# Antiangiogenic iridoids from *Stachys ocymastrum* and *Premna resinosa**

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**Abstract**

In this paper, the isolation of one new iridoid glucoside, 6β-acetoxyipolamiide (**1**) and thirteen (**2-14**) known congeners from two Lamiaceae species, *Stachys ocymastrum* and *Premna resinosa* leaves extracts, is reported. The structural determination of the isolated compounds was performed by mono- and bidimensional NMR spectroscopic analysis, as well as MS experiments. The isolates were assayed for their antiangiogenic activity by two *in vivo* models, zebrafish embryos and chick chorioallantoic membrane assays. The compounds with a significant antiangiogenic activity in both assays were β-hydroxyipolamiide (**2**), ipolamiide (**3**), and buddlejoside $A_5$ (**8**). 6-*O*-α-L-(3”-*O*-*p*-Methoxycinnamoyl-4”-*O*-acetyl)rhamnopyranosyl catalpol (**13**) and 6-*O*-α-L-(2”-*trans*-caffeoyl)rhamnopyranosyl catalpol (**6**) showed the best antiangiogenic response on blood vessel growth in zebrafish embryos, whereas saccatoside (**10**) and 6-*O*-α-L-(2”-*O*-*p*-methoxycinnamoyl-3”-*O*-acetyl)rhamnopyranosyl catalpol (**14**) resulted in a strong reduction of capillary formation in the CAM assay.

**Key words**

*Stachys ocymastrum*, *Premna resinosa*, Lamiaceae, iridoids, antiangiogenic activity, chick chorioallantoic membrane, zebrafish

## Introduction

Naturally occurring iridoids are secondary metabolites found in a large number of plant families usually as glycosides. Chemotaxonomically they are useful as markers of several genera, and they have been regarded as defense chemicals against herbivores and pathogens, due to their antifeedant and growth inhibitory activities against insects [1]. Additionally, many iridoids are associated with a wide range of pharmacological activities such as cardio- and neuroprotective, anti-inflammatory, and antitumour [2]. In our previous reports we found that some iridoids showed antiangiogenic activity in the chick embryo chorioallantoic membrane (CAM) and zebrafish embryos assays [3,4]. The number of antiangiogenic compounds from natural origin is increasing constantly [5] and some plant families, such as Lamiaceae, have been found to be good sources for their isolation [6]. Accordingly, two species, *Stachys ocymastrum* (L.) Briq. (syn. *Stachys hirta* L.) and *Premna resinosa* (Hochst.) Schauer. from the family Lamiaceae, widely known for its iridoid content, were selected for phytochemical investigation and assessment of their iridoid constituents on angiogenesis. *S. ocymastrum* is a Mediterranean semi-arid grass of low height (12 to 70 cm), exhibiting simple or ramified hairy stems [7]. To the best of our knowledge, in the literature there are only few preliminar phytochemical studies on this plant, reporting the presence of iridoids and flavonoids in the aerial parts [8,9]. *P. resinosa*, a shrub or small bushy tree, with whitish stems and coriaceous pleasant smelling leaves [10], is used in Indian traditional medicine, as laxative agent and to treat bronchitis [11]. Phytochemical works on the genus *Premna* resulted in the isolation of many secondary metabolites including iridoids and their glycosides [12]. In addition, *P. resinosa* extracts and its flavonoid constituents showed cytotoxic, antituberculosis, and antimicrobial activities [11,13], and a previous study on some Saudi and Jordanian plants has revealed that the methanolic extract of *P. resinosa* possesses a selective antiangiogenic effect in rat aortic ring assay [14].

In the present study, the isolation and structural characterization of one new (**1**) and four known iridoid glycosides (**2-5**) from *S. ocymastrum*, and nine known iridoid diglycosides (**6-14**) from *P. resinosa* are reported (**Fig. 1**). The isolates were assayed in two *in vivo* models, zebrafish embryos and chick chorioallantoic membrane (CAM) assays, to evaluate their potential antiangiogenic effects.

## Results and discussion

The leaves of *S. ocymastrum* and *P. resinosa* were extracted with solvents of increasing polarity. After separation by Sephadex LH-20 and centrifugal partition chromatography (CPC), reverse phase HPLC was employed to obtain the pure iridoids. Compounds **1-5** were isolated from *S. ocymastrum*, while compounds **6-14** were obtained from *P. resinosa* (**Fig. 1**).

Compound **1**, was a yellow residue and had a molecular formula of $C_{19}H_{28}O_{13}$ as deduced from the molecular ion $[M + Na]^+$ at *m/z* 487.0646 observed in the HRESIMS. A fragment peak observed at *m/z* 427.0852 $[M + Na – 60]^+$ revealed the loss of one acetyl group. The $^1$H NMR spectroscopy data (**Table 1**) indicated that **1** was a 4-methoxycarbonyl iridoid glycoside, displaying the presence of one oxygenated methine proton at δ 6.19 (1H, br s, H-1), one olefinic methine proton at δ 7.62 (1H, s, H-3), another oxymethine proton at δ 4.38 (1H, t, *J* = 5.0 Hz, H-6), one methylene group at δ 2.16 (2H, d, *J* = 5.0 Hz, H-7), and one methoxycarbonyl group at δ 3.76 (3H, s, 11-OMe). Additionally, a characteristic anomeric proton resonance at δ 4.63 (d, *J* = 8.0 Hz, H-1’), along with signals in the region of δ 3.23–3.95, suggested that **1** contained a β-glucopyranoside unit. Moreover, signals for an acetyl group were evident. All these assignments were confirmed by COSY, HSQC, and HMBC spectra. Particularly, the HMBC cross peak observed between δ 1.44 (Me-10) and 86.0 (C-8), was in agreement with the presence of a tertiary methyl group and a hydroxyl substituent geminally attached to C-8. The comparison of the NMR spectroscopic data with those reported in the literature for similar compounds [15] suggested that **1** possessed an ABX system in the cyclopentane ring, in

which the methylene group at C-7 was adjacent to the oxygenated methine group at C-6, as in 6β-hydroxyipolamiide [16]. This assumption was confirmed by the HMBC correlations between H-6—C-8, H-6—C-9, and between $H_2$-7—C-5, $H_2$-7—C-8, and $H_2$-7—C-9. The chemical shifts observed for H-6 ($\delta_H$ 4.38) and C-6 ($\delta_C$ 74.9 ppm) in the HSQC spectrum indicated that C-6 was the position of esterification. The relative configuration of **1** was established on the basis of 1D NOESY data and comparison with the literature [15,17]. NOESY interactions between δ 1.44 (Me-10) and 6.19 (H-1), and between δ 1.44 (Me-10) and 4.38 (H-6) indicated the α-orientation of Me-10 and H-6, and the consequential β-orientation of 6-$OCOCH_3$. Moreover, δ 2.94 (H-9) correlated with δ 1.44 (Me-10), suggesting that H-9 and Me-10 were on the same side of the molecule. On the basis of the above results, the structure of 6β-acetoxyipolamiide was assigned to **1**.

Compounds **2**-**14** were known iridoids identified as 6β-hydroxyipolamiide (**2**) [16], ipolamiide (**3**) [18], ipolamiidoside (**4**) [19], and lamiide (**5**) [18], 6-*O*-α-L-(2”-*trans*-caffeoyl)rhamnopyranosyl catalpol (**6**) [20], 6-*O*-α-L-(3”-*O*-*p*-methoxycinnamoyl)rhamnopyranosyl catalpol (**7**) [21], buddlejoside $A_5$ (**8**) [22], 6-*O*-α-L-(4”-*trans*-caffeoyl)rhamnopyranosyl catalpol (**9**) [23], saccatoside (**10**) [24], 6-*O*-α-L-(4”-*O*-*p*-coumaroyl)rhamnopyranosyl catalpol (**11**) [24], scopolioside A (**12**) [25], 6-*O*-α-L-(3”-*O*-*p*-methoxycinnamoyl-4”-*O*-acetyl)rhamnopyranosyl catalpol (**13**) [21], and 6-*O*-α-L-(2”-*O*-*p*-methoxycinnamoyl-3”-*O*-acetyl)rhamnopyranosyl catalpol (**14**) [26] by comparison of their NMR and MS data with literature values. Compounds **7**, **11**, **13**, and **14** were isolated as *cis* and *trans* isomers in equilibrium.

All isolated iridoids **1**-**14** were assayed for their *in vivo* antiangiogenic effects in zebrafish embryos. The zebrafish is a suitable model for identification of new angiogenesis inhibitors, since development of blood vessels in early transparent embryos is easily monitored by quantification of endogenous alkaline phosphatase (EAP) activity released by the endothelial cells as a marker of vessel growth. As shown in **Table 2**, among the isolates, compounds **13** > **6** > **2** > **3**, exhibited the best antiangiogenic activity at 2 μM, reducing significantly ($P < 0.05$ and $P < 0.01$) vessel growth

in treated embryos as compared to control (41.59, 37.27, 35.20, 34.66% of inhibition, respectively). It is also evident that treatment with 4 μM did not further increase the antiangiogenic activity as compared to 2 μM. A significant response on EAP activity was also observed for compounds **8**, **4**, and **5** (31.20, 31.00 and 24.86% of inhibition, respectively). The effects on angiogenesis of isolated compounds were compared with that of 2-methoxyestradiol, an endogenous metabolite of 17β-estradiol having known antiangiogenic and antitumor properties. This compound led to an inhibition of 49.50% at 2 μM in our experimental conditions, as it was previously reported, while higher concentrations completely abolished vessel growth [27].

The CAM assay was also performed to explore the antiangiogenic potential of the isolates. Two different doses (10 and 20 μM) were used to evaluate the efficacy of the isolated iridoids as antiangiogenic agents. The results, summarized in **Table 3**, showed a good and dose-dependent antiangiogenic response for compounds **8** > **10** > **3** > **2** ($P < 0.05$ and $P < 0.01$). Compound **14** demonstrated the best activity (72.22%) at 10 μM but at 20 μM an increase of antiangiogenic activity was not observed. The other tested iridoids showed mild antiangiogenic effects with inhibition values in the range of 10-15%. Retinoic acid (3 μM) was used as positive control (54.92%). The images of representative microscopical observations of the CAMs treated with the active compounds are shown in **Figure 2**. After six days of incubation, CAM control eggs evidenced the presence of a rich vascular network. Conversely strong inhibitory effects on capillary formation are induced by retinoic acid. In the CAMs treated with the active compounds, the microvasculature seems to be less dense (**Figures 2 A-E**). Overall, the images illustrate the progressive decrease of vessel branch points at increased concentrations of tested compounds. The inhibitory effects are particularly evident in the CAMs treated with compounds **8** and **14** (**Figure C** and **E**), which showed to be potent even at lower concentration (10 μM).

In conclusion, compounds **2**, **3**, and **8** demonstrated a good antiangiogenic activity in both assays; these results are in accordance with recent reports showing the antiangiogenic activity of iridoid derivatives such as catalpol [28] and with previous results that showed ipolamiide (**3**) to be a new

natural antiosteoporotic agent on the basis of its effect on alkaline phosphatase activity in MC3T3-E1 osteoblast cells as a marker [29].

## Material and Methods

### General experimental procedures

Optical rotations were measured on an Atago AP-300 digital polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. 1D and 2D NMR experiments were recorded at 300 K in $CD_3OD$ on a Bruker DRX-500 and Bruker Avance 250 spectrometers (Bruker BioSpin GmBH) [30]. HRESIMS were acquired in the positive ion mode on a Q-TOF premier mass spectrometer (Waters). ESI-MS spectra were obtained from an LCQ Advantage ThermoFinnigan spectrometer (ThermoFinnigan), equipped with Xcalibur software. Column chromatography was carried out over Sephadex LH-20 (40–70 μm, Pharmacia). High-Performance Centrifugal Partition Chromatography (HPCPC) was performed on an Everseiko CPC240 instrument equipped with 3136 cells (total volume, 240 mL). HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C18 μ-Bondapak column (30 cm x 7.8 mm, 10 μm Waters, flow rate 2.0 mL/min). TLC separations were carried out using silica gel 60 $F_{254}$ (0.20 mm thickness) plates (Merck) and cerium sulphate (Sigma-Aldrich) as spray reagent.

### Plant material

*S. ocymastrum* leaves were collected and identified by one of the authors, Dr. S. Amira, in June 2013 in the region of Jijel, 15 km from the North East coast of Algeria. A voucher specimen has been deposited at the Herbarium Horti Botanici Pisani, Pisa, Italy (n. 7281 *Stachys ocymastrum*/1, Nuove Acquisizioni).

*P. resinosa* leaves were collected at Al-Kurr, Makkah Province, Saudi Arabia, in June 2016 and identified by one of the authors, Prof. A. Bader. A voucher specimen was deposited at the Herbarium of Pharmacognosy Lab at the Faculty of Pharmacy, Umm Al-Qura University (n. UQU-SA-109).

**Extraction and isolation**

The dried leaves of *S. ocymastrum* (1 kg) were extracted with solvents of increasing polarity, *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH, by exhaustive maceration to give 2.6, 10.5, 14.3, and 18.5 g of the respective residues. The MeOH extract was partitioned between *n*-BuOH and $H_2O$ to afford a *n*-BuOH residue (3.5 g), that was submitted to Sephadex LH-20 column chromatography (flow rate 1 mL/min), using MeOH as eluent and collecting eight major fractions (1-8) grouped after TLC analysis. Fraction 1 (120.0 mg) was subjected to RP-HPLC with MeOH-$H_2O$ (3:7) as eluent, to give compounds **1** (2.6 mg, $t_R$ 9 min) and **4** (3.5 mg, $t_R$ 26 min). Part of the $CHCl_3$-MeOH extract (4.5 g) was subjected to Sephadex LH-20 column chromatography (flow rate 1 mL/min, collection volume 15 mL) using MeOH as eluent and collecting nine major fractions (A-I) grouped by TLC. Fraction B (312.9 mg) was subjected to HPCPC (flow rate 3 mL/min, collection volume 3 mL), using $CHCl_3$-MeOH-$H_2O$-*n*-PrOH (9:12:8:1) as solvent system, in which the stationary phase consisted of the lower phase, and collecting eight major sub-fractions in ascending mode ($B_1$-$B_8$). Fractions $B_2$ (70.3 mg) and $B_3$ (75.9 mg) were submitted to RP-HPLC with MeOH-$H_2O$ (3:7) as eluent, to give compounds **5** (1.2 mg, $t_R$ 8 min), **2** (1.7 mg, $t_R$ 9 min), and **3** (2.5 mg, $t_R$ 12 min) from fraction $B_2$ and compounds **2** (1.1 mg, $t_R$ 9 min) and **3** (1.1 mg, $t_R$ 12 min) from fraction $B_3$. Fractions $B_5$ and $B_7$ yielded compounds **1** (12.8 mg) and **4** (6.5 mg), respectively.

The air-dried powdered leaves of *P. resinosa* (200.0 g) were extracted for 48 h at room temperature with solvents of increasing polarity: *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH, by exhaustive maceration (3 x 2 L) to yield 4.7, 9.6, 6.7, and 30.0 g of the respective extracts. The MeOH extract was partitioned between *n*-BuOH and $H_2O$ and part of the obtained *n*-BuOH extract

(5.0 g) was subjected to Sephadex LH-20 column (flow rate 1 mL/min), using methanol as eluent, to obtain 4 major fractions (AA-DD), grouped by TLC. Fractions BB (1.0 g) and CC (690.0 mg) were subjected to HPCPC, eluting with $CHCl_3$-MeOH-$H_2O$-*n*-PrOH (9:12:8:1) as solvent system, in which the stationary phase consisted of the lower phase, and collecting nine major sub-fractions ($BB_1$-$BB_9$ and $CC_1$-$CC_9$). Fraction $BB_2$ (138.4 mg) was purified by RP-HPLC with MeOH-$H_2O$ (2:3) to obtain compound **7** (10.8 mg, $t_R$ 16 min). Fraction $BB_6$ (21.2 mg) and $CC_6$ (52.2 mg) were purified by RP-HPLC with MeOH-$H_2O$ (1:1) to yield compounds **14** (3.0 mg, $t_R$ 14 min) and **8** (3.7 mg, $t_R$ 16 min) from fraction $BB_6$, and compound **13** (4.3 mg, $t_R$ 17 min) from fraction $CC_6$. Fraction $BB_9$ (18.2 mg) was purified by RP-HPLC with MeOH-$H_2O$ (5.5:6.5) to yield compound **12** (3.9 mg, $t_R$ 18 min). Fraction $CC_2$ (122.0 mg) was purified by RP-HPLC with MeOH-$H_2O$ (3:7) to give compounds **6** (7.0 mg, $t_R$ 12 min) and **9** (4.0 mg, $t_R$ 40 min). Fraction $CC_4$ (84 mg) was purified by RP-HPLC with MeOH-$H_2O$ (3.5:6.5) to give compounds **10** (5.3 mg, $t_R$ 17 min) and **11** (5.6 mg, $t_R$ 36 min).

*Compound* **1**: yellow amorphous powder; $[\alpha]_D$ – 23.1 (*c* 0.1, MeOH); $^1H$ and $^{13}C$ NMR data, see **Table 1**; ESIMS *m/z* 487 $[M + Na]^+$, 427 $[M + Na – 60]^+$, 409 $[M + Na – 60 – 18]^+$; HRESIMS *m/z* 487.0646 $[M + Na]^+$ (calcd. for $C_{19}H_{28}NaO_{13}$, 487.1428), 427.0852 $[M + Na – 60]^+$.

**Zebrafish embryo generation and treatment protocol**

Zebrafish embryos were obtained from wild type fishes bought from a local pet store and maintained in flow through aquaria at 28.5 °C on a 14/10 h (light/dark) photoperiod. Embryos were generated by natural mating as described by Kimmel et al. (1995) [31] and they were cultured in water at 28.5 °C. The ethical guidelines, described in the National Institutes of Health Guide for Care and Use of Laboratory Animals, were followed throughout the experiments. Then, healthy and regular embryos were selected at 24 h post fertilization (hpf), manually dechorionated with forceps, distributed in 96 single well microplates (one embryo per well) and finally incubated with 100 µL of embryo water containing test compounds (2 or 4 µM) or 2-methoxyestradiol (ME, 2 µM),

employed as a standard antiangiogenic substance. DMSO (0.2% v/v) was used as a vehicle for the treatments. Control group received only DMSO (0.2% v/v). All treated embryos (10 for each group) were incubated from 24 hpf to 72 hpf (total 48 hours of exposure).

**Quantitative determination of endogenous alkaline phosphatase (EAP) activity**

Quantitative determination of EAP activity was performed as described by Germanò et al. (2015) [32]. Treated embryos at 72 hpf were dehydrated with increasing concentrations of ethanol, then they were washed three times with diethanolamine buffer (1 M, pH 9.8), and incubated with the substrate containing 0.5 mg/mL *p*-nitrophenyl phosphate disodium salt (Sigma Aldrich) for 30 min at room temperature. NaOH (2 M) was added to stop the reaction. The optical density (OD) of soluble EAP substrate was measured at 405 nm using a microplate reader (Mutiskan GO, Thermo Scientific). Vessel growth was expressed as a percentage of change in OD compared with control. The results obtained were finally expressed as percent values. Each assay was repeated at least three times. The significance of the differences was assesses on the basis of the *t*-test, considering the differences for $P < 0.05$ and $P < 0.01$, and finally calculated versus control embryos.

**Chorioallantoic membrane assay (CAM)**

CAM assay was performed following the method of Certo et al. (2017) [33]. Fertilized chicken eggs were incubated at 37 °C. The eggs were positioned horizontally and rotated for several times. After 4 days of incubation, a window (1 $cm^2$) was carefully created on the broad side of the egg to assess the extent of embryonic blood vessels. The development of the embryos was checked by a visual inspection. Malformed or dead embryos were excluded. Then, isolated compounds were tested at 10 and 20 µM (100 µL/egg). Ten eggs were used for each group. DMSO (0.2% v/v) in Tris buffer (pH 7.4) was used as a vehicle for the treatments. Retinoic acid (3 µM) was used as positive control. After treatment, the eggs were reincubated for other two days. At the end of incubation, each egg was observed under a stereomicroscope (SMZ-171 Series, Motic) to visualize the microvasculature. CAM images were acquired by a digital camera (Moticam 5 plus). After counting the number of

blood vessel branch points in a standardized area, the antiangiogenic activity was calculated using an equation 1-*T/C,* where *T* represents the number of vessel branch points in the treated CAMs, whereas *C* indicates the number of vessel branch points in control samples. The results obtained were finally expressed as percent values. Each experiment was repeated three times. The significance of the differences was assessed on the basis of the *t*-test, considering the differences for $P < 0.05$ and $P < 0.01$, and finally calculated versus control samples.

**Supporting information**

NMR spectra of compound **1** are available as Supporting Information.

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**Conflict of Interest**

The authors declare no conflict of interest.

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**Legends for figures**

**Fig. 1** Structures of compounds **1**–**14**.

**Fig. 2** Representative images of CAMs exposed to different concentrations (10 and 20 µM) of active isolated compounds; (A) **2**= 6β-hydroxyipolamiide, (B) **3** = ipolamiide, (C) **8** = buddlejoside A5, (D) **10** = saccatoside, (E) **14** = 6-*O*-β-L-(2”-*O*-*p*-methoxycinnamoyl-3”-*O*-acetyl)rhamnopyranosyl catalpol. CAMs were observed under a stereomicroscope (SMZ-171 Series, Motic). The images of CAMs were acquired by a digital camera (Moticam 5 plus).

**Table 1** 1H and 13C NMR data of compound **1** (CD3OD, 500 MHz, *J* in Hz).

| position | **1** | |
|---|---|---|
| | $\delta_H$ | $\delta_C$ |
| 1 | 6.19 br s | 95.3 |
| 3 | 7.62 s | 155.0 |
| 4 | | 112.3 |
| 5 | | 72.6 |
| 6 | 4.38 t (5.0) | 74.9 |
| 7a | 2.16 d (5.0) | 45.8 |
| 7b | 2.16 d (5.0) | |
| 8 | | 86.0 |
| 9 | 2.94 br s | 57.6 |
| 10 | 1.44 s | 21.6 |
| 11 | | 167.8 |
| O-Me | 3.76 s | 52.7 |
| 6-OCO$\underline{CH_3}$ | 2.05 s | 21.6 |
| 6-O$\underline{CO}CH_3$ | | 172.9 |
| 1’ | 4.63 d (8.0) | 100.2 |
| 2’ | 3.23 dd (8.5, 8.0) | 74.4 |
| 3’ | 3.36 t (8.5) | 78.3 |
| 4’ | 3.35 t (8.5) | 71.6 |
| 5’ | 3.42 m | 77.5 |
| 6a’ | 3.95 dd (11.5, 3.0) | 62.8 |
| 6b’ | 3.73 dd (11.5, 5.0) | |

*J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments.

**Table 2** Antiangiogenic activity (% inhibition versus control) of iridoids **1-14** in zebrafish embryo endogenous alkaline phosphatase (EAP) assay.

| | Concentration | |
|---|---|---|
| compound | 2 μM | 4 μM |
| **1** | 15.09 ± 2.07 | 24.94 ± 1.98 |
| **2** | 35.20 ± 2.85* | 46.52 ± 1.62** |
| **3** | 34.66 ± 1.20* | 30.28 ± 0.86 |
| **4** | 31.00 ± 2.01 | 25.01 ± 1.56 |
| **5** | 24.86 ± 0.87 | 24.65 ± 2.04 |
| **6** | 37.27 ± 1.14* | 28.42 ± 1.83 |
| **7** | 8.01 ± 2.53 | 8.51 ± 2.89 |
| **8** | 31.20 ± 1.79 | 27.73 ± 1.84 |
| **9** | 5.25 ± 1.26 | 7.53 ± 2.36 |
| **10** | 13.48 ± 3.01 | 27.53 ± 0.94 |
| **11** | 20.00 ± 2.41 | 11.89 ± 1.69 |
| **12** | 12.47 ± 2.06 | 19.00 ± 2.18 |
| **13** | 41.59 ± 1.09** | 36.74 ± 1.46* |
| **14** | 9.11 ± 2.31 | 18.42 ± 1.79 |
| 2-methoxyestradiol | 49.50 ± 0.90** | |

Control embryos received only DMSO (0.2% v/v). All treated embryos (10 for each group) were incubated from 24 hours post fertilization to 72 hours post fertilization (total 48 hours of exposure). Each experiment was repeated three times. *$P < 0.05$ and **$P < 0.01$vs control: Student's *t*-test.

**Table 3** Antiangiogenic activity (% inhibition versus control) of iridoids **1-14** in the chorioallantoic membrane (CAM) assay.

| | Concentration | |
|---|---|---|
| compound | 10 μM | 20 μM |
| **1** | 12.18 ± 0.32 | 14.94 ± 2.04 |
| **2** | 28.02 ± 1.07 | 48.01 ± 0.27* |
| **3** | 44.00 ± 0.66* | 64.42 ± 0.78** |
| **4** | 11.00 ± 2.03 | 13.22 ± 0.94 |
| **5** | 10.42 ± 0.34 | 11.56 ± 0.75 |
| **6** | 11.51 ± 0.62 | 13.22 ± 2.14 |
| **7** | 10.32 ± 0.54 | 11.67 ± 2.03 |
| **8** | 68.84 ± 1.32** | 76.96 ± 0.92** |
| **9** | 12.05 ± 0.92 | 10.03 ± 1.98 |
| **10** | 48.22 ± 2.67* | 80.08 ± 0.64** |
| **11** | 11.45 ± 1.06 | 12.67 ± 0.38 |
| **12** | 10.38 ± 0.87 | 12.49 ± 0.86 |
| **13** | 12.02 ± 0.78 | 11.37 ± 1.32 |
| **14** | 72.22 ± 2.05** | 70.02 ± 2.24** |

Retinoic acid (3 μM), positive control, % of inhibition = 54.92 ± 0.45**.

Antiangiogenic activity = 1-*T/C, T* = number of vessel branch points in the treated CAMs; *C* = number of vessel branch points in control CAMs. Ten eggs were used for each group. Vehicle = DMSO (0.2% v/v) in Tris buffer (pH 7.4). Each experiment was repeated three times. *$P < 0.05$ and **$P < 0.01$ vs control: Student's *t*-test.