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SHORT COMMUNICATION

Antiinflammatory and Lipoxygenase Inhibitory Compounds from *Vitex agnus-castus*

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Several secondary metabolites, artemetin (1), casticin (2), 3,3′-dihydroxy-5,6,7,4′-tetramethoxy flavon (3), penduletin (4), methyl 4-hydroxybenzoate (5), *p*-hydroxybenzoic acid (6), methyl 3,4-dihydroxybenzoate (7), 5-hydroxy-2-methoxybenzoic acid (8), vanillic acid (9) and 3,4-dihydroxybenzoic acid (10) were isolated from a folkloric medicinal plant, *Vitex agnus-castus*. The structures of compounds 1–10 were identified with the help of spectroscopic techniques. Compounds 3–10 were isolated for the first time from this plant. These compounds were screened for their antiinflammatory and lipoxygenase inhibitory activities. Compounds 6, 7 and 10 were found to have significant antiinflammatory activity in a cell-based contemporary assay, whereas compounds 1 and 2 exhibited a potent lipoxygenase inhibition. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Vitex agnus-castus; Verbenaceae; antiinflammatory activity; lipoxygenase inhibitory activity; flavonoids; phenolic derivatives.

INTRODUCTION

Vitex agnus-castus Linn. (Verbenaceae) is locally known as 'Hub-el-faked' and 'Sumbhalu-ke-bij'. It is also called as 'Chaste tree'. It is a very well known plant, which has immense medicinal importance. It is abundantly found in the Mediterranean region through South-West Asian countries up to Baluchistan in Pakistan and also cultivated in gardens (Chadha, 1976). It is traditionally used as an emmenagogue, sedative, anaphrodisiac and galactagogue (Bruneton, 1993). *Vitex agnus-castus* regulates hormones and increases breast milk production (Thomas, 2000). An ethanol extract of the *Vitex agnus-castus* is used as a homeopathic drug (agnus castus) for the treatment of impotence and central nervous system disorders (Schwabe, 1987). In Unani medicine, the seeds are taken for dropsy and inflammation, to purify the brain and liver, and as a contraceptive (McKenna *et al.*, 2002). Flowers are effective in diarrhoea, liver affections and as a cardiac tonic. The powder of green parts is useful as internal antihemorrhage (Usmanghani *et al.*, 1997).

Inflammation occurs as a defensive response, which induces physiological adaptations to limit tissue damage and to remove pathogenic infections. Inflammation is characterized by the immediate infiltration of a specific site or lesion with neutrophils, followed by monocytes and finally lymphocytes. As a first line of defense, activated neutrophils play a crucial role in the destruction of foreign antigens and the breakdown and remodeling of injured tissues. For this purpose, they are equipped with various defensive mechanisms using different proteins and signaling pathways related to chemotaxis, phagocytosis, exocytosis and bactericidal activities contained in granules and the generation of toxic oxygen metabolites including reactive oxygen species (ROS) by various pathways (Roussin *et al.*, 1997). ROS are formed subsequent to the assembly and activation of the phagocyte-specific enzyme, NADPH oxidase. This process is initiated by the production of superoxide anion (O^{2-}), during a 'respiratory burst' of non-mitochondrial oxygen uptake by an NADPH oxidase system (Tan and Berridge, 2000).

In this study, a tetrazolium salt (WST-1) was used to measure the superoxide production by neutrophils activated by opsonized zymosan, which induces phagocytic activation of neutrophils. This technique is more sensitive and reliable than other available techniques and can be adapted in microplate format. WST-1 is reduced by NADPH oxidase via superoxide. It is a very useful assay for measuring the neutrophil functions and anti-inflammatory response (Costantino *et al.*, 1998).

Lipoxygenases constitute a family of non-haem iron containing dioxygenases that are widely found in animals and plants. In mammalian cells, these are the key enzymes involve the biosynthesis of a variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines (Land, 1985). Lipoxygenase products play an important role in a variety of disorders such as bronchial asthma, inflammation (Steinhilber, 1999), autoimmune diseases and tumor angiogenesis (Nie and Honn, 2002). Lipoxygenases are therefore potential targets for rational drug design and

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the discovery of mechanism-based inhibitors for the treatment of these diseases.

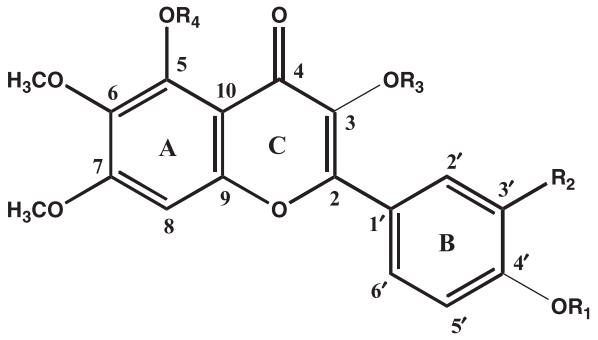
Based on the potent medicinal importance of *Vitex agnus-castus*, a phytochemical study was conducted on this plant and four flavonoids **1–4**, and six phenolic derivatives, methyl 4-hydroxybenzoate **5–10** were isolated. The structures of these compounds were deduced by comparison of their spectral data with those reported in the literature. Compounds **3–10** were isolated for the first time from this plant. The aim of this study was to evaluate the antiinflammatory activities of these compounds with the help of an *in vitro* assay and to explore their potential as non-steroidal antiinflammatory agents. Lipoxygenase inhibitory activity of compounds **1–10** was also studied.

MATERIAL AND METHODS

Analytical methods. The ^{1}H-NMR spectra were recorded in $CDCl_3$ and CD_3OD on Bruker AM-400 and AMX-500 NMR spectrometers with TMS as an internal standard using a UNIX operating system at 400 and 500 MHz, respectively. The ^{13}C-NMR spectra were recorded in $CDCl_3$ and CD_3OD at 125 MHz on a Bruker AMX-500 NMR spectrometer. HREI-MS were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The IR spectra were recorded on a Jasco A-302 spectrophotometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. The optical rotations were measured on a JASCO DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried out on silica gel columns (70–230 mesh). The purity of the samples was checked by pre-coated silica gel TLC (GF-254, 20 × 20 cm, 0.25 mm thick, Merck) and were detected under UV light (254 and 366 nm), while ceric sulphate was used as a spraying reagent. WST-1 was purchased from Dojindo Laboratories (Kumamoto, Japan). Zymosan A was purchased from Sigma Chemicals (St Louis, MO, USA), while Ficoll paque was purchased from the Pharmacia Biotech Amersham (Uppsala, Sweden). Lipoxygenase (EC. 1.13.11.12) (5-LOX) type I-B (isolated from soybean) and linoleic acid were purchased from Sigma (St Louis, MO, USA). All reagents were of analytical grade. Deionized water was used in all experimental procedures. Absorbances were measured on a SpectraMax 384 microplate reader (Molecular Devices, USA).

Plant material. The aerial parts of *Vitex agnus-castus* Linn. (40 kg) were collected in September 1997 from Khost near Quetta (Pakistan) and were dried in air. The plant was identified by Dr Rasool Bakhsh Tareen, Department of Botany, Baluchistan University, Quetta, Pakistan. A herbarium specimen of this plant (VS # 1445) was deposited at the Department of Botany, University of Baluchistan, Quetta.

Extraction and isolation of compounds. Air-dried plant of *Vitex agnus-castus* (17 kg) was extracted with methanol (50 L) at room temperature (30 °C) for 15 days. After evaporation of the solvent, a crude extract (800 g) was obtained, which was dissolved in distilled H_2O (3 L) and defatted with petroleum ether (9 L). The defatted

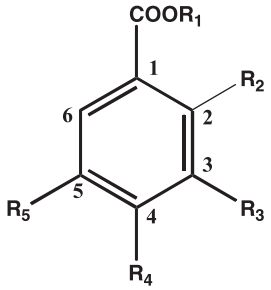


1. $R_1 = R_3 = CH_3$, $R_2 = OCH_3$, $R_4 = H$

2. $R_1 = R_3 = CH_3$, $R_2 = OH$, $R_4 = H$

3. $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$

4. $R_1 = R_2 = R_4 = H$, $R_3 = CH_3$



5. $R_1 = CH_3$, $R_2 = R_3 = R_5 = H$, $R_4 = OH$

6. $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$

7. $R_1 = CH_3$, $R_2 = R_5 = H$, $R_3 = R_4 = OH$

8. $R_1 = R_3 = R_4 = H$, $R_2 = OCH_3$ $R_5 = OH$

9. $R_1 = R_2 = R_5 = H$, $R_3 = OCH_3$, $R_4 = OH$

10. $R_1 = R_2 = R_5 = H$, $R_3 = R_4 = OH$

Figure 1. Structures of compounds **1–10** isolated from methanol extract of *Vitex agnus-castus*.

aqueous extract was further fractionated using various solvent mixtures (chloroform, ethyl acetate and butanol, each 9 L) to obtain $CHCl_3$, EtOAc and butanol fractions, respectively.

The resulting $CHCl_3$ fraction (41.2 g) was loaded onto a silica gel (70–230 mesh, 500 g) column and eluted with about 8 L of $CHCl_3$: petroleum ether to $CHCl_3$: MeOH mixtures with increasing polarity (from 1:9 to 9:1, respectively) to afford eight major fractions (CH-1 to CH-8). Four compounds, **1** (52.8 mg, petroleum ether: $CHCl_3$, 3.2:6.8), **2** (612.8 mg, petroleum ether: acetone, 8.5:1.5), **3** (8.6 mg, petroleum ether: acetone, 9:1) and **4** (12.2 mg, petroleum ether: $CHCl_3$, 4.7:5.3), were obtained from these column fractions by using repeated column chromatography.

The EtOAc fraction (87.5 g) was also subjected to column chromatography on silica gel (70–230 mesh, 950 g) and eluted with petroleum ether: EtOAc to EtOAc: MeOH mixtures (from 9:1 to 9:1, respectively,

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10 L) to afford nine major fractions (EA-1 to EA-9). These fractions were subjected to repeated column chromatography (silica gel) with the gradient solvent systems to obtain six compounds, i.e. **5** (22.6 mg, petroleum ether: EtOAc, 9:1), **6** (28.4 mg, petroleum ether: EtOAc, 5.7:4.3), **7** (26.3 mg, $CHCl_3$), **8** (12.8 mg, $CHCl_3$: CH_3OH, 9.5:0.5), **9** (7.2 mg, petroleum ether: EtOAc, 8.4:1.6) and **10** (13.5 mg, $CHCl_3$: CH_3OH, 9.2:0.8).

Isolation of human neutrophils. Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated by the method of Siddiqui *et al.* (1995). Whole blood was mixed with Ficoll paque and left for the sedimentation of unwanted red blood cells. After 30 min the buffy coat was layered on the Ficoll paque in the centrifuge tube. It was centrifuged for 30 min at 1500 rpm. After discarding the supernatant, RBCs were lysed by mixing with the hypotonic ammonium chloride solution (0.83%). It was centrifuged again and the neutrophils were washed with MHS (modified Hank's solution, pH 7.4) and resuspended at a concentration of 1×10^7 cells/mL.

Antiinflammatory assay. Antiinflammatory activity of test compounds was determined using a modified assay of Tan and Berridge (2000). This *in vitro* assay is based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Antiinflammatory activity was determined in a total volume of 250 µL MHS (pH 7.4) containing 1.0×10^4 neutrophils/mL, 500 µM WST-1 and various concentrations of test compounds. The control contained buffer, neutrophils and WST-1. All compounds were equilibrated at 37 °C and the reaction was initiated by adding opsonized Zymosan A (15 mg/mL), which was prepared by mixing it with human pooled serum, followed by centrifugation at 3000 rpm and the pellet was resuspended in PBS buffer. The absorbance was measured at 450 nm. Aspirin and indomethacin were used as positive controls, which are widely used as non-steroidal antiinflammatory drugs (NSAIDs) (MacDonald *et al.*, 2002; Martin *et al.*, 2002). The IC_{50} values were calculated in comparison with the DMSO as a blank and expressed as % inhibition of superoxide anions produced.

Lipoxygenase inhibition assay. Lipoxygenase (EC. 1.13.11.12) inhibiting activity was conveniently measured by modifying the spectrophotometric method developed by Tappel (1962). The reaction mixture, containing 165 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of test compound solution and 20 µL of lipoxygenase solution, was incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 µL linoleic acid (substrate) solution. With the formation of (9*Z*, 11*E*)-(13*S*)-13-hydroperoxyoctadeca-9,11-dienoate, the changes in absorbance at 234 nm were measured for 6 min. Test compounds and the control were dissolved in methanol. All the reactions were performed in triplicate in 96-well microplates in SpectraMax 384 Plus (Molecular Devices, USA). The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA). The percentage (%) inhibition was calculated as follows:

$$\% \text{ Inhibition} = (E - S)/E \times 100$$

where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

RESULTS AND DISCUSSION

The study on the methanol extract of *Vitex agnus-castus* of Pakistani origin resulted in the isolation and characterization of compounds **1–10**. The structures of these compounds were identified on the basis of spectroscopic methods as artemetin (**1**) (Atta-ur-Rahman *et al.*, 1988), casticin (**2**) (Iinuma *et al.*, 1980), 3,3′-dihydroxy-5,6,7,4′-tetramethoxy flavon (**3**) (Wagner *et al.*, 1965), penduletin (**4**) (Wang *et al.*, 1989), methyl 4-hydroxybenzoate (**5**) (Scott, 1970), *p*-hydroxybenzoic acid (**6**) (Scott, 1972), methyl 3,4-dihydroxybenzoate (**7**) (Scott, 1970), 5-hydroxy-2-methoxybenzoic acid (**8**) (Scott, 1970), vanillic acid (**9**) (Scott, 1972) and 3,4-dihydroxybenzoic acid (**10**) (Scott, 1972). All compounds, except **1** and **2**, were isolated for the first time from this plant.

The antiinflammatory activities of these compounds were evaluated using a contemporary assay (Tan and Berridge, 2000). Indomethacin and aspirin, clinically used antiinflammatory drugs were used as positive controls. Compounds **6**, **7** and **10** showed significant activities, compared with the standard at 400 µg/mL concentration, while compounds **2** and **4** were moderately active. Compounds **1**, **3**, **5**, **8** and **9** on the other hand, did not exhibit any activity even at the 1000 µg/mL concentration. Table 1 summarizes the IC_{50} values (the concentration of compound at which the superoxide production suppresses up to 50%) and the percentage inhibition of reduction of WST-1 compared with the positive control.

Compounds **1–10** were also tested for lipoxygenase inhibitory activity. Baicalein was used as a positive control. The result of the enzyme inhibition study is

Table 1. IC_{50} (µg/mL) values and percentage inhibition of reduction of WST-1 by NADPH oxidase via superoxides in the presence of test compounds 1–10 and positive controls, using freshly isolated human neutrophils. Cells were induced by opsonized zymosan at various concentrations of test compounds

Compound	% Inhibition (400 mg/mL)	IC_{50} (µg/mL) ± SEM
1	31.77	>400
2	69.51	302.10 ± 0.01
3	38.25	>400
4	59.82	342.65 ± 3.48
5	32.82	>400
6	76.62	167.52 ± 1.52
7	72.68	182.26 ± 2.34
8	41.38	>400
9	41.04	>400
10	82.26	156.34 ± 0.35
Indomethacin[a]	58.82	271.21 ± 5.90
Aspirin[a]	70.45	50.30 ± 4.42

IC_{50}, concentration of a test compound at which 50% inhibition of superoxide radicals was observed with reference to the blank. SEM, standard error of the mean of five assays.
[a] Positive control used in the assay.

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presented in Table 2. Compound **2**, which is the major constituent of the plant, showed the most potent activity of all the compounds. Compound **1** was found to be significantly active, whereas compounds **6** and **9** had moderate activity as previously reported (Richard-Forget *et al.*, 1995). Compounds **3–5**, **7**, **8** and **10** did not exhibit any activity against the enzyme.

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Table 2. *In vitro* quantitative inhibition of lipoxygenase by compounds 1–10

Compound	IC_{50} (μM) ± SEM against lipoxygenase
1	54.6 ± 3.3
2	26.0 ± 0.5
3	>400
4	>400
5	>400
6	197.37 ± 34.16
7	>400
8	>400
9	85.59 ± 3.50
10	>400
Baicalein[a]	22.7 ± 0.5

IC_{50}, concentration of a test compound.
SEM, standard error of the mean of five assays.
[a] Positive control used for the inhibition of lipoxygenase.

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