Egypt. The collection was taken under the permission of Saint Katherine protectorate for scientific purposes.

3.3. Extraction and isolation

Aerial parts (2 kg) of *S. aegyptiaca* were powdered and extracted with CH_2Cl_2-MeOH (1:1) at room temperature. The extract was concentrated *in vacuo* to obtain a residue of 118 g. The residue was fractionated on a silica gel column (6 × 120 cm) eluting with *n*-hexane (3000 mL) followed by a gradient of *n*-hexane-$CHCl_3$ up to 100% $CHCl_3$ and $CHCl_3$-MeOH up to 100% MeOH (2000 mL each of the solvent mixture). The *n*-hexane-$CHCl_3$ (1:3) fraction (15.77 g) and 100% CH_2Cl_2 (7.5 g) were added together due to same chromatographic pattern then chromatographed on a ODS column (3 × 90 cm) eluted with 80%, 90% (MeOH:H_2O) then washing with 100% MeOH. Fractions were obtained as two main portions: A (6.5 g), and B (6.3 g). Sub-fraction A was re-purified by reversed-phase HPLC using MeOH/H_2O (65–35% 2500 mL) to afford **1** (14 mg), **2** (16 mg), **7** (5 mg) and **13** (15.5 mg). (Sub-fraction B was re-purified by reversed-phase HPLC using MeOH:H_2O (70:30%, 1000 mL) to afford **3** (8.6 mg), **9** (3.5 mg), **11** (5.3 mg), **12** (3.4 mg) and **14** (4.5 mg). The 5% MeOH fraction (8.5 g) was chromatographed on ODS column (3 × 90 cm) eluted with 80%, 90% (MeOH:H_2O) then washed with MeOH. Fractions were obtained as one main portions (2.5 g), which was re-purified by reversed-phase HPLC using MeOH:H_2O (80:20%, 2500 mL) to afford **4** (7.5 mg) and **6** (65.8 mg).

The 15% MeOH fraction (7.9 g) was chromatographed on ODS column (3 × 90 cm) eluted with 80%, 90% (MeOH:H_2O) then washing with MeOH. Fractions were obtained as one main portion(3.6 g), which was re-purified by reversed-phase HPLC using MeOH/H_2O (70–30%, 2500 mL) to afford **5** (5.75 mg), **8** (4 mg) and **10** (3.8 mg).

3.3.1. 2-Oxo-neo-cleroda-3, 13(16), 14-trien-7-ol (Stachaegyptin A, 1)

Colorless crystals; [α]25*D* −19(c 0.01, MeOH)); FT-IR (KBr) υ_{max}: 3450, 2933, 1745, 1455, and 1220 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 325.2140 (M + Na); (calcd. for $C_{20}H_{30}O_2Na$).

3.3.2. X-ray crystallography data

Single crystal X-ray analysis established the complete structure and relative configuration of compound **1** and the crystal data are summarized as follows: $C_{20}H_{30}O_2$, formula wt 302.46, Orthorhombic, space group $P4_32_12_1$, a = 9.6965(9) Å, b = 9.7317(8) Å, c = 38.076(3) Å, V = 3580.0(6) $Å^3$, Z = 8, D_{cacld} = 1.122 g/cm^3, crystal size 0.300 × 0.280 × 0.070 mm^3. All diagrams and calculations were performed using Rigaku R-AXIS RAPID diffractometer, using graphite monochromated Mo-Ka (radiation (λ = 0.71075 Å). The structures were refined by full matrix least squares on F^2 using Brucker SHELX-97.[22] The final *R* and R_w were 0.1147 and 0.1766, respectively. CCDC 1546725 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

3.3.3. 2-Oxo-neo-cleroda-3, 12, 14-trien-7-ol (Stachaegyptin B, 2)

Colorless oil; [α]25D −6 (c 0.001, MeOH)); FT-IR (KBr) υ_{max}: 3450, 2933, 1745, 1455, and 1220 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 325.2141 (M+Na); (calcd. for $C_{20}H_{30}O_2Na$)

3.3.4. 2-Oxo-neo-cleroda-3, 13-dien-7-ol- 15,16-endoperoxide (Stachaegyptin C, 3)

Colorless oil; [α]25D −17 (c 0.01, MeOH); FT-IR (KBr) υ_{max}: 3450, 2933, 1745, 1455, and 1220 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 357.2033 (M+Na); (calcd. for $C_{20}H_{30}O_4Na$).

3.4. Cell culture

RAW 264.7 murine macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified incubator. Cells were routinely sub-cultured by scrapping from the culture vessels when they are 75%–80% confluent.

3.5. Anti-inflammatory assay

Murine macrophage RAW 264.7 cells (0.5 × 10^6/ml) were seeded onto 96-well plates and incubated for 24 h under 37 °C, 95% Air and 5% CO_2 in a Certomat® 20 incubator (Sartorius Stedim Biotech GmbH, Gemany). To test the inhibitory action of compounds on NO release, cells were co-treated with 0.1% DMSO or an isolated compound (100 μM) in the presence of 100 ng/ml lipopolysaccharides (LPS, from *E.coli* serotype O111:B4, Sigma-Aldrich, Germany) for 48 h. Nitric oxide (NO) release in culture supernatants was then estimated using Griess reaction by adding equal volumes of supernatant and Griess reagent (Baeuerle, 1998; Xie, 2001). Absorbance was then measured at 540 nm (OD_{540nm}) on a Fluostar Optima microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Data were presented as %Inhibition of NO release calculated from the following equation:

%NO inhibition = $100 - \left(100 \times \frac{T}{C}\right)$ where T = mean of $OD_{540\ nm}$ recorded for the supernatants from cells co-treated with test sample + LPS, C = mean $OD_{540\ nm}$ recorded for the supernatants from LPS only-treated cells.

IC_{50} values (concentrations of samples that inhibit 50% of LPS-induced NO) were statistically derived from the concentration-response curves plotted on Graphpad Prism v6.0 (Graphpad Software, San Diego, USA) by fitting the curves to non-linear regression model.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2017.06.003.

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