PHYTOTHERAPY RESEARCH
Phytother. Res. **21**, 391–394 (2007)
Published online 30 January 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2021

SHORT COMMUNICATION

Diterpenoids and Flavonoids from the Fruits of *Vitex agnus-castus* and Antioxidant Activity of the Fruit Extracts and Their Constituents

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From the *n*-hexane fraction of the fruits of *Vitex agnus-castus*, two labdane-type diterpenes, vitetrifolin B and C, were isolated by means of multiple chromatographic separations, together with the previously identified rotundifuran, vitexilactone and the sesquiterpene spathulenol. From the EtOAc fraction, eupatorin was identified for the first time, besides the known casticin, penduletin, vitexin and orientin. The *n*-hexane, EtOAc and MeOH–H_2O fractions of the MeOH extract of *Agni-casti fructus* were subjected to *in vitro* antioxidant assays. The EtOAc extract displayed a significant concentration-dependent effect when tested by 1,1-diphenyl-2-picrylhydrasyl (DPPH) free radical assay (IC_{50} = 68 μg/mL) and against the autooxidation of a standard rat brain homogenate (IC_{50} = 14 μg/mL). The MeOH–H_2O fraction was less active with 3643 μg/mL (DPPH test) and IC_{50} = 125 μg/mL (rat brain homogenate), while the *n*-hexane phase proved to be inactive. The main flavonoid constituents of the EtOAc extract, casticin, vitexin and orientin were assayed for antioxidant activity and found that only casticin possesses a marked lipid peroxidation inhibitory effect (IC_{50} = 0.049 mM) compared with that of the positive control ascorbic acid (IC_{50} = 0.703 mM). Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Vitex agnus-castus; Verbenaceae; labdane diterpenoids; flavonoids; antioxidant activity.

INTRODUCTION

Vitex agnus-castus L. (Verbenaceae) (chaste tree) is a small tree or shrub, widely distributed in the Mediterranean region of Europe and in Central Asia. The fruits of *V. agnus-castus* are traditionally used for the treatment of different female conditions, e.g. premenstrual problems, menopause and disrupted lactation. Clinical studies, including double-blind placebo-controlled investigations, have confirmed the beneficial effects of the fruit extracts on psychic and somatic symptoms of the premenstrual syndrome (Wuttke *et al*., 2003). The extracts of the fruits are important constituents of herbal medicinal products in European phytomedicine because of their experimentally demonstrated dopaminergic action. Furthermore, in recent publications the cytotoxic (Hirobe *et al*., 1997), estrogenic (Jarry *et al*., 2003) and apoptosis-inducing activities (Ohyama *et al*., 2003) of the fruit extracts have been reported. Previous phytochemical work revealed the presence of iridoid glycosides (Kuruüzüm-Uz *et al*., 2003), flavonoids (Hirobe *et al*., 1997; Wollenweber and Mann, 1983), diterpenoids (Hoberg *et al*., 1999), ecdysteroids (Ramazanov, 2004), and essential and fatty oils (Sorensen and Katsiotis, 2000) in the fruits of *V. agnus-castus*.

The present paper reports on the isolation and characterization of vitetrifolin B (**1**), vitetrifolin C (**2**) and eupatorin (**6**) for the first time from the fruits of *V. agnus-castus*, together with the previously identified rotundifuran (**3**), vitexilactone (**4**), spathulenol (**5**), casticin (**7**) and penduletin (**8**). By means of TLC investigations, the presence of vitexin (**9**) and orientin (**10**) was also detected. Further, the radical scavenging activity and the protective effects of the fruit extracts against enzyme-independent lipid peroxidation were studied.

MATERIALS AND METHODS

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C), and on a Bruker Avance DRX 400 spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C). The signals of the deuterated solvents were taken as reference. EIMS and HREIMS spectra were obtained on a Finnigan MAT

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Contract/grant sponsor: National Research and Development Programme (Hungary) (NKFP); contract/grant number: 4/0037/2002.

Received 1 October 2005
Revised 19 April 2006
Accepted 10 September 2006

95 S spectrometer. For vacuum liquid chromatography (VLC), silica gel (Kieselgel GF_{254} 15 μm, Merck) was used. Preparative thin-layer chromatography (TLC) was performed on polyamide (Macherey Nagel DC 6), silica gel (Merck 5715) and silica gel RP-18 F_{254s} (Merck 5559) plates. Gel chromatography was carried out on Sephadex LH-20 (Pharmacia Fine Chemicals AB). Vitexin and orientin were purchased from Fluka and Riedel-de Haen (reference no. 49513) and from Extrasynthese (Genay, France, reference no. 1054 S), respectively.

Two experiments were performed: for the first, the fruits of *V. agnus-castus* were purchased from Caesar and Lorentz GmbH (Hilden, Germany) (HAB 2000, No. 21.11.00 Ch-B.: 04103471 D. Froehlingsdorf); in the second experiment, a sample purchased from Müggenburg GmbH (Hamburg, Germany) was applied.

Extraction and isolation. In the first experiment, 100 g *Agni-casti fructus* was extracted in an ultrasonic bath with 6 × 250 mL MeOH at room temperature. After evaporation, the MeOH extract was dissolved in 25 mL 90% MeOH and extracted with 7 × 20 mL *n*-hexane. The *n*-hexane phase (6.0 g) was subjected to VLC, using silica gel and a gradient system of *n*-hexane–EtOAc (100:0, 49:1, 19:1, 9:1, 4:1, 7:3, 1:1 and 0:100). Fraction 5, eluted with *n*-hexane–EtOAc (4:1), was fractionated by TLC on silica gel with the use of benzene–$CHCl_3$–diethyl ether (8:2:1). From the plates, three bands (I–III) were removed, and eluted with chloroform. Band I was repeatedly chromatographed on silica gel with *n*-hexane–Me_2CO (9:1) as the developing system, and then on RP-silica gel with MeOH–H_2O (9:1), to afford vitetrifolin B (**1**) (8 mg). Band II was purified on silica gel plates with benzene–EtOAc (19:1), yielding rotundifuran (**3**) (25 mg). Band III contained two compounds, spathulenol (**5**) (11 mg) and vitetrifolin C (**2**) (6 mg), which were separated by TLC on silica gel, using *n*-hexane–Me_2CO (19:1) as the mobile phase.

In the next experiment, 230 g *Agni-casti fructus* was extracted with 7 L MeOH. The MeOH extract was concentrated and partitioned between 50% MeOH (150 mL), *n*-hexane (10 × 175 mL) and EtOAc (10 × 175 mL). The *n*-hexane phase (24.59 g) was separated by VLC using a gradient system of *n*-hexane–EtOAc–EtOH (100:0:0, 49:1:1, 19:1:0, 9:1:0, 4:1:0, 7:3:0, 1:1:0 and 1:1:1). The fraction obtained with the last eluent was subjected to gel chromatography, using MeOH as the mobile phase, yielding three subfractions with different compositions. From subfraction 2, vitexilactone (**4**) (5 mg) was isolated by means of TLC on silica gel with $CHCl_3$–MeOH (49:1). The EtOAc phase (6.3 g) of *V. agnus-castus* was fractionated by TLC on polyamide, using toluene–petroleum ether–Me_2CO–MeOH (12:6:2:1), and the fractions obtained here were then rechromatographed on silica gel in two steps: first $CHCl_3$–MeOH (97:3) and then *n*-hexane–Me_2CO (7:3) was applied as the developing system. These separations afforded eupatorin (**6**) (3 mg), casticin (**7**) (29 mg) and penduletin (**8**) (4 mg). In the EtOAc phase of the MeOH extract the presence of vitexin (**9**) and orientin (**10**) were detected by TLC on silica gel using authentic samples and EtOAc–HCOOH–H_2O (17:2:1) and EtOAc–HCOOH–CH_3COOH–EtMeCO–H_2O (50:7:3:30:10) as developing systems. The compounds were detected in UV light after spraying with $AlCl_3$ reagent.

The isolated compounds were identified by spectral analyses, including HREIMS, EIMS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. The experimental data were in good agreement with the respective literature data, identifying vitetrifolin B (**1**), vitetrifolin C (**2**), rotundifuran (**3**), vitexilactone (**4**) (Ono *et al.*, 2000, 2001), spathulenol (**5**) (Venkateshwar *et al*., 2002), eupatorin (**6**) (Adams and Lewis, 1977), casticin (**7**) (Horie *et al.*, 1989) and penduletin (**8**) (Barberá *et al.*, 1986).

Vitexilactone **4**. HREIMS *m/z*: 378.24062 $[M]^+$ calc. for $C_{22}H_{34}O_5$ 378.24062. EIMS *m/z* (rel. int. %): 349 $[M–HCO]^+$ (4), 334 $[M–COO]^+$ (6), 318 $[M–CH_3COOH]^+$ (100), 303 (21), 289 (7), 275 (7), 262 (11), 245 (6), 194 (14), 181 (32), 168 (27), 150 (38), 135 (40), 123 (56), 109 (51), 95 (41), 81 (32), 69 (50), 57 (25), 55 (24), 43 (31), 41 (14), 28 (13).

Casticin **7**. ^{13}C-NMR (100 MHz, $CDCl_3$): 179.6 (C-4), 156.3 (C-9), 159.5 (C-7), 153.4, 153.0 (C-2, C-5), 149.5 (C-4′), 146.2 (C-3′), 139.7 (C-3), 132.9 (C-6), 124.2 (C-1′), 122.2 (C-6′), 115.0 (C-2′), 111.1 (C-5′), 107.3 (C-10), 91.0 (C-8), 61.5, 60.8, 57.0, 56.7 (4xOMe).

Preparation of the extracts. 100 g pulverized *Agni-casti fructus* was percolated with 3 L MeOH. The extract was evaporated and dissolved in 80 mL 50% MeOH, and then extracted with 8 × 130 mL *n*-hexane, followed by 4 × 130 mL EtOAc. The fractions obtained were concentrated *in vacuo* to yield 8.07 g (*n*-hexane phase), 2.24 g (EtOAc phase) and 4.36 g (MeOH–H_2O phase) dry material.

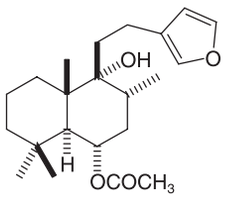
Measurement of radical scavenging activity by DPPH assay. The effect of extracts and flavonoids from *Vitex agnus-castus* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assayed as described earlier (Krings and Berger, 2001). Briefly, different amounts of the extracts and compounds were added to a final volume of 3 mL 0.1 mM DPPH dissolved in ethanol. The mixture was shaken vigorously and allowed to stand for 30 min, and then the absorbance of the solution was measured at 517 nm.

Measurement of lipid peroxidation inhibitory activity. The antioxidant effects of the extracts were measured by means of inhibition of the autooxidation of unsaturated fatty acids present in animal brain tissue (Zupkó *et al*., 2001). Briefly, a lipid-rich fraction was prepared from the brain of male Sprague-Dawley rats (250–300 g) by homogenization and centrifugation. The fatty acids in this fraction were spontaneously oxidized during an incubation of 1 h at 37 °C, a process which can be inhibited by antioxidants. The oxidized products were determined by spectrophotometry after reaction with thiobarbituric acid. Experiments were carried out in duplicate and sigmoid curves were fitted to the results. IC_{50} values were calculated by means of GraphPad Prism 2.01 (GraphPad Software, San Diego, CA, USA). All experimental animal protocols satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

RESULTS AND DISCUSSION

The MeOH extract of the dried fruits of *Vitex agnus-castus* was extracted with *n*-hexane and EtOAc. The

DOI: 10.1002/ptr



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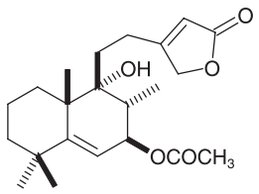

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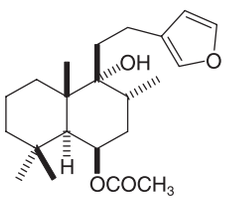

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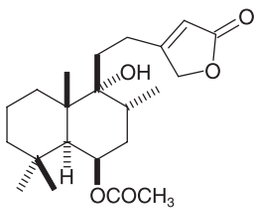

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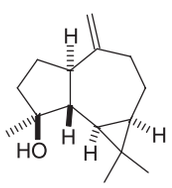

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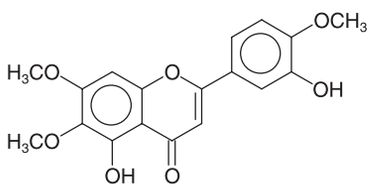

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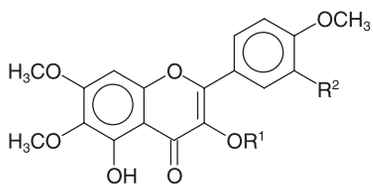

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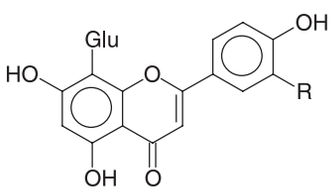
8	H	H



	R
9	H
10	OH

n-hexane fraction was subjected to VLC and preparative TLC, and gel-filtration was performed to afford the diterpenes **1**–**4** and the sesquiterpene **5**. From the EtOAc fraction, the flavonoids **6**–**8** were isolated, by means of multistep TLC separations on polyamide and on silica gel. On the basis of ^{1}H-NMR, ^{13}C-NMR, EIMS and HREIMS investigations, and by comparison of the spectral data with those published in the literature, the compounds were determined to be vitetrifolin B (**1**), vitetrifolin C (**2**), rotundifuran (**3**), vitexilactone (**4**), spathulenol (**5**) eupatorin (**6**), casticin (**7**) and penduletin (**8**). Vitetrifolin B (**1**) and C (**2**) are labdane-type diterpenes containing a furan ring. Moreover, vitetrifolin B (**1**) is the C-6 epimer of the known compound, rotundifuran (**3**). Vitetrifolin B (**1**) and C (**2**), identified here for the first time from *V. agnus-castus*, were previously described from the fruits of *V. trifolia* L. (Ono *et al*., 2000). The now-isolated sesquiterpene spathulenol (**5**), was earlier detected by GC-MS in the essential oil of *Agni-casti fructus* (Sorensen and Katsiotis, 2000). Eupatorin (**6**), obtained from the EtOAc fraction, is the first identified methylated flavone of this plant. Apigenin, luteolin, glycosylated flavones and highly methylated flavonols were previously isolated from *V. agnus-castus* (Hirobe *et al*., 1997; Jarry *et al.*, 2003; Wollenweber and Mann, 1983). TLC examination of the EtOAc fraction revealed the presence of vitexin (**9**) and orientin (**10**), which were described earlier from the fruit of *Vitex agnus-castus* (Gomaa *et al*., 1978; Jarry *et al.*, 2003).

DOI: 10.1002/ptr

The *n*-hexane, EtOAc and MeOH–H_2O fractions of the MeOH extract were tested by DPPH free radical assay and against the autooxidation of a standard rat brain homogenate. The calculated IC_{50} value of the DPPH scavenging activity of ascorbic acid was found to be 0.0138 mM. The extracts exhibited the following order of potency concerning the DPPH-assay: EtOAc fraction (IC_{50} = 68 μg/mL) > the MeOH–H_2O fraction (IC_{50} = 3643 μg/mL) > the *n*-hexane fraction (IC_{50} not determined). In the rat brain homogenate assay ascorbic acid also was used as a standard; its IC_{50} value was found to be 0.703 mM. The extracts exhibited antioxidant effects in the sequence of potency of the EtOAc fraction (IC_{50} = 14 μg/mL) > the MeOH–H_2O fraction (IC_{50} = 125 μg/mL) > the *n*-hexane fraction (IC_{50} not determined) (Fig. 1).

In order to identify the compounds responsible for the antioxidant activity, flavonoids present in the EtOAc extract [casticin (**7**), vitexin (**9**) and orientin (**10**)] were tested. Only casticin (**7**) showed marked inhibitory activity (IC_{50} = 0.049 mM) against lipid peroxidation in rat brain homogenate, but proved to be inactive in the DPPH assay. Vitexin (**9**) and orientin (**10**) were inactive up to a dose of 0.03 mg/mL. The results suggest that casticin (**7**) is partly responsible for the high activity and further unidentified antioxidant compounds must be present in the EtOAc extract. The *n*-hexane fraction, from which diterpenes (**1**–**4**) and spathulenol (**5**) were obtained, did not exhibit anti-lipid peroxidant activity, indicating that these compounds do not have significant antioxidant effects.

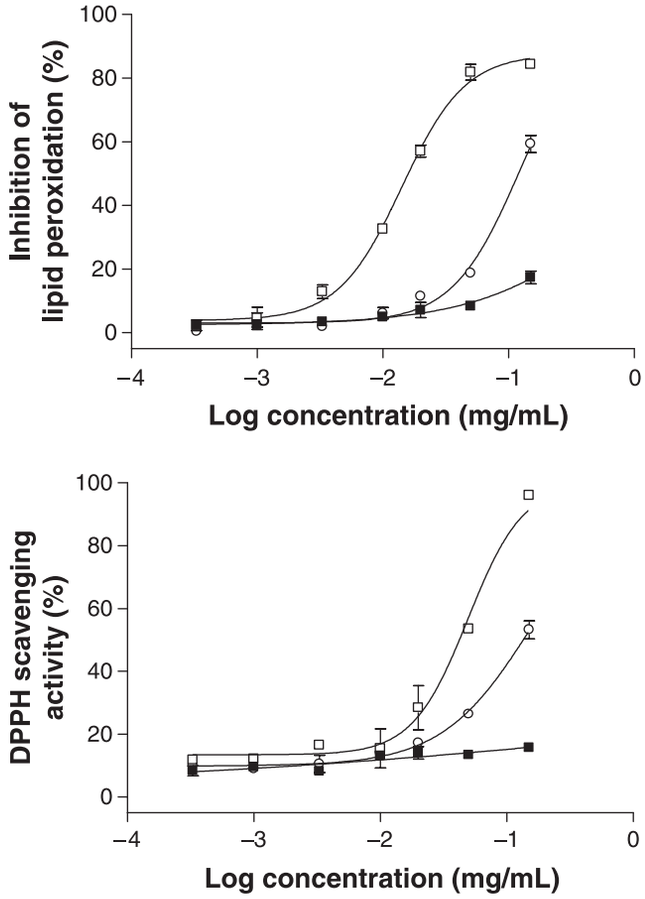


Figure 1. Antioxidant effects of the tested extracts on rat brain homogenate (upper panel) and DPPH radicals (lower panel). ■ *n*-hexane phase □ EtOAc phase ○ MeOH–H_2O phase of the MeOH extract of *Agni-casti fructus.* The data are mean of the experiments performed in duplicate ± SD.

Acknowledgements

Financial support of the National Research and Development Programme (NKFP) 4/0037/2002 is gratefully acknowledged.

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