Full Length Research Paper

Biological activities of a new compound isolated from the aerial parts of *Vitex agnus castus* L.

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A new compound trivially named vitexcarpan was isolated from the ethyl acetate fraction of *Vitex agnus castus*. The structure of compound was elucidated with the help of spectroscopic techniques: 13C NMR, 1H NMR, heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC), nuclear overhauser effect spectroscopy (NOESY) and correlation spectroscopy (COSY). The isolated compound was screened for possible urease, chymotrypsin and anti-inflammatory activities. The results showed that the compound possess moderate inhibitory activity against urease (43.3 %) and chymotrypsin (39.8 %) enzymes. Vitexcarpan also showed moderate (48 %) *in vitro* anti-inflammatory activity using activated human neutrophils.

Key words: *Vitex agnus castus*, vitexcarpan urease, chymotrypsin, anti-inflammatory.

INTRODUCTION

Vitex agnus castus (*Verbernaceae*) commonly known as chaste tree, chaste berry, or monk's pepper is a native of the Mediterranean region. Locally, the plant is used as insect repellent and insecticide. A wide range of medicinal applications are also shown by other plants of this family as berries are considered as tonic supplement for male and female reproductive system. Flowers, berries and leaves are used in various decoction, traditional tincture, cider vinegar tincture, syrup and elixir that can simply be eaten as medicinal food (Hartung, 2000; Chevallier, 2000). The CO_2 extract of the seeds of *V. agnus castus* is reported to be used as repellent for mosquitoes, biting flies and fleas from animals and humans (Heinz et al., 2005). Antifungal activity of seeds of *Vitex negundo* has been reported (Shaukat et al., 2009) and this is used in different preparations for treatment of various skin diseases (Amann, 1975). Similarly, anti-inflammatory and anti-androgenic activities have been reported (Bhargava, 1989).

A wide range of biological effects of phytoestrogens and other compounds are contained in several members of the plant genus Vitex e.g., *V. negundo, Vitex doniana, Vitex polygama, Vitex trifolia, Vitex rotundifolia, Vitex altissima* and *Vitex peduncularis* (Eckman and Hines, 1993; Goncalves et al., 2001; Li et al., 2005; Okuyama et al., 1998a; Sridhar et al., 2005; Suksamrarn et al., 2002). These plants along with their constituents have been reported for their effectiveness in anti-inflammatory (Chawla et al., 1992), antitrypanosomal (Kiuchi et al., 2004), anticonvulsant (Tandon and Gupta, 2005), antivenin (Alam and Gomes, 2003), profertility agents (Eckman and Hines, 1993), antidiarrheal (Agunu et al., 2005), antihistamine (Alam et al., 2002), analgesic (Dharmasiri et al., 2003), and antiviral (Goncalves et al., 2001; Okuyama et al., 1998a). Various flavonoidal com-

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Abbreviations: EtOAc, Ethyl acetate; **BuOH,** butanol; **IR, infrared**; **CC,** column chromatography; **UV,** ultraviolet; **DMSO,** dimethyl sulfoxide; **MHS,** modified Hank's solution; **PBS,** phosphate buffered saline; **NMR,** nuclear magnetic resonance; **HMBC,** heteronuclear multiple bond correlation; **HSQC,** heteronuclear single quantum coherence.

pounds have been isolated from the root bark of *V. agnus castus* (Okuyama et al., 1998a; You and Kim, 1999). New ecdysteroid compounds have also been isolated from *Vitex polygama* (Goncalves et al., 2001). From the fruits of *V. agnus castus,* a triterpenoid and labdane diterpene alkaloids have been reported (Ono et al., 1998; Sridhar et al., 2005). Two ecdysteroids were isolated from the bark of *Vitex glabrata* and *Vitex strickeri*, while leaves and flowers of *V. agnus castus* were reported for ketosteroid hormones (Eckman and Hines, 1993; Suksamrarn et al., 2002; Zhang et al., 1991; Saden et al., 1991). From chloroform fraction of *V. agnus castus*, compounds casticin, vitexilactone, pinnatasterone and 17-OH-pro-gesterone were isolated (Suksamrarn et al., 2000). From fruits of *V. rotundifolia*, labdane type diterpene glycosides were reported (Ibrahim et al., 2006). Abietane-type diterpenes were reported from the fruits of *V. trifolia* L. (Hoberg et al., 1999). *Vitex canescens* contains highly oxygenated ecdysteroids (Zhang et al., 2002). Based on the above reported literature of the plant and other plants of the family, we isolated compounds from *V. agnus castus* and screened the isolated compounds for possible pharmacological/biological activities.

MATERIALS AND METHODS

Plant material

Aerial parts of *V. agnus castus* (*Verbenaceae*) were collected from Hazara division, KPK, Pakistan, from December to January, 2007. The plant was identified by Professor Dr. Habib Ahmad, Plant Taxonomist, Hazara University, KPK, Pakistan.

Extraction

The shade dried plant materials of *V. agnus castus* were chopped into small pieces and grinded to fine powder by using electric grinder. The powdered plant material of *V. agnus castus* (9 kg) was soaked in commercial grade methanol for 15 days at room tempe-rature with occasional shaking. After 15 days, methanol soluble materials were filtered off. All filtrates were combined and concentrated under vacuum at 40°C using a rotary evaporator till a blackish crude extract of about 900 g was obtained.

Fractionation

The crude methanolic extract (850 g) of *V. agnus castus* was sus-pended in distilled water (400 ml) and partitioned with *n*-hexane (3 x 400 ml), CHCl₃ (3 x 400 ml), ethyl acetate (EtOAc) (3 x 400 ml) and butanol (BuOH) (3 x 400 mm) to yield *n*-hexane (100 g), CHCl₃ (80 g), EtOAc (140 g), BuOH (80 g) and aqueous (155 g) fractions. About 50 g of crude methanolic extract was reserved for pharma-cological/biological screenings.

General experimental conditions

HREI-MS were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The ^{1}H- nuclear magnetic resonance (NMR) spectra were recorded in CDCl₃ and CD₃OD 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 CDCl₃ and CD₃OD at 125 MHz on a Bruker AMX-500 NMR spectrometer. The infrared (IR) spectra were recorded on a Jasco A-302 spectrophotometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. Silica gel columns (230 to 270 mesh) were used for column chromatography. Pre-coated silica gel TLC (GF-254, 20 × 20 cm, 0.25 mm thick, Merck) were used to check the purity of the compound and were observed under ultraviolet (UV) light (254 and 366 nm), while ceric sulphate was used as a spraying reagent.

Isolation

The ethyl acetate fraction of *V. agnus castus* was subjected to column chromatography and sequentially sub-fractionated in increasing order of polarity, with solvent system of ethyl acetate/n-hexane (0.5 / 9.5 (i), 1.0 / 9.0 (ii), 1.5 / 8.5 (iii), 2.0 / 8.0 (iv), 2.5 / 7.5 (v), 3.0 / 7.0 (vi), 4.0 / 6.0 (vii), 5.0 / 5.0 (viii), 6.0 / 4.0 (ix), 7.0 / 3.0 (x), 2.0 / 8.0 (xi), 1.0 / 9.0 (xii) and 100% (xiii) ethyl acetate) mixture with increasing order of polarity. The isolation was further preceded and we selected the sub-fraction (vii) by subjecting it to flash column chromatography. Pure compound was isolated by eluting the fraction with solvent system of ethyl acetate: *n*-hexane (4.5:5.5%). Structural elucidation was performed using different spectroscopic techniques mentioned in general experimental conditions.

Chymotrypsin inhibitory activity

The chymotrypsin inhibitory activity of the compound was perfor-med according to the procedure of Cannel et al. (1988). Chymotrypsin (9 units/ml of 50 mM Tris–HCl buffer pH 7.6; Sigma Chemical Co. USA) was preincubated with the compounds for 20 min at 25°C. 100 µl of substrate solution (1 mg *of N*-succinyl-phenylalanine-*p*-nitroanilide / ml of 50 mM Tris–HCl buffer pH 7.6) was added to start the enzyme reaction. The absorbance of free *p*-nitroaniline was endlessly monitored at 410 nm until a considerable color change was achieved. The final dimethyl sulfoxide (DMSO) concentration in the reaction mixture was 7%. The percentage (%) inhibition was calculated as follows

Percentage (%) inhibition = $(E - S) / E \times 100$

Where, E is the activity of the enzyme without test compound and S is the activity of enzyme in the presence of the test compound.

Urease inhibition assay

The urease inhibitory activity was performed according to the procedure of Akhtar et al. (2008). A solution containing 25 µl of Jack bean urease, 100 mM urea and 55 µl of buffer were incubated with 5 µl (0.5 mM conc.) of the test compounds at 30°C for 15 min in a 96 well microtiter plate. The ammonia production was mea-sured by indophenol method and used to determine the urease inhibitory activity. The alkali reagents (70 µl, 0.5% w/v sodium hydroxide and 0.1% NaOCl) and phenol reagents (45 µl, 1% w/v phenol and 0.005% w/v sodium nitroprusside) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). The change in absorbance per min was noted and the results processed using Soft-Max Pro software (Molecular Device, USA). All the tests were performed in triplicate. The assays were

performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1.0 mM EDTA and 0.01 M $LiCl_2$). The percentage inhibition was calculated from the formula:

$$100 - (OD_{test\ well} / OD_{control}) \times 100$$

Thiourea was used as the standard inhibitor.

Anti-inflammatory activity

Isolation of human neutrophils

Human neutrophils were isolated by the modified method of Siddiqui et al. (1995). Briefly describing, fresh heparinized blood was collected from healthy volunteers and diluted with an equal volume of modified Hank's solution (MHS), pH 7.4. After 20 min, mixture of blood and MHS was centrifuged at 1500 rpm. The upper leukocyte layer was collected and layered over Ficoll opaque and the pellets were resuspended with MHS. A cell count was performed by using the improved Neubaur chamber. The viability of cells determined by the Trypan Blue method was above 97% (Siddiqui et al., 1995).

Respiratory burst assay

Anti-inflammatory activity of the test samples were determined by using a modified assay (Tan and Berridge, 2000). This *in vitro* assay was based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Anti-inflammatory activity was determined in a total volume of 200 µl MHS (pH 7.4) containing 1.0×10^4 neutrophils per ml, 250 µM WST-1 with various concentrations of test compounds. The negative control contained buffer, neutrophils and WST-1. The compounds were equilibrated at 37°C and the reaction was initiated by the addition of opsonized zymosan A (15 mg/ml), which was prepared by mixing with human pooled serum, followed by centrifugation at 3000 rpm and the pellet was resuspended in phosphate buffered saline (PBS) buffer. Absorbance was measured at 450 nm. Aspirin and indomethacin were used as positive controls. The percentage inhibitory activity by the samples was determined against a DMSO blank using the formula:

% Inhibition = 100 - {(OD test compound/OD control) x100}

RESULTS AND DISCUSSION

The compound was obtained as white crystals from the EtOAc fraction of the aerial parts of *V. agnus castus*. Its optical rotation $\{[\alpha]_D^{25} = + 17.230$ (MeOH, C = 0.26)$\}$, indicated the presence of chiral center in molecule. The HREI-MS displayed M^+ at 324.1460 which is consistent with the molecular formula $C_{20}H_{20}O_4$ (calculated for $C_{20}H_{20}O_4 = 324.1452$) with eleven degrees of unsaturation. The IR max (KBr) cm^{-1} indicated OH at 3461, C=C at 1609 and C–O at 1138 functionalities when compared with Gomotsang et al. (2002).

The ^{1}H-NMR spectrum (Table 1) showed four proton signals at δ 3.51 (1H, dd, $J_{7ax,7eq}$ = 10.4 Hz, $J_{7ax,\ 6b}$ 9.0 Hz), 4.23 (1H, dd, $J_{7eq,\ 7ax}$ = 10.4 Hz, $J_{7eq\ ,\ 6b}$ = 4.5 Hz), 3.50 (1H, ddd, $J_{6b,\ 7ax}$ = 9.0 Hz, $J_{6b,\ 12b}$ = 7.0 Hz, $J_{6b,\ 7eq}$ = 4.5 Hz) and 5.48 (1H,d, $J_{12b,\ 6b}$ = 7.0 Hz) characteristic of the H-7_{ax}, H-7_{eq}, H-6b and H-12b, -O-CH2-CH-CH-O-

unit forming the rings B and C of a pterocarpan nucleus (Rukachaisirikul et al., 2007). The ^{1}H-NMR spectrum further revealed the five proton signals in the aromatic region, at δ 6.24 (1H, d, $J_{5,\ 6}$ = 8.4 Hz), 7.02 (1H, d, $J_{6,\ 5}$= 8.4 Hz), 6.34 (1H, d, $J_{9,\ 11}$= 2.4 Hz), 6.54 (1H, dd, $J_{11,\ 12}$ = 8.4 Hz , $J_{11,\ 9}$ = 2.4 Hz) and 7.31 (1H, d, $J_{12,11}$ = 8.4 Hz) were assigned to the H-5, H-6, H-9, H-11 and H-12, respectively. Presence of signals on ^{1}H-NMR spectrum at δ 2.55 and 2.61 (1H each, m), 1.74 (2H, t, $J_{2,\ 1}$ = 6.8 Hz), 1.34 (3H, s), 1.36 (3H, s) were attributed to the methylene protons of C-1, methylene protons of C-2, methyl protons of C-14 and methyl protons of C-15, respectively. This indicated the presence of dimethyl dihydropyran ring. The placement of dimethyl dihydro-pyran substituent was deduced with the help of splitting pattern of the protons of the aromatic rings, ^{13}C-NMR values and Heteronuclear Multiple Bond correlations (HMBC).

Strong NOESY correlation (Figure 1) between H-12b and H-7_{ax} revealed that , H-12b is axially oriented, coupling constant value of H-12b with H-6b (7.0 Hz) indicated that they are trans (both are axially oriented) to each other. *Trans* ring fusion at carbon number 6b and 112b was further confirmed by the comparison of its optical rotation $\{[\alpha]_D^{25} = + 17.70\}$ and negative cotton effect at 342 nm on CD spectrum, with reported ptero-carpan skeleton which has *cis* ring fusion with negative optical rotation and positive cotton effect (Osterberg et al., 1990). Proton carbon connectivities were determined by Heteronuclear Single Quantum Coherence (HSQC), while long range proton carbon interactions were predicted by using HMBC interactions (Figure 2). ^{13}C-NMR spectrum broad band and distortion-less enhancement by polarization transfer (DEPT) (Table 1) displayed twenty carbon signals, including two methyl, three methylene, seven methine and eight quaternary carbons. From the above spectral discussion, the structure of the compound is (6b R, 12b R)-3,3-dimethyl-2,3,6b,12b-tetrahydro-1H,7H-chromeno{6',5':4,5} furo {3,2-c} chromen-10-ol, which is trivially named vitex-carpan (Figure 3). Con-sequently, the isolated compound was screened for chymotrypsin, urease and anti-inflammatory activity.

Urease

Urease serves as a powerful immunogen; it is the most important protein component of the *Helicobacter pylori* (Osterberg et al., 1990; Gattermann and Marre, 1989; Cover et al., 1995; Dunn et al., 1990). Patients with active gastritis due to *H. pylori* infection show significantly elevated serum IgG and IgA levels along with elevated urease levels as compared to the normal levels (Evans et al., 1991). In this study, the isolated new compound was screened against urease enzyme and the results showed moderate (43.3%) urease inhibitory activity as given in

Table 1. ^{1}H- and ^{13}C- NMR chemical shift values of the compound (ppm, C_3D_6O, 400 and 100 MHz, respectively).

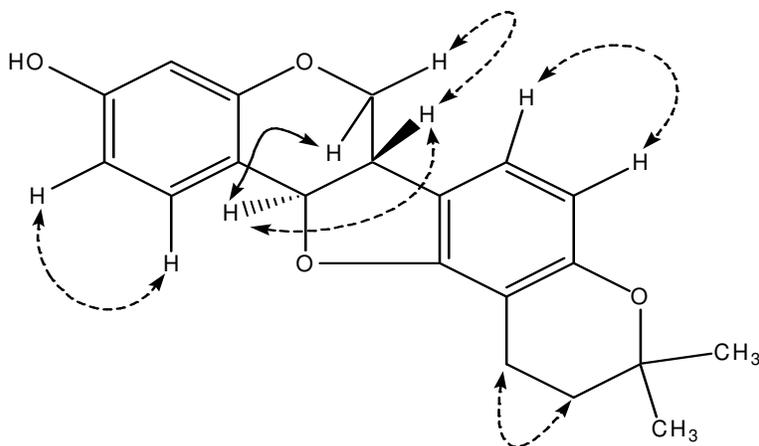| C. No	δc	δ$_H$ (J, Hz)	Multiplicity (DEPT)	HMBC
1	17.6	2.55 m, 2.61 m	CH$_2$	C-13b
2	32.5	1.74 t (6.8)	CH$_2$	------
3	74.5	-	C	------
4a	157.4	-	C	------
5	109.1	6.24 d (1H, d, $J_{5,6}$ = 8.4 Hz)	CH	C-6a
6	125.0	7.02 (1H, d, $J_{6,5}$ = 8.4 Hz)	CH	C-5, C-6a
6a	117.0	-	C	------
6b	40.8	3.50 (1H, ddd, $J_{6b, 7ax}$ = 9.0 Hz, $J_{6b, 12b}$ = 7.0 Hz, $J_{6b, 7eq}$ = 4.5 Hz)	CH	C-7, C-12b, C-6a
7	67.2	3.51 (1H, dd, $J_{7ax,7eq}$ = 10.4 Hz, $J_{7ax,6b}$ 9.0 Hz), 4.23 (1H, dd, $J_{7eq,7ax}$ = 10.4 Hz, $J_{7eq, 6b}$ = 4.5 Hz)	CH$_2$	C-8a, C-4a, C-12b
8a	158.9	-	C	------
9	103.9	6.34 (1H, d, $J_{9, 11}$= 2.4 Hz)	CH	C-8a, C-10
10	159.7	-	C	------
11	110.4	6.54 (1H, dd, $J_{11,12}$ = 8.4 Hz , $J_{11,9}$ = 2.4 Hz)	CH	C-12, C-10
12	133.1	7.31 (1H, d, $J_{12,11}$ = 8.4 Hz)	CH	C-12a, C-12b
12a	118.2	-	C	------
12b	79.3	5.48 (1H,d, $J_{12b, 6b}$ = 7.0 Hz)	CH	C-6b, C-12a
13a	155.8	-	C	------
13b	113.0	-	C	------
14	26.9	1.34 s	CH$_3$	C-3, C-2
15	26.9	1.36 s	CH$_3$	C-3

Figure 1. Key COSY and NOESY interactions.
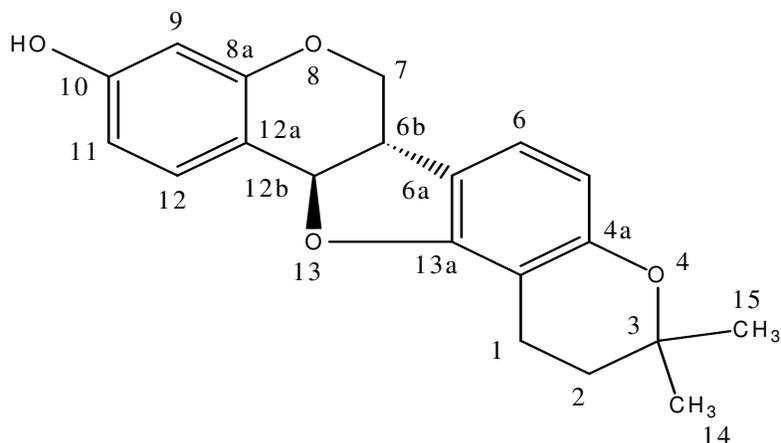
Table 2.

Chymotrypsin

The physiological role of serine proteases inhibitors has undoubtedly been recognized and it has been projected that they are part of plants natural defense system, by inhibiting insect proteinases against insect predation. Due to this inhibitory action, these inhibitors have gained attention as possible sources of engineered resistance against harmful pests and pathogens for transgenic plants expressing heterologous inhibitors (Boulter et al., 1989, Masoud et al., 1993, Hilder et al., 1987). The lignans isolated from the roots of *V. negundo* Linn. Were found to be active, noncompetitive and competitive inhibitors for α-chymotrypsin, respectively (Lodhi et al., 2008). The compound isolated from the aerial parts of the plant shows moderate (39.8 %) inhibitory activity against the chymotrypsin enzyme as shown in Table 2.
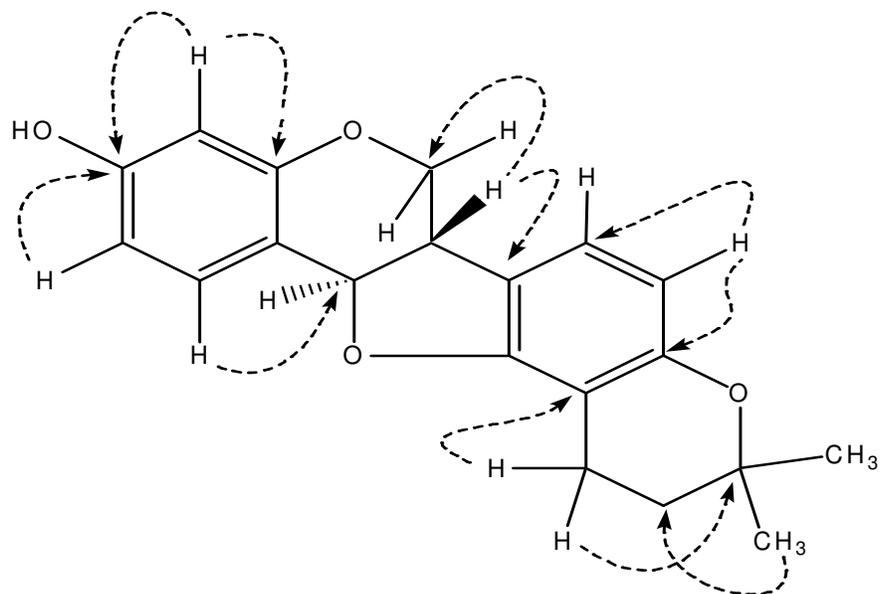
Figure 2. Key HMBC interactions.

Figure 3. Structure of vitexcarpan.

Table 2. Enzyme inhibition and anti-inflammatory activities of isolated compound.

Test	Urease inhibition (%)	Chymotrypsin inhibition (%)	Anti-inflammatory activity (%)
Sample	43.3	39.8	48
Standard	96.8***	94.3**	98*

***Thiourea was used as the standard inhibitor for urease; **chymostatin was used as the standard inhibitor in chymotrypsin inhibition; *indomethacin was used as positive controls for anti-inflammatory activity.

Anti-inflammatory activity

Different compounds, that is, casticin, p-hydroxybenzoic acid, methyl 3,4-dihydroxybenzoate and 3,4-dihydroxy-benzoic acid, isolated from the aerial parts of the *V. agnus castus* possess significant anti-inflammatory activity with the minimum inhibitory concentration required to inhibit the growth of 50% of organisms (MIC_{50} in the range of 156 to 400 µg (Choudhary et al., 2009). The compound screened for anti-inflammatory shows moderate (48 %) activity as shown in Table 2.

From the above results, it is concluded that the com-

pound isolated from the EtOAc fraction of *V. agnus castus* showed moderate chymotrypsin urease inhibitory and anti-inflammatory activities.

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