Pharmacological Reports
2013, 65, 410–420
ISSN 1734-1140

Effects of sesquiterpene, flavonoid and coumarin types of compounds from *Artemisia annua* L. on production of mediators of angiogenesis

Xiaoxin X. Zhu[1], Lan Yang[1], Yujie J. Li[1], Dong Zhang[1], Ying Chen[1], Petra Kostecká[2], Eva Kmoníčková[3], Zdeněk Zídek[2]

[1]Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Nanxiaojie 16, Dongzhimen Nei Avenue, Beijing 100700, China

[2]Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic

[3]Institute of Pharmacology and Toxicology, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic

Correspondence: Zdeněk Zídek, e-mail: zidekz@biomed.cas.cz

Abstract:
Background: In addition to recognized antimalarial effects, *Artemisia annua* L. (Qinghao) possesses anticancer properties. The underlying mechanisms of this activity are unknown. The aim of our experiments was to investigate the effects of distinct types of compounds isolated from *A. annua* on the immune-activated production of major mediators of angiogenesis playing a crucial role in growth of tumors and formation of metastasis.
Methods: Included in the study were the sesquiterpene lactones artemisinin and its biogenetic precursors arteannuin B and artemisinic acid. The semi-synthetic analogue dihydroartemisinin was used for comparative purposes. The flavonoids were represented by casticin and chrysosplenol D, the coumarin type of compounds by 4-methylesculetin. Their effects on the lipopolysaccharide (LPS)-induced *in vitro* production of nitric oxide (NO) were analyzed in rat peritoneal cells using Griess reagent. The LPS-activated production of prostaglandin E_2 (PGE_2) and cytokines (VEGF, IL-1β, IL-6 and TNF-α) was determined in both rat peritoneal cells and human peripheral blood mononuclear cells using ELISA.
Results: All sesquiterpenes (artemisinin, dihydroartemisinin, artemisinic acid, arteannuin B) significantly reduced production of PGE_2. Arteannuin B also inhibited production of NO and secretion of cytokines. All NO, PGE_2 and cytokines were suppressed by flavonoids casticin and chrysosplenol D. The coumarin derivative, 4-methylesculetin, was ineffective to change the production of any of these factors.
Conclusions: The inhibition of immune mediators of angiogenesis by sesquiterpene lactones and flavonoids may be one of the mechanisms of anticancer activity of *Artemisia annua* L.

Key words:
Artemisia annua L., nitric oxide, prostaglandins, cytokines, angiogenic factors

Abbreviations: IL- – interleukin-, LPS – lipopolysaccharide, NO – nitric oxide, PBMC – peripheral blood mononuclear cells, PGE_2 – prostaglandin E_2, TNF – tumor necrosis factor, VEGF – vascular endothelial growth factor

Introduction

Artemisia annua L. (Qinghao), a medicinal plant of the traditional Chinese medicine, is a recognized

source of artemisinin (Qinghaosu) which is broadly used in the treatment of malaria [13]. Artemisinin and its semi-synthetic derivatives have also been found effective against growth of a plethora of tumors including leukemia, prostate, breast, lung, colon and pancreatic cancer, pituitary macroadenoma, and uveal melanoma [3, 6, 13, 17, 26, 39]. It has been well established that the essential role in many pathological events including growth of tumors and formation of metastasis is played by angiogenesis [10, 40]. It is under the control of many factors such as cytokines, e.g., vascular endothelial growth factor (VEGF) [38, 56] and nitric oxide (NO).

The anticancer effects of *A. annua* have been suggested to depend at least in part on the anti-angiogenic mode of action [51]. Except of VEGF which is suppressed by artemisinin derivatives [20, 49, 55], data on possible effects of these and other secondary metabolites isolated from *A. annua* on production of other pro-angiogenic factors have been missing.

The aim of this study was to analyze the effects of *Artemisia*-derived compounds on the *in vitro* production of major angiogenic factors such as NO, PGE_2 and cytokines VEGF, IL-1β, IL-6 and TNF-α. Included in the study were sesquiterpene lactone artemisinin and its biogenetic precursors arteannuin B and artemisinic acid [7, 9, 43]. The flavonoids were represented by casticin and chrysosplenol D, which have been reported to reduce the proliferation and growth of animal and human cancer cells and suggested as promising anti-cancer agents [4, 16, 30, 36]. The coumarin family of compounds was represented by 4-methylesculetin.

Materials and Methods

Animals and cell culture

Female Wistar rats, 8–10 weeks old, were purchased from Velaz (Prague, Czech Republic). They were maintained in transparent plastic cages in groups of four, and housed in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France) under controlled 12/12 h light/dark cycle (lights on 6.00 a.m.), temperature (22 ± 2°C), and relative humidity (50 ± 10%) conditions.

Animals, killed by cervical dislocation, were intraperitoneally injected with 16 ml of sterile saline. Peritoneal cells were washed, resuspended in complete culture medium and seeded into 96-well round-bottom microplates (Costar) in final 100-µl volumes, 2×10^6 cells/ml. Cultures were maintained at 37°C, 5% CO_2 in humidified Heraeus incubator. The complete RPMI-1640 culture medium contained 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma-Aldrich). All experimental variants were run in duplicate.

The animal welfare and all experimental procedures have been approved by the Institution Animal Ethics Committee (no. 045/2010).

Isolation and culture of human peripheral blood mononuclear cells (PBMC)

Normal PBMC, provided by the Institute of Clinical and Experimental Medicine, Prague, were obtained from healthy human volunteers. In accordance with the Helsinki Convention (1964), informed consent was obtained regarding blood collection for immunological examination. Peripheral blood was withdrawn from a forearm vein. PBMC were separated from the buffy coat by Ficoll-Paque (Amersham Biosciences) gradient centrifugation and then washed three times in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated FBS. They were cultured at a final density of 1.5×10^6 cells/ml in complete RPMI-1640 (100 µl per well).

Compounds

Artemisinin, its biogenetic precursors (arteannuin B and artemisinic acid), flavonoids casticin and chrysosplenol D, and a coumarin type compound 4-methylesculetin were isolated from *Artemisia annua* L. The procedures were in part described previously (54). Briefly, the air-dried leaves of *A. annua* were extracted with ethanol (100%) at room temperature. After concentration in vacuum at 50°C, the residue was fractionated with ethyl acetate (EtOAc). The EtOAc-soluble fraction was subjected to column chromatography on silica gel and eluted with petroleum ether, increasing the amounts of EtOAc up to 6 : 4 (petroleum ether : EtOAc, v/v). Thin layer chromatography (TLC) was used to obtain the fractions from the column. Based on the results of TLC, the

fractions were separately combined and purified by recrystallization to afford artemisinin, artemisinic acid, arteannuin B, casticin, chrysosplenol D and 4-methylesculetin. Structures were confirmed by UV, NMR, and MS data. The semi-synthetic derivative dihydroxyartemisinin was purchased from Chongqing Holley Wuling Mountain Pharmaceutical Co. (Batch No 20100304, Quality Standard: Chinese Pharmacopoeia 2005). Stock solutions (200 mM) of the compounds were prepared in DMSO and kept at –25°C until use.

NO production

The *in vitro* effects of compounds on NO production were investigated in rat peritoneal cells. The compounds were applied simultaneously with the NO-activating stimulus, i.e., LPS (*Escherichia coli* 0111:B4, Sigma; 1 ng/ml). The nitrite concentration in cell supernatants, taken as a measure of NO production [31], was determined at the interval of 24 h. It was detected in individual cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of the Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to μM nitrite.

Cytokine and PGE_2 production

Secretion of cytokines IL-1β, IL-6 and TNF-α by rat peritoneal cells was induced by LPS (1 ng/ml). Secretion of VEGF was activated by LPS (10 ng/ml) plus rat recombinant IFN-γ (5 ng/ml). Production of cytokines by human PBMC was stimulated by LPS (1 μg/ml).

Production of PGE_2 by rat peritoneal cells and human PBMC was activated by LPS (5 and 1 μg/ml, respectively).

The compounds were applied simultaneously with the immune stimuli.

Concentration of cytokines and PGE_2 in supernatants of rat and human cells was determined at the interval of 24 h of culture using ELISA (R&D Systems, Minneapolis, MN, USA).

Great interindividual variation of constitutive and LPS-activated PGE_2 and cytokine production by human PBMC was observed. Therefore, the effects of compounds were expressed as a percent change related to the effect of LPS. The response to LPS was taken as 100%. The inhibitory effects of compounds were estimated according to the formula:

$$\{[(X - \text{control}) - (\text{LPS} - \text{control})]/(\text{LPS} - \text{control})\} \times 100$$

where X is the value of a compound effect.

Expression of iNOS mRNA and COX-2 mRNA

Semi-quantitative reverse transcriptase-polymerase chain reaction was used to analyze expression of genes *iNOS*, *COX-2*, and as an internal control gene *GAPDH*. Total RNA was isolated from 4×10^6 cells per sample using the RNeasy mini kit and RNAse free DNAse I set (Qiagen, Hilden, Germany). RNA and its purity were quantified spectrophotometrically by measuring the optical density at 260 and 280 nm (BioPhotometer, Eppendorf AG, Hamburg, Germany). Reverse transcription catalyzed by Moloney murine leukemia virus reverse transcriptase in the presence of RNAse inhibitor was used to prepare cDNA. PCR of iNOS gene was performed with synthesized primers from Metabion (Martinsried, Germany); F: 5-ACA-ACAGGAACCTACCAGCTCA-3; R: 5-GATGTT-GTAGCGCTGTGTGTCA-3 (651 bp, annealing temperature: 61°C). Primers for rat COX-2 were obtained from Sigma (Prague, Czech Republic); F: 5-ACA-CTCTATCACTGGCATCC-3; R: 5-GAAGGGACA-CCCTTTCACAT-3 (584 bp, annealing temperature: 59°C). All other chemicals used in the PCR protocol were obtained from Top-Bio (Prague, Czech Republic). The polymerase chain reaction products were identified by electrophoresis on agarose gel (1.5%) containing ethidium bromide and photographed under UV light.

Cell viability

Viability of cells was determined using a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Diagnostics, Mannheim, Germany). The cells (1×10^6/ml) were cultured as described above. After the 24-h culture, the WST-1 was added and the cells were kept in the Heraeus incubator at 37°C for additional 3 h. Optical density at 450/690 nm was recorded.

Statistical analysis

Analysis of variance (ANOVA) with subsequent Dunnett or Bonferroni multiple comparison tests, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

Results

Effects of compounds on NO production by rat cells

While the constitutive production of NO by rat peritoneal cells was negligible, it was substantially enhanced in the presence of LPS. Supernatant concentrations of nitrites reached the value of approximately 70 μM at the interval of 24 h of culture (Fig. 1). The NO formation remained uninfluenced by artemisinic acid and 4-methylesculetin. Artemisinin and dihydroartemisinin had only marginal, statistically not significant effect at the highest concentration (100 μM) used [F (7, 15) = 2.10, p = 0.15; and F (7, 15) = 1.48, p = 0.30, respectively]. Arteannuin B completely abolished formation of NO at the 20 μM concentration. Flavonoids casticin and chrysosplenol D significantly decreased production of NO ($p < 0.01$) at the 10 μM concentration.

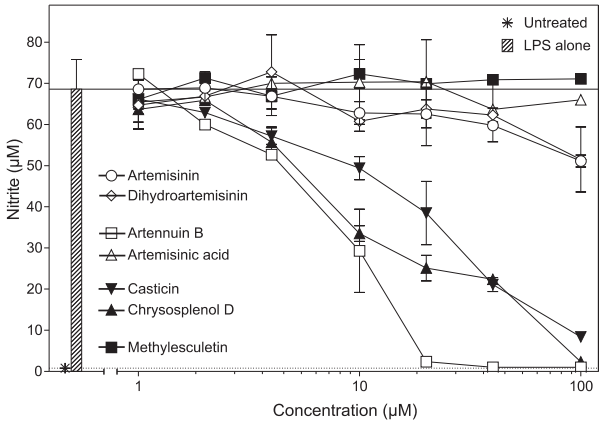

Fig. 1. Effect of the compounds on *in vitro* production of NO by rat (n = 6) peritoneal cells cultured 24 h in presence of LPS (1 ng/ml). Concentration of nitrites in cell supernatants was determined by Griess reagent. Each point is the mean ± SEM. The data are representative of two identical experiments. The IC_{50} estimates are shown in Table 1

The IC_{50} estimates (Tab. 1) ranged within the interval of 7–22 μM.

The flavonoids casticin and chrysosplenol D inhibited the expression of the LPS-activated iNOS mRNA (Fig. 2). Dihydroxyartemisinin which did not inhibit production of NO had no influence on the iNOS mRNA transcription.

Tab. 1. Effectiveness of compounds to inhibit the immune-induced production of NO, PGE_2 and cytokines in rat peritoneal cells. The data are IC_{50}s (μM) with 95% limits of confidence in parentheses

	NO	PGE_2	IL-1β	IL-6	TNF-α	VEGF
Artemisinin	> 100	37.59 (33.22 – 42.53)	> 100	> 100	> 100	> 100
Dihydroartemisinin	> 100	31.94 (15.40 – 66.26)	> 100	> 100	> 100	> 100
Arteannuin B	7.04 (4.18 – 1.87)	4.45 (1.39 – 14.27)	1.06 (0.61 – 1.85)	1.53 (0.77 – 3.04)	4.41 (2.89 – 6.73)	1.21 (0.69 – 2.12)
Artemisinic acid	> 100	24.30 (19.35 – 30.51)	> 100	> 100	> 100	> 100
Casticin	21.10 (16.65 – 6.73)	3.16 (2.01 – 4.99)	6.94 (5.38 – 8.94)	8.69 (5.66 – 13.35)	14.95 (9.67 – 13.13)	3.36 (2.52 – 5.40)
Chrysosplenol D	12.71 (9.70 – 16.66)	14.54 (4.81 – 43.97)	5.46 (2.79 – 10.70)	10.30 (4.76 – 22.27)	8.67 (4.48 – 16.80)	11.25 (7.71 – 16.41)
4-Methylesculetin	> 100	> 100	> 100	> 100	> 100	> 100

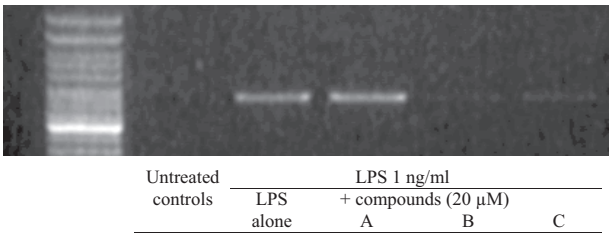

Fig. 2. Expression of iNOS mRNA in rat peritoneal cells cultured 2 h in the presence of LPS (1 ng/ml) plus dihydroartemisinin (A), casticin (B), and chrysosplenol D (C)

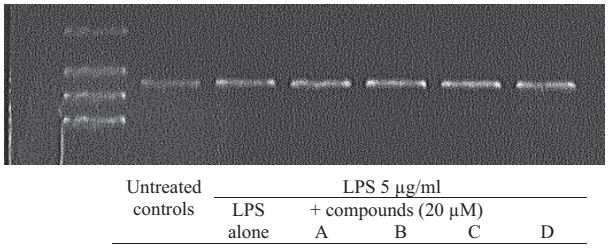

Fig. 4. Expression of COX-2 mRNA in rat peritoneal cells cultured 4 h in the presence of LPS (5 μg/ml) plus dihydroartemisinin (A), casticin (B), chrysosplenol D (C) and artemisinin (D)

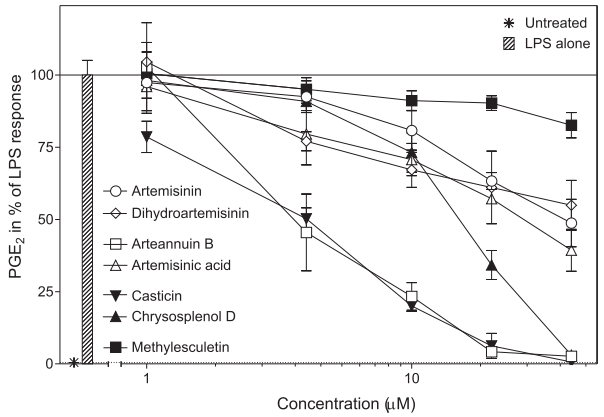

Fig. 3. Effect of the compounds on *in vitro* production of PGE_2 by rat (n = 7) peritoneal cells cultured 24 h in the presence of LPS (5 μg/ml). Concentration of PGE_2 was determined by ELISA. Each point is the mean ± SEM showing the average of two identical experiments. Because the concentration of PGE_2 differed between them, the data were transformed to percent values. The IC_{50} estimates are shown in Table 1

Effects of compounds on PGE_2 production by rat cells

Two identical experiments were done. Both constitutive and the LPS-activated PGE_2 production differed between them. The concentration of PGE_2 in supernatants of control cells was 44.1 ± 18.0 and 114.9 ± 54.7 pg/ml in experiments 1 and 2, respectively (the means ± SEM). It increased up to 15.39 ± 0.29 and 18.09 ± 1.50 ng/ml, respectively, upon the exposure of the cells to LPS (5 μg/ml). The effects of compounds were therefore evaluated as percent changes.

Except 4-methylesculetin, the compounds dose-dependently suppressed production of PGE_2 (Fig. 3). Arteannuin B and casticin reduced PGE_2 by about 50% at concentration of 4 μM ($p < 0.05$). The onset of significant inhibitory effects of artemisinin, dihydroartemisinin, artemisinic acid (p 0.05) and chrysosplenol D ($p < 0.001$) was observed with concentration of 20 μM. No significant difference between the effects of artemisinin and dihydroartemisinin was found [$F(1, 18) = 0.80$, $p = 0.38$]. The difference between the effects of arteannuin B and artemisinic acid was statistically highly significant [$F(1, 18) = 36.71$, $p < 0.0001$]. The IC_{50} estimates are shown in Table 1.

Artemisinin, dihydroartemisinin, casticin, chrysosplenol D did not change the expression of COX-2 mRNA expression in cells cultured in the presence of LPS (5 μg/ml) for 4 h (Fig. 4) or 24 h (data not shown).

Effects of compounds on cytokine secretion by rat cells

The secretion of cytokines was dose-dependently inhibited by arteannuin B, and by flavonoids casticin and chrysosplenol D (Fig. 5). Dihydroartemisinin, artemisinic acid, and methylesculetin remained ineffective even at the highest dose used, i.e., 40 μM. The IC_{50} estimates were within the range of 1–15 μM for all cytokines (Tab. 1).

Effects of compounds on production of PGE_2 and cytokines by human PBMC

The constitutive production of PGE_2 and the response of PBMC to the PGE_2-augmenting effect of LPS differed among individual donors (n = 4). The constitutive values were 130, 64, 52, and 426 pg/ml in donors I, II, III, and IV, respectively. They increased to 16,062, 8,069, 4,362, and 27,651 pg/ml (donors I, II, III, and IV, respectively) after the LPS treatment.

Interindividual differences were also found in production of cytokines. Concentrations of TNF-α in control PBMC were 149, 89, 235, and 284 pg/ml in

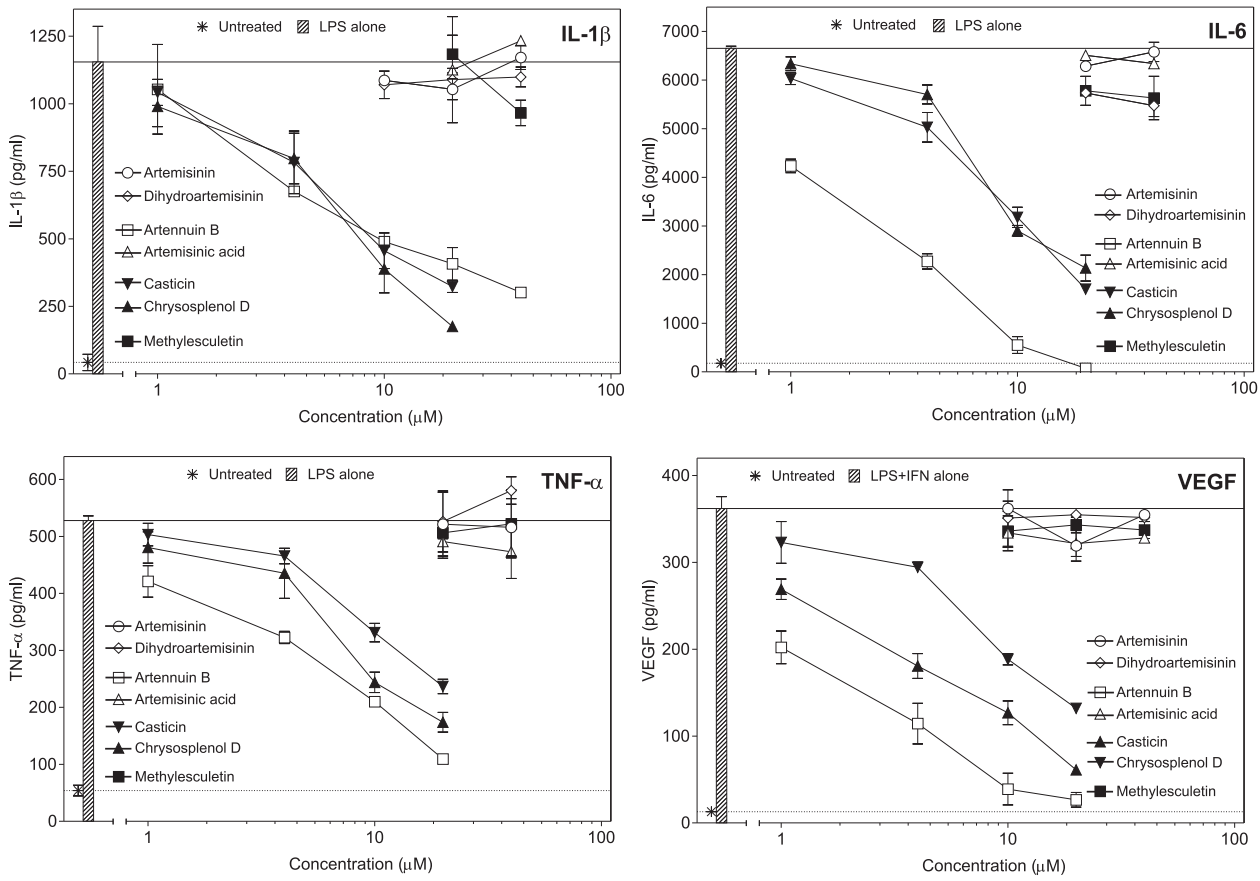

Fig. 5. *In vitro* effect of test compounds on immune-activated secretion of cytokines by rat (n = 5) peritoneal cells. Production of IL-1β, IL-6 and TNF-α was activated by LPS (1 ng/ml). VEGF secretion was activated by LPS (10 ng/ml) plus rat recombinant IFN-γ (5 ng/ml). Cytokine concentrations were evaluated at the interval of 24 h of culture, using ELISA. Each point is the mean ± SEM. The data represent one of two identical experiments. The IC_{50} estimates are shown in Table 1

donors I, II, III, and IV, respectively. Those in the LPS-activated PBMC reached the values of 3,325, 3,138, 5,999, and 8,178 pg/ml in donors I, II, III, and IV, respectively. The following constitutive and LPS-augmented concentrations of IL-6 were observed in PBMC from individual donors: 2,175 and 5,141 pg/ml (donor I), 1,663 and 4,376 pg/ml (donor II), 1,126 and 3,816 pg/ml (donor III), and 1,996 and 3,721 pg/ml (donor IV), respectively.

The effects of compounds, expressed as percent changes, are shown in Figure 6. The highest concentration (20 μM) of dihydroartemisinin suppressed significantly production of PGE_2 ($p < 0.01$), but it did not affect the cytokine secretion. As low concentration of artennuin B as 5 μM completely inhibited production of PGE_2 and reduced secretion of TNF-α and IL-6 by approximately 50% at the same dose ($p < 0.01$). Flavonoids casticin and chrysosplenol B inhibited dose-dependently production of both PGE_2 and cytokines, the effect on PGE_2 being more pronounced. While the 10 μM dose was sufficient to reduce significantly secretion of TNF-α, higher concentration (20 μM) was required to suppress IL-6 by about 30% ($p < 0.01$).

Cytotoxicity

Artemisinin, dihydroartemisinin, artemisinic acid, and 4-methylesculetin were devoid of any signs of cytotoxicity up to the highest (40 μM) concentration used (Fig. 7). Flavonoids casticin and chrysosplenol D applied at 40 μM concentration suppressed the viability of cells by approximately 70% ($p < 0.01$). Significant suppressive effect of arteannuin B was exhibited with the concentration of 10 μM ($p < 0.01$), and it was further enhanced in dependence on increasing doses.

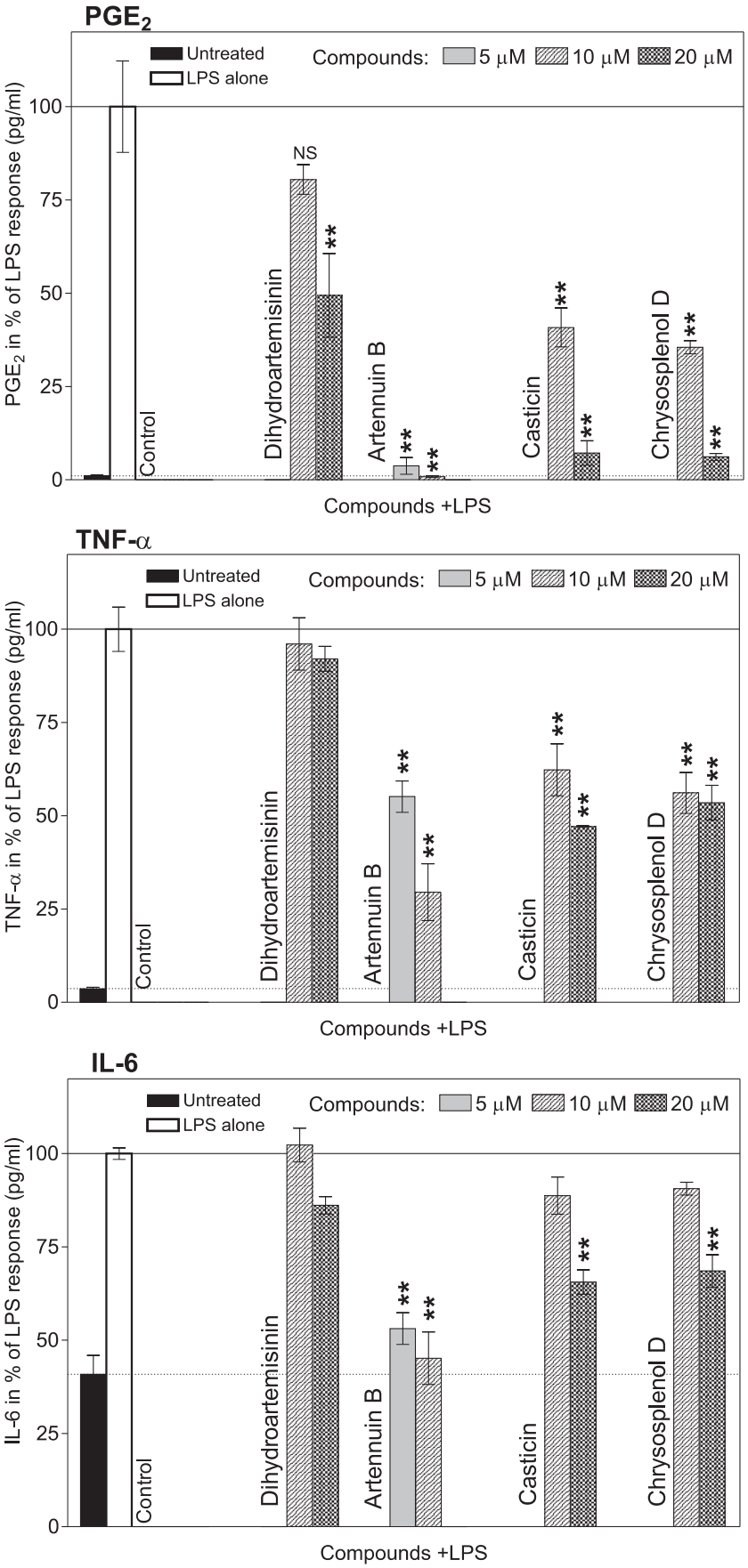

Fig. 6. Effect of test compounds on *in vitro* production of PGE_2 and cytokines TNF-α and IL-6 by human PBMC cultured 24 h in the presence of LPS (1 μg/ml). Concentration of the analytes was determined by ELISA. Each point is the mean ± SEM. The effects have been confirmed in another identical experiment. ** $p < 0.01$

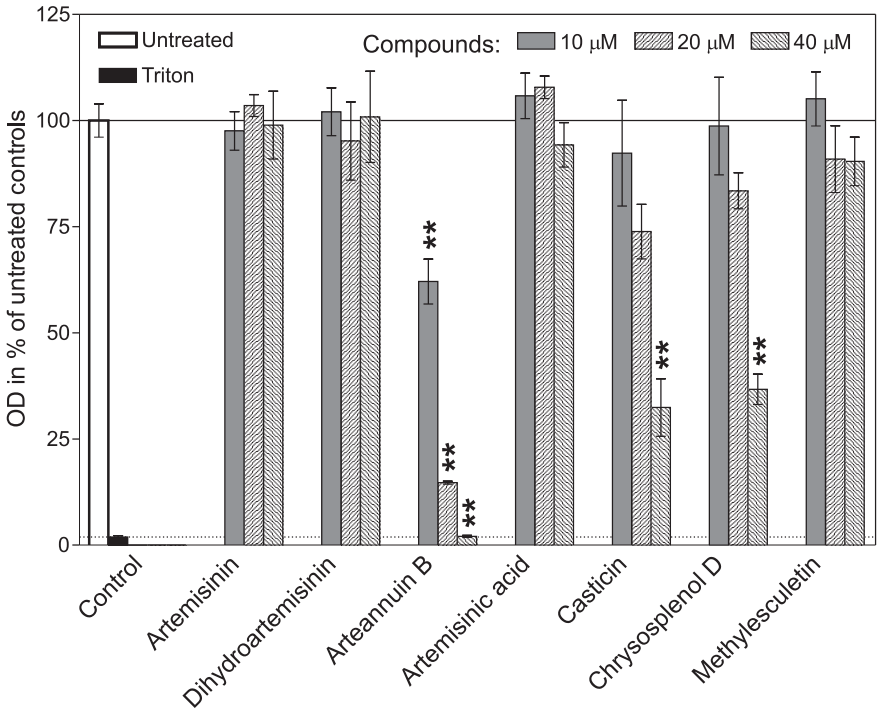

Fig. 7. Effect of test compounds on viability of rat (n = 4) peritoneal cells. The effects on mitochondrial respiration were evaluated at the interval of 24 h of culture. Optical density (OD) of untreated control cells (i.e., cultured in the absence of any compound) was taken as 100%. Triton was used to kill all cells. The control OD value was 1.935. Each bar is the mean ± SEM. The data are representative of two identical experiments. ** $p < 0.01$

Discussion

Characteristic feature of *Artemisia annua* L. phytochemistry is a large number of biologically active chemical entities with prevailing occurrence of sesquiterpenoids, flavonoids, terpene allylic hydroperoxides and endoperoxides [9, 53]. Despite the crucial importance of Artemisia in the development of antimalarials, only limited data are available on immunobiological properties of Artemisia metabolites including artemisinin itself. With regard to the fact that the plant possesses anticancer potential, we have explored the effects of selected pure compounds isolated from *A. annua* on major mediators of angiogenesis such as NO [50, 57], PGE_2 [12, 41] and cytokines [5, 28].

Several lines of evidence suggested the effects of artemisinin and dihydroartemisinin on NO formation. Artemisinin itself, applied at concentration of 4 μg/ml inhibited moderately (by approximately 38%) the LPS/IFN-γ-activated production of NO in mouse RAW 264.7 macrophage cell line. The same concentration of artesunate, a semi-synthetic derivative of artemisinin, was about twice-more effective [25]. Artemisinin (10 μM) was also found to inhibit the NOS activity and NO production in human astrocytoma T67 cells activated by cytokines and LPS [2]. A novel artemisinin derivative SM905 (10 μM) decreased the iNOS mRNA expression and production of NO in the LPS-stimulated murine macrophage RAW 264.7 cell line [46]. In contrast, NO production was greatly enhanced by artesunate in hepatoma cell line HepG2 when applied at concentration of 50 μM, but it was ineffective to alter NO formation at higher doses [52].

Artemisinin and artesunate were found to attenuate the release of TNF-α and IL-6 induced by CpG oligodeoxynucleotide, LPS, or *Escherichia coli* in murine peritoneal macrophages and RAW 264.7 macrophage cell line [29, 45]. Artesunate also lowered the expression of VEGF in tumor cells [20].

Obviously, the present data are in variance with these findings. We have found no substantial effect of artemisinin and dihydroartemisinin on immune-stimulated production of NO in rat peritoneal cells. Neither the secretion of cytokines IL-1β, IL-6, TNF-α and VEGF, produced by both rat cells and human PBMC, was substantially influenced by these compounds. The source of the discrepancy is unclear but it cannot be excluded that it may result from different responsiveness of distinct cell types.

Whereas not inhibiting NO and cytokines, both artemisinin and dihydroartemisinin did inhibit production of PGE_2 in cells of both animal and human

origin. The present findings are the first evidence demonstrating the ability of these animalarials to interfere with prostaglandin biosynthesis.

Immunobiological activities of sesquiterpene artemisinin precursors, arteannuin B and artemisinic acid differed substantially. Similarly though more effectively than artemisinin and dihydroartemisinin, both arteannuin B and artemisinic acid suppressed production of PGE_2 (IC_{50}s 4.45 and 24.30 μM, respectively). However, only arteannuin B inhibited production of NO and cytokines (IL-1β, IL-6, TNF-α, VEGF) with the IC_{50}s < 5 μM. Artemisinic acid was entirely ineffective. It cannot be excluded that the high inhibitory effectiveness of arteannuin B might be partially influenced by enhanced cytotoxicity. While the 10 μM concentration virtually completely abolished production of IL-6 and VEGF, the viability of cells decreased by 40% at this dose. Immunosuppressive mode of action of arteannuin B was exhibited in the cells of both rat and human origin. It was especially effective in inhibiting the production of PGE_2 in immune-activated human PBMC.

4-Methylesculetin, a coumarin-type compound isolated from *A. annua*, did not alter any of the parameters investigated. Its reported effectiveness to reduce the symptoms of experimental colitis in rats [48] thus seems unlikely to depend on the interaction with the disease-associated immune mechanisms. It should be mentioned that many analogues of coumarin possess immunomodulatory properties. They are usually suppressive [23, 32, 37] though esculetin (i.e., the 6,7-dihydroxy derivative of coumarin) has been reported to augment production of NO in LPS-treated macrophages [27].

Like many other flavonoids [18, 19, 24, 35], both casticin and chrysosplenol D, included in the present study, were found to be effective inhibitors of NO, PGE_2 and cytokine production. Casticin was slightly less effective than chrysosplenol D in suppressing NO, but it was 3-4-fold more effective in inhibiting production of PGE_2 (IC_{50}s: 3.2 and 14.5 μM, respectively) and VEGF (IC_{50}s: 3.4 and 11.3 μM, respectively). Their effectiveness to reduce secretion of cytokines IL-1β, IL-6, and TNF-α was nearly identical, the IC_{50}s being approximately 10 μM. They both potently inhibited PGE_2 and less prominently though statistically significantly the cytokine production also in human PBMC. Importantly, neither of them exhibited significant cytocidal effects up to the concentration of 20 μM. Importantly, casticin has also been found to inhibit another angiogenic factor, transforming growth factor-β1 (TGF-β) [14].

All sesquiterpenes encompassed in the study, including artemisinin, inhibited production of PGE_2 but only arteannuin B inhibited NO and cytokines in addition. The most remarkable inhibitory potential was possessed by flavonoids casticin and chrysosplenol D that inhibited all these mediators. Most importantly, they also suppressed secretion of VEGF which plays a crucial role in physiologic and pathologic angiogenesis [22] and thus has become the most important target in anti-cancer therapy. Several small-molecular weight compounds inhibiting the VEGF receptor signaling have been developed and used in the treatment of renal cell carcinoma, imatinib-resistant gastrointestinal stromal tumors [15], non-small-cell lung carcinoma, colon cancer [11], and malignant pleural mesothelioma [33]. VEGF has been shown to be upregulated by NO [50] and PGE_2 [8]. Whether or not the angiogenic property of NO and PGE_2 totally depends on enhanced VEGF secretion is unclear, however. In any case, PG inhibitors play a role in eradication and prevention of tumors [1, 21, 47], and also inhibitors of NO biosynthesis have been found to exhibit anticancer effects [42, 44].

Our findings suggest that the immunomodulatory effects of flavonoids casticin and chrysosplenol D depend on distinct modes of action. We have observed that while the interaction with transcription events is the most plausible explanation for reduced NO production, the inhibition of PGE_2 depends obviously on post-transcriptional mechanisms. The exact mechanism(s) remain unclear at the present stage of experimental findings.

To summarize, the present study demonstrates that several metabolites produced by *A. annua* are inhibitors of major pro-angiogenic factors such as NO, PGE_2, and cytokines VEGF, IL-1β, IL-6 and TNF-α. Noteworthy, *A. annua* derived flavonoids casticin and chrysosplenol D are potent dual inhibitors of both NO and PGE_2. Angiogenesis, the development of new capillaries, is associated with the development and progression of many diseases, and it is a rate-limiting step in tumor development [34]. The present data contribute to the better understanding of plausible mechanisms of anticancer effects of *Artemisia annua* L.

Acknowledgments:
This work was supported by the bilateral grant from the Ministry of Education, Youth and Sports of the Czech Republic (No. ME10116), and by the International Science & Technology Cooperation Program of the People's Republic of China (No. 2011DFA30870).

References:

1. Al-Waili NS: A potential concept in the management of tumors with modulation of prostaglandin, nitric oxide and antioxidants. ScientificWorldJournal, 2007, 7, 466–478.
2. Aldieri E, Atragene D, Bergandi L, Riganti C, Costamagna C, Bosia A, Ghigo D: Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-κB activation. FEBS Lett, 2003, 552, 141–144.
3. Aung W, Sogawa C, Furukawa T, Saga T: Anticancer effect of dihydroartemisinin (DHA) in a pancreatic tumor model evaluated by conventional methods and optical imaging. Anticancer Res, 2011, 31, 1549–1558.
4. Awale S, Linn TZ, Li F, Tezuka Y, Myint A, Tomida A, Yamori T et al.: Identification of chrysoplenetin from *Vitex negundo* as a potential cytotoxic agent against PANC-1 and a panel of 39 human cancer cell lines (JFCR-39). Phytother Res, 2011, 25, 1770–1775.
5. Benelli R, Lorusso G, Albini A, Noonan DM: Cytokines and chemokines as regulators of angiogenesis in health and disease. Curr Pharm Des, 2006, 12, 3101–3115.
6. Berger TG, Dieckmann D, Efferth T, Schultz ES, Funk JO, Baur A, Schuler G: Artesunate in the treatment of metastatic uveal melanoma-first experiences. Oncol Rep, 2005, 14, 1599–1603.
7. Bertea CM, Freije JR, van der Woude H, Verstappen FW, Perk L, Marquez V, De Kraker JW et al.: Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. Planta Med, 2005, 71, 40–47.
8. Bradbury D, Clarke D, Seedhouse C, Corbett L, Stocks J, Knox A: Vascular endothelial growth factor induction by prostaglandin E_2 in human airway smooth muscle cells is mediated by E prostanoid EP_2/EP_4 receptors and SP-1 transcription factor binding sites. J Biol Chem, 2005, 280, 29993–30000.
9. Brown GD: The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). Molecules, 2010, 15, 7603–7698.
10. Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. Nature, 2000, 407, 249–257.
11. Cascone T, Gridelli C, Ciardiello F: Combined targeted therapies in non-small cell lung cancer: a winner strategy? Curr Opin Oncol, 2007, 19, 98–102.
12. Ding YB, Shi RH, Tong JD, Li XY, Zhang GX, Xiao WM, Yang JG et al.: PGE_2 up-regulates vascular endothelial growth factor expression in MKN28 gastric cancer cells via epidermal growth factor receptor signaling system. Exp Oncol, 2005, 27, 108–113.
13. Ferreira JF, Luthria DL, Sasaki T, Heyerick A: Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules, 2010, 15, 3135–3170.
14. Freitas S, Costa S, Azevedo C, Carvalho G, Freire S, Barbosa P, Velozo E et al.: Flavonoids inhibit angiogenic cytokine production by human glioma cells. Phytother Res, 2011, 25, 916–921.
15. Grandinetti CA, Goldspiel BR: Sorefanib and sunitinib: novel targered therapies for renal cell cancer. Pharmacotherapy, 2011, 27, 1125–1144.
16. Haďdara K, Zamir L, Shi QW, Batist G: The flavonoid casticin has multiple mechanisms of tumor cytotoxicity action. Cancer Lett, 2006, 242, 180–190.
17. He Q, Shi J, Shen XL, An J, Sun H, Wang L, Hu YJ et al.: Dihydroartemisinin upregulates death receptor 5 expression and cooperates with TRAIL to induce apoptosis in human prostate cancer cells. Cancer Biol Ther, 2010, 9, 819–824.
18. Higa S, Hirano T, Kotani M, Matsumoto M, Fujita A, Suemura M, Kawase I, Tanaka T: Fisetin, a flavonol, inhibits TH2-type cytokine production by activated human basophils. J Allergy Clin Immunol, 2003, 111, 1299–1306.
19. Hirano T, Higa S, Arimitsu J, Naka T, Shima Y, Ohshima S, Fujimoto M et al.: Flavonoids such as luteolin, fisetin and apigenin are inhibitors of interleukin-4 and interleukin-13 production by activated human basophils. Int Arch Allergy Immunol, 2004, 134, 135–140.
20. Chen HH, Zhou HJ, Wu GD, Lou XE: Inhibitory effects of artesunate on angiogenesis and on expressions of vascular endothelial growth factor and VEGF receptor KDR/flk-1. Pharmacology, 2004, 71, 1–9.
21. Chen T, Hwang H, Rose ME, Nines RG, Stoner GD: Chemopreventive properties of black raspberries in N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis: down-regulation of cyclooxygenase-2, inducible nitric oxide synthase, and c-Jun. Cancer Res, 2006, 66, 2853–2859.
22. Iqbal S, Lenz HJ: Integration of novel agents in the treatment of colorectal cancer. Cancer Chemother Pharmacol, 2004, 54, Suppl 1, S32–S39.
23. Jackson SA, Sahni S, Lee L, Luo Y, Nieduzak TR, Liang G, Chiang Y et al.: Design, synthesis and characterization of a novel class of coumarin-based inhibitors of inducible nitric oxide synthase. Bioorg Med Chem, 2005, 13, 2723–2739.
24. Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP: Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem Pharmacol, 1999, 58, 759–765.
25. Konkimalla VB, Blunder M, Korn B, Soomro SA, Jansen H, Chang W, Posner GH et al.: Effect of artemisinins and other endoperoxides on nitric oxide-related signaling pathway in RAW 264.7 mouse macrophage cells. Nitric Oxide, 2008, 19, 184–191.
26. Krishna S, Bustamante L, Haynes RK, Staines HM: Artemisinins: their growing importance in medicine. Trends Pharmacol Sci, 2008, 29, 520–527.
27. Leung KN, Leung PY, Kong LP, Leung PK: Immunomodulatory effects of esculetin (6,7-dihydroxycoumarin) on murine lymphocytes and peritoneal macrophages. Cell Mol Immunol, 2005, 2, 181–188.
28. Lewis CE, Leek R, Harris A, McGee JO: Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. J Leukoc Biol, 1995, 57, 747–751.
29. Li B, Zhang R, Li J, Zhang L, Ding G, Luo P, He S et al.: Antimalarial artesunate protects sepsis model mice

against heat-killed *Escherichia coli* challenge by decreasing TLR4, TLR9 mRNA expressions and transcription factor NF-κB activation. Int Immunopharmacol, 2008, 8, 379–389.
30. Li WX, Cui CB, Cai B, Wang HY, Yao XS: Flavonoids from *Vitex trifolia* L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells. J Asian Nat Prod Res, 2005, 7, 615–626.
31. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS: Macrophage oxidation of L-arginine to nitrite and nitrate. Biochemistry, 1988, 27, 8706–8711.
32. Matsuda H, Morikawa T, Ohgushi T, Ishiwada T, Nishida N, Yoshikawa M: Inhibitors of nitric oxide production from the flowers of *Angelica furcijuga*: structures of hyuganosides IV and V. Chem Pharm Bull (Tokyo), 2005, 53, 387–392.
33. Ogino H, Yano S, Kakiuchi S, Yamada T, Ikuta K, Nakataki E, Goto H et al.: Novel dual targeting strategy with vandetanib induces tumor cell apoptosis and inhibits angiogenesis in malignant pleural mesothelioma cells expressing RET oncogenic rearrangement. Cancer Lett, 2008, 265, 55–66.
34. Oklu R, Walker TG, Wicky S, Hesketh R: Angiogenesis and current antiangiogenic strategies for the treatment of cancer. J Vasc Interv Radiol, 2010, 21, 1791–1805.
35. Olszanecki R, Gębska A, Kozlovski VI, Gryglewski RJ: Flavonoids and nitric oxide synthase. J Physiol Pharmacol, 2002, 53, 571–584.
36. Ono M, Yanaka T, Yamamoto M, Ito Y, Nohara T: New diterpenes and norditerpenes from the fruits of *Vitex rotundifolia*. J Nat Prod, 2002, 65, 537–541.
37. Raghav SK, Gupta B, Shrivastava A, Das HR: Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1β through suppression of NF-κB activation by 3-(1'-1'-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from *Ruta graveolens* L. Eur J Pharmacol, 2007, 560, 69–80.
38. Sakurai T, Kudo M: Signaling pathways governing tumor angiogenesis. Oncology, 2011, 81, Suppl 1, 24–29.
39. Singh NP, Panwar VK: Case report of a pituitary macroadenoma treated with artemether. Integr Cancer Ther, 2006, 5, 391–394.
40. Song X, Chen Y, Sun Y, Lin B, Qin Y, Hui H, Li Z et al.: Oroxylin A, a classical natural product, shows a novel inhibitory effect on angiogenesis induced by lipopolysaccharide. Pharmacol Rep, 2012, 64, 1189–1199.
41. Toomey DP, Murphy JF, Conlon KC: COX-2, VEGF and tumour angiogenesis. Surgeon, 2009, 7, 174–180.
42. Uneda S, Hata H, Matsuno F, Nagasaki A, Harada N, Mitsuya Y, Matsuzaki H, Mitsuya H: A nitric oxide synthase inhibitor, N^G-nitro-l-arginine-methyl-ester, exerts potent antiangiogenic effects on plasmacytoma in a newly established multiple myeloma severe combined immunodeficient mouse model. Br J Haematol, 2003, 120, 396–404.
43. Wallaart TE, Pras N, Beekman AC, Quax WJ: Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Med, 2000, 66, 57–62.
44. Wang GY, Ji B, Wang X, Gu JH: Anti-cancer effect of iNOS inhibitor and its correlation with angiogenesis in gastric cancer. World J Gastroenterol, 2005, 11, 3830–3833.
45. Wang J, Zhou H, Zheng J, Cheng J, Liu W, Ding G, Wang L et al.: The antimalarial artemisinin synergizes with antibiotics to protect against lethal live *Escherichia coli* challenge by decreasing proinflammatory cytokine release. Antimicrob Agents Chemother, 2006, 50, 2420–2427.
46. Wang JX, Hou LF, Yang Y, Tang W, Li Y, Zuo JP: SM905, an artemisinin derivative, inhibited NO and pro-inflammatory cytokine production by suppressing MAPK and NF-κB pathways in RAW 264.7 macrophages. Acta Pharmacol Sin, 2009, 30, 1428–1435.
47. Watanabe K, Kawamori T, Nakatsugi S, Wakabayashi K: COX-2 and iNOS, good targets for chemoprevention of colon cancer. Biofactors, 2000, 12, 129–133.
48. Witaicenis A, Seito LN, Di Stasi LC: Intestinal anti-inflammatory activity of esculetin and 4-methylesculetin in the trinitrobenzenesulphonic acid model of rat colitis. Chem Biol Interact, 2010, 186, 211–218.
49. Wu XH, Zhou HJ, Lee J: Dihydroartemisinin inhibits angiogenesis induced by multiple myeloma RPMI8226 cells under hypoxic conditions via downregulation of vascular endothelial growth factor expression and suppression of vascular endothelial growth factor secretion. Anticancer Drugs, 2006, 17, 839–848.
50. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG: The role of nitric oxide in cancer. Cell Res, 2002, 12, 311–320.
51. Yance DRJ, Sagar SM: Targeting angiogenesis with integrative cancer therapies. Integr Cancer Ther, 2006, 5, 9–29.
52. Zeng Q-P, Zhang P-Z: Artesunate mitigates proliferation of tumor cells by alkylating heme-harboring nitric oxide synthase. Nitric Oxide, 2011, 24, 110–112.
53. Zhai DD, Zhong JJ: Simultaneous analysis of three bioactive compounds in *Artemisia annua* hairy root cultures by reversed-phase high-performance liquid chromatography-diode array detector. Phytochem Anal, 2010, 21, 524–530.
54. Zhang D, Yang L, Yang L-X, Wang M-Y, Tu Y-Y: Determination of artemisinin, arteannuin B and artemisinic acid in Herba Artemisiae Annuae by HPLC-UV-ELSD. Acta Pharmaceut Sinica, 2007, 42, 978–981.
55. Zhou HJ, Wang WQ, Wu GD, Lee J, Li A: Artesunate inhibits angiogenesis and downregulates vascular endothelial growth factor expression in chronic myeloid leukemia K562 cells. Vascul Pharmacol, 2007, 47, 131–138.
56. Zhu Z, Witte L: Inhibition of tumor growth and metastasis by targeting tumor-associated angiogenesis with antagonists to the receptors of vascular endothelial growth factor. Invest New Drugs, 1999, 17, 195–212.
57. Ziche M, Morbidelli L: Molecular regulation of tumour angiogenesis by nitric oxide. Eur Cytokine Netw, 2009, 20, 164–170.

Received: May 15, 2012; **in the revised form:** November 15, 2012; **accepted:** November 22, 2012.