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Published in:
Planta Medica

Publication date:
2003

Document version
Publisher's PDF, also known as Version of record

Terpenoids of *Salvia hydrangea*: Two New, Rearranged 20-Norabietanes and the Effect of Oleanolic Acid on Erythrocyte Membranes

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Original Paper

Abstract

Four abietane-type terpenoids, including two known royleanones and two new, rearranged 20-norabietanes, were isolated from the roots of the Iranian medicinal plant *Salvia hydrangea* DC. ex Bentham (Lamiaceae), which is used as an anthelmintic and antileishmanial remedy. Their structures were established using COSY, NOESY, HSQC, and HMBC spectral data. The possible identity of one of the 20-norabietanes with demethylmulticauline, previously reported from a different *Salvia* species, is discussed. A moderate *in vitro* antiplasmodial effect of the extract of *S. hydrangea* flowers was found to be associated with the presence of large amounts of pentacyclic triterpenes, mainly oleanolic acid. The observed antiplasmodial activity of oleanolic acid is apparently due to its incorporation into the erythrocyte membrane, which adversely affects the growth of *Plasmodium falciparum* parasites. Thus, oleanolic acid caused transformation of erythrocytes into stomatocytes in the concentration range where the *in vitro* antiplasmodial activity was observed.

Key words
Salvia hydrangea · Lamiaceae · norabietanes · *Plasmodium falciparum* · royleanones · stomatocytes · erythrocyte membrane

Introduction

Some *Salvia* species (Lamiaceae), notably the Chinese red-rooted sage *Salvia miltiorrhiza* Bunge, are well-known medicinal plants [1], [2], [3]. The medicinal properties of *S. miltiorrhiza* are believed to be associated with the presence of tanshinones, which are 20-norditerpenoids that have a quinone moiety in the C-ring [4], [5]. Some tanshinones exhibit leishmanicidal and antiplasmodial activity [6]. Not all *Salvia* species produce tanshinones [7], [8], and only a very few examples of the occurrence of tanshinones outside *Salvia* are known [6]. The majority of *Salvia* species synthesise royleanone-type diterpenes in which C-20 is preserved [9]. The distribution of tanshinones in *Salvia* species is therefore of considerable interest.

Salvia hydrangea DC. ex Bentham, endemic to Iran, has been widely used in traditional Iranian medicine [10]. Among others, the flowers (inflorescences) have a reputation as an anthelmintic and antileishmanial remedy [10]. Essential oils of leaves of *S. hydrangea* have been studied [11], [12], but there are no reports of investigations of the non-volatile constituents of the plant. Herein, we describe bioactivity-directed fractionation of an extract of the inflorescences of *S. hydrangea* using the *in vitro Plasmodium falciparum* (3D7 strain) toxicity assay, because the extract exhibited moderate antiplasmodial activity in initial tests (IC_{50} < 12.5 μg/mL). Although no significant antiplasmodial activity of extracts of roots was found, the roots were investigated as part of our ongoing studies of tanshinone-producing plants.

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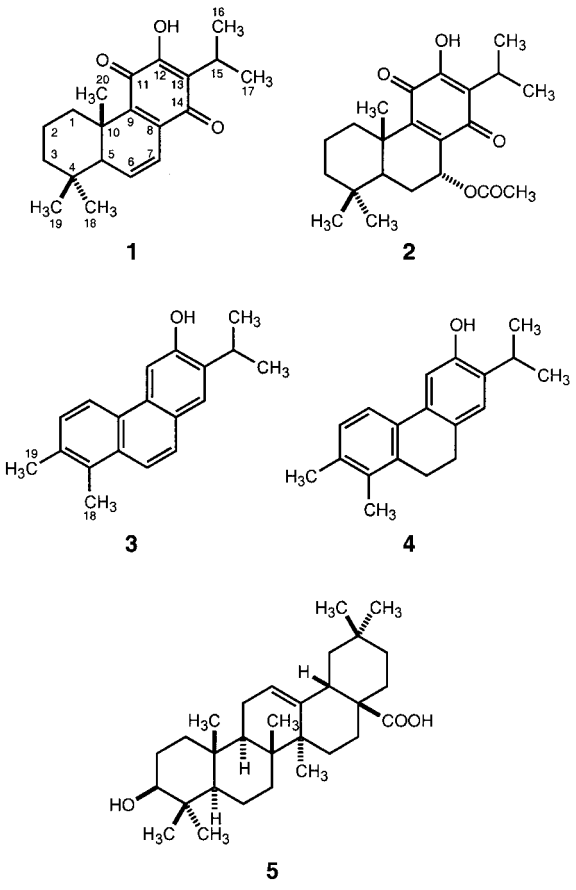
Received January 28, 2003 · **Accepted** March 11, 2002

Bibliography
Planta Med 2003; 69: 846–850 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943



Materials and Methods

General procedures

Optical rotations were measured on a Perkin Elmer 241 polarimeter. NMR spectra were recorded on a Bruker AMX400 or a Varian Mercury Plus 300 MHz spectrometer, using $CDCl_3$ as solvent and TMS as internal reference. High-resolution mass measurements for exact mass determination were carried out using a Bruker APEX III Fourier transform mass spectrometer equipped with a 7 Tesla superconducting magnet and an external electrospray ion source (Apollo source). The spectra were externally calibrated with a capillary skimmer dissociation spectrum of LHRH (luteinising hormone releasing hormone). The samples were introduced using a 250 μL syringe with a flow of 2 μL/min. Vacuum liquid chromatography (VLC) was performed using Merck silica gel 60 H. Preparative HPLC separations were performed on a chromatograph consisting of a Gynkotek P 580 pump, Rheodyne 7725 injector, a Shimadzu SPD-10AV spectrophotometric detector operating at 254 nm, and a recorder, using a 25 cm × 1.6 cm I. D. column packed with Lichrosorb Si-60 (7 μm), eluted with heptane/EtOH (9 : 1) at 6 mL/min. Melting points were determined in capillaries and are uncorrected.

Plant material

Roots and aerial parts of *Salvia hydrangea* DC. ex Bentham (Persian name: aruone) were collected at the altitude of 2500 – 2800 m in the area of Chadegan, Isfahan, Iran, and identified by M. Norouzi and N. Etemadi, Isfahan Research Centre of Natural Resources and Animal Science, Isfahan, Iran. A voucher specimen (no. 999) was deposited in the herbarium of this institute.

Extraction and isolation

Dried roots (256 g) were extracted with EtOAc (4 × 2 L) by overnight soaking at room temperature, and the crude product (6.5 g) was fractionated on a VLC column (8 cm × 10 cm I. D., Merck silica gel 60 H), eluted with toluene to give fraction A (1.3 L) and fraction B (4.5 L). Crystallisation of fraction A (0.46 g) from heptane gave **1** (68 mg). The mother liquid gave, after repeated purification by preparative HPLC, an additional amount of **1** (42 mg, Rt 7 min), impure **4** (12 mg, Rt 17 min), and **3** (5 mg, Rt 22 min). Crystallisation of fraction B (2.46 g) from heptane/EtOH (99 : 1) gave **2** (194 mg). An additional amount of **2** (100 mg) was obtained from fraction B by preparative HPLC (Rt 9 min) of the mother liquid. Pure **4** (2 mg) was obtained by HPLC on a Waters Symmetry 300 C_{18} column, 5 μm, 250 mm × 4.6 mm I. D., eluted with $MeCN/H_2O$ (3 : 1).

Dried inflorescences (140 g) were extracted with EtOAc (4 × 1 L) as above, the combined extracts were evaporated, and the residue (5 g) was fractionated by VLC (8 cm × 10 cm I. D. column, Merck silica gel 60 H) using toluene (2 L) followed by EtOAc (2.5 L). The toluene fraction (2.3 g) showed antiplasmodial activity (IC_{50} < 12.5 μg/mL), whereas the EtOAc fraction (2.7 g) was inactive (IC_{50} > 50 μg/mL). Further fractionation of a portion of the toluene fraction (1 g) by preparative HPLC (Rt 11 min) afforded 450 mg of oleanolic acid [(**5**), 450 mg, 0.73 % of dry weight of plant material]; $[\alpha]_D^{25}$: + 72° (*c* 0.2, $CHCl_3$); ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) identical with those obtained with authentic material obtained from Sigma-Aldrich Co.

6,7-Dehydroroyleanone (**1**): red crystals, total yield 110 mg (0.04 %); m. p. 160 – 164 °C (from heptane), lit. [13] 167 – 168 °C; $[\alpha]_D^{25}$: –508° (*c* 0.17, $CHCl_3$), lit. [14] –586° (*c* 0.09, $CHCl_3$); ^{1}H- and ^{13}C-NMR spectra as reported [14].

7α-Acetoxyroyleanone (**2**): yellow crystals, total yield 294 mg (0.11 %); m. p. 194 – 198 °C (from heptane), lit. [15] 212 – 214 °C; $[\alpha]_D^{25}$: –8° (*c* 0.04, $CHCl_3$), lit. [15] –7° ($CHCl_3$); ^{1}H- and ^{13}C-NMR spectra as reported [14].

7,8-Dimethyl-2-(1-methylethyl)phenanthren-3-ol (**3**): yellowish gum, yield 5 mg (0.002 %); HRMS: *m/z* = 264.15113 ($M^{+\cdot}$), $C_{19}H_{20}O^{+\cdot}$ requires 264.15087; ^{1}H- and ^{13}C-NMR: see Table **1**.

9,10-Dihydro-7,8-dimethyl-2-(1-methylethyl)phenanthren-3-ol (**4**): yellowish gum, yield 2 mg (0.0008 %); HRMS: *m/z* = 266.16664 ($M^{+\cdot}$), $C_{19}H_{22}O^{+\cdot}$ requires 266.16652; ^{1}H- and ^{13}C-NMR: see Table **1**.

Assay for antiplasmodial activity

The assay for antiplasmodial activity was performed with *Plasmodium falciparum* strain 3D7 essentially as previously described [6], [16]. For screening, the fractions were tested at 12.5, 25, 50, and 100 μg/mL. For determination of IC_{50} values, eight different concentrations of the test substance were tested in duplicate, and the determination was repeated twice with independent samples. The IC_{50} value for oleanolic acid was 8.8 ± 0.4 μg/mL (19.3 ± 0.8 μM). The IC_{50} value of chloroquine used as a reference was 16.9 ± 0.5 ng/mL (32.7 ± 0.9 nM). Microscopic investigation of the membrane effects of the test compounds was performed at concentrations 2, 10, 20, 50, and 100 μg/mL (4.4, 21.9,

Table **1** ^{1}H- and ^{13}C-NMR spectral data for compounds **3** and **4** in $CDCl_3$[a]

	Compound 3		*Compound 4*	
Position	***^{1}H***	***^{13}C***	***^{1}H***	***^{13}C***
1	8.29 (d, $^{3}J_{1,2}$ 8.5 Hz)	119.97	7.43 (d, $^{3}J_{1,2}$ 7.9 Hz)	121.00
2	7.42 (d, $^{3}J_{1,2}$ 8.5 Hz)	128.53	7.09 (d, $^{3}J_{1,2}$ 7.9 Hz)	128.00
3	–	133.97	–	135.80[b]
4	–	132.18	–	133.69[c]
5	–	130.84	–	135.90[b]
6	7.85 (d, $^{3}J_{6,7}$ 9.2 Hz)	120.34	2.84 (m)	25.59
7	7.69 (d, $^{3}J_{6,7}$ 9.2 Hz)	126.30	2.78 (m)	28.28
8	–	126.14	–	129.29
9	–	130.09	–	133.84[c]
10	–	127.66	–	132.05
11	7.93 (s)	106.78	7.11 (s)	110.64
12	–	152.32	–	151.61
13	–	135.37	–	133.04
14	7.68 (s)	125.88	7.04 (s)	125.60
15	3.40 (sp, $^{3}J_{15,16}$ 6.9 Hz)	27.55	3.21 (sp, $^{3}J_{15,16}$ 6.9 Hz)	26.99
16, 17	1.42 (d, $^{3}J_{15,16}$ 6.9 Hz)	22.81	1.29 (d, $^{3}J_{15,16}$ 6.9 Hz)	22.71
18	2.66 (s)	15.23	2.25 (s)	15.43
19	2.53 (s)	21.10	2.33 (s)	20.88
OH	5.14 (br s)	–	4.60 (br s)	–

[a] Abbreviations: s singlet, d doublet, sp septet, m multiplet, br broad.

[b,c] Due to close proximity of the signals, these assignments could not be unequivocally derived from HMBC spectra and may be interchanged.

43.8, 109.5, and 218.9 μM, respectively) as previously described, using light microscopy [16], [17].

Results

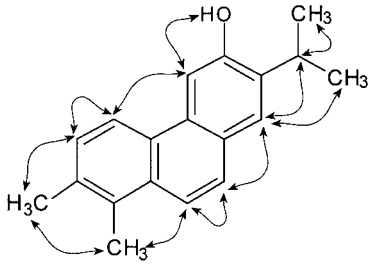
Fractionation of the extracts of the roots of *S. hydrangea* by VLC and preparative HPLC afforded 6,7-dehydroroyleanone (**1**) and 7α-acetoxyroyleanone (**2**), as well as two new phenanthrene derivatives **3** and **4**. The structures of all compounds were elucidated using COSY, NOESY, HSQC, and HMBC experiments. The NMR and optical rotation data of **1** and **2** corresponded well to those reported in the literature [13], [14], [15]. The ^{1}H- and ^{13}C-NMR data for **3** and **4** are given in Table **1**. NOESY and HMBC experiments provided firm proof of the structures **3** and **4**. The observed NOESY and HMBC connectivities, which established the structure of **3** and **4** unambiguously, are shown graphically in Figs. **1** and **2**.

Bioactivity-guided fractionation of the extract of the flowers (inflorescences), based on *in vitro* toxicity against *Plasmodium falciparum* 3D7 strain, yielded oleanolic acid (**5**) as a major constituent accounting for about 0.7 % of plant material (dry weight) or 21 % of material extractable with EtOAc. The IC_{50} value of purified **5** was 8.8 ± 0.4 μg/mL (19.3 ± 0.8 μM). However, microscopic examination of erythrocytes exposed to oleanolic acid (**5**) disclosed membrane curvature changes towards stomatocytic forms at active concentrations. Thus, after incubation of non-parasitised erythrocytes with 10 μg/mL (21.9 μM) of oleanolic acid for 48 h as in the assay procedure, formation of type 1 and type 2 stomatocytes (according to the nomenclature by Bessis [18]) was observed by light microscopy. At 20 μg/mL (43.8 μM), half of the cells were transformed. From 50 μg/mL and above ($\geq$ 109.5 μM), all cells were transformed with pronounced formation of spherostomatocytes.

Discussion

Fractionation of the extract of roots of *S. hydrangea* led to the isolation of the royleanones **1** and **2**, as well as the 20-norabietane derivatives **3** and **4**, but no tanshinones. Compounds **1** and **2** are relatively common constituents of *Salvia* species.

The structure of **3** was unambiguously assigned based on connectivities from NOESY and HMBC experiments, as shown in Figs. **1** and **2**. A compound assigned structure **3** was previously reported from *S. multicaulis* and named demethylmulticauline [19]. The ^{1}H-NMR data reported for demethylmulticauline are somewhat similar to those found in the present work (Table **1**), but only if the chemical shifts of H-2 and H-14, reported at δ = 7.60 and 7.57, respectively [19], are corrected to δ = 7.66 and 7.67 (A. Ulubelen, personal communication), and if the reported assignments of H-2 and H-7 are interchanged. However, the reported ^{13}C-NMR chemical shifts [19], recorded in the same solvent as in the present work ($CDCl_3$), are completely different from those established for **3** (Table **1**). For example, demethylmulticauline was reported to give two quaternary carbon resonances close to δ = 148 [19], whereas only one signal above δ = 140 is observed in the spectrum of **3** (the hydroxylated carbon C-12, δ = 152.3; Table **1**). The reported ^{13}C-NMR data for demethylmulticauline [19] might correspond to an isomer of **3** in



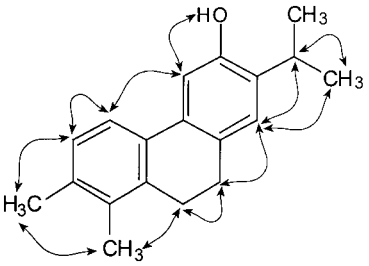
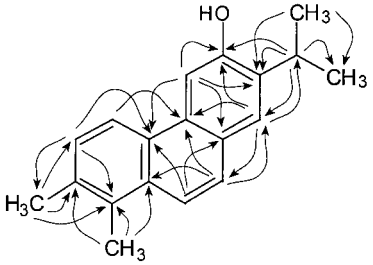


Fig. **1** NOE correlations for **3** (left) and **4** (right) for mixing time 500 ms (400 MHz).



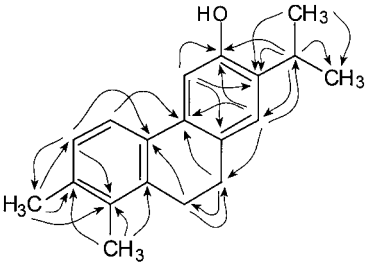


Fig. **2** Network of HMBC connectivities observed at 300 MHz for **3** (left) and **4** (right) with 70 ms delay for evolution of small C,H couplings; arrows point from H to C.

which the hydroxy group is attached to C-11 rather than to C-12. In this case, C-13 is not affected by *ortho*-shielding effect of the 12-hydroxy group, and is therefore expected to appear around δ = 148. This, together with the hydroxylated C-11, would give two quaternary carbon resonances at low field. Comparison with the published ^{13}C-NMR spectrum of 2-methyl-4-anthrenol [20] with the expected effect of replacement of the methyl group by an isopropyl group [21] confirms this prediction. On the other hand, if the *S. multicaulis* isolate were the 11-hydroxy isomer, there should be a small but observable *meta* coupling between H-12 and H-14 [22], not reported in the original work [19]. Thus, a final conclusion about the identity of the isolate published as demethylmulticauline cannot be reached, but the reported ^{13}C-NMR spectrum [19] does not correspond to the structure **3**. Original samples of demethylmulticauline were no longer available (A. Ulubelen, personal communication) for a direct comparison with **3**.

Compound **4** is new. A structure alternative to that of **4**, in which the A_2B_2 pattern observed in the ^{1}H-NMR spectrum corresponds to the protons at C-1 and C-2, rather than to those at C-6 and C-7, was excluded by a NOESY experiment. Thus, an aromatic proton singlet observed in the ^{1}H-NMR spectrum showed an NOE to one of the two CH_2 groups, as well as to the isopropyl group, whereas the other CH_2 group showed an NOE to one of the methyl groups present in the molecule. The second aromatic proton singlet showed a NOE to the hydroxy proton present, as well as to an aromatic proton, which was part of an AB system (Fig. **1**). This establishes the structure **4** and excludes the alternative structure with a saturated bond between C-1 and C-2. Compound **4** slowly converted to **3** when kept in a chloroform solution; **3** may therefore be an artefact formed by the oxidation of **4** with atmospheric oxygen.

The major *in vitro* antiplasmodial agent of *S. hydrangea* flowers was found to be oleanolic acid (**5**). Moderate *in vitro* antiplasmodial activity of this compound has previously been reported [23]. However, in the present study it was found that oleanolic acid (**5**) causes transformation of erythrocytes (discocytes) into stomatocytes at active concentrations. The extent of incorporation of **5** into the erythrocyte membrane, assessed as the extent of stomatocytogenesis observed microscopically, correlates well with its IC_{50} value for the *in vitro* inhibition of *Plasmodium* parasite growth. Similar effects were previously described and studied in more detail with dehydroabietinol and lupeol [16], [17]. It was concluded that stomatocytogenic agents and membrane-modifying compounds in general may show *in vitro* antiplasmodial activity via an indirect effect on the membrane of the host-cell [16]. Recent studies have linked erythrocyte membrane cholesterol content with malarial infection [24], and incorporation of other chemicals into the erythrocyte membrane may well have similar effects. Genuine antiplasmodial agents, e. g., chloroquine, inhibit *Plasmodium* parasites without visible changes of the normal erythrocyte cell shape at active concentrations [16]. The present findings strongly suggest that the *in vitro* antiplasmodial effect observed with **5** results from its incorporation into the erythrocyte membrane, similarly as described for dehydroabietinol and lupeol [16], [17]. Previously, **5** was reported to have a strong condensing effect on liposomal membranes [25], which is in agreement with the erythrocyte membrane modifying effect observed here.

Comparison of the IC_{50} value of pure **5** (8.8 ± 0.4 μg/mL or 19.3 ± 0.8 μM) with that of the crude extract (IC_{50} < 12.5 μg/mL), which has a very high content of **5**, shows a good correspondence, although other pentacyclic triterpenes may well contribute to the effect. Thus, the evidence is accumulating that common plant constituents may give a positive response in the *in vitro Plasmodium falciparum* toxicity assay via membrane effects. This emphasizes the need of caution in interpretation of results of the assay with plant extracts and isolates, in particular with amphiphilic and lipophilic constituents [16], [17].

Acknowledgements

We thank Prof. Ayhan Ulubelen, Faculty of Pharmacy, University of Istanbul, for corrections to the ^{1}H-NMR data reported in ref.

[19], and Ms. Dorte Brix, Ms. Katja Maj Nielsen, and Ms. Birgitte Simonsen, Danish University of Pharmaceutical Sciences, for technical assistance.

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