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Radical scavenging potential of compounds isolated from *Vitex agnus-castus*

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Nine secondary metabolites—artemetin (**1**), casticin (**2**), 3-hydroxy-5,6,7,4′-tetramethoxy flavone U(**3**), penduletin (**4**), *p*-hydroxybenzoic acid (**5**), methyl 3,4-dihydroxybenzoate (**6**), methyl isovanillate (**7**), vanillic acid (**8**), and 3,4-dihydroxybenzoic acid (**9**)–were isolated from a folkloric medicinal plant, *Vitex agnus-castus*. The structures of compounds **1-9** were identified using spectroscopic techniques. Compound **7** was isolated for the first time from this plant. These compounds were screened for their antioxidant activity. Compounds **6** and **9** exhibited significant activity against the DPPH free radical.

Key Words: *Vitex agnus-castus*, Verbenaceae, antioxidant, DPPH radical scavenging activity, flavonoids.

Introduction

Vitex agnus-castus Linn. (Verbenaceae) is locally known as Hub-el-faked and Sumbhalu-ke-bij. It is a very well-known plant that is abundant in Pakistan.[1] It is traditionally used as an emmenagogue, sedative, anaphrodisiac, and galactagogue.[2] An ethanolic extract of *Vitex agnus-castus* is used as a homeopathic drug (agnus castus) for the treatment of impotence and central nervous system disorders.[3] Flowers of the plant are used for diarrhea and liver infections, and as a cardiac tonic. The powder of its green parts is used as an internal antihemorrhagic[4] agent.

As an antioxidant agent, it can prevent or inhibit the oxidation of auto-oxidizable materials in living cells. As a result of oxidation, free radicals are formed,[5] which are highly reactive species that may be involved

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in oxidative damage of vital molecules, such as cancers of the lungs, cervix, skin, stomach, prostate, colon, and esophagus. Free radicals may also cause many other diseases, such as heart disease, diabetes, arteriosclerosis, and arthritis,[6] as all cells of the body are susceptible to the adverse effects of free radicals. Antioxidants retard premature aging,[7] cancer,[8] and heart disease[9] in humans by inhibiting or quenching free radicals and reactive oxygen species (ROS).[5] They can also increase the shelf life and quality of foods and oils.

Based on the medicinal importance of *Vitex agnus-castus*, we conducted a phytochemical study on this plant and isolated 4 flavonoids (**1-4)** and 5 derivatives of benzoic acid (**5-9)**. The structure of these compounds was deduced by comparison of their spectral data with those reported in the literature. Compound **7** was isolated for the first time from this plant. The aim of this study was to evaluate the antioxidant activity of these compounds using an in vitro assay, and to explore their potential as non-steroidal antioxidant agents.

Experimental

General experimental procedures

^{1}H-NMR spectra were recorded in $CDCl_3$ 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. ^{13}C-NMR spectra were recorded in $CDCl_3$ at 125 MHz on a Bruker AMX-500 NMR spectrometer. HREI-MS was recorded on Jeol JMS 600 and HX 110 mass spectrometers, with the DA 5000 data system. IR spectra were recorded on a Jasco A-302 spectrophotometer. UV spectra were recorded on a Hitachi U-3200 spectrophotometer. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. Melting points were determined on a Buchi 510 apparatus. Column chromatography (CC) was carried out on silica gel columns (70-230 mesh). Purity of the samples was checked by TLC on pre-coated silica gel GF-254 preparative plates (20 × 20 cm × 0.25 mm thick, Merck) and were detected under UV light (254 and 366 nm), while cerium(IV) sulfate was used as a spraying reagent. All reagents were of analytical grade. Deionized water was used in all experimental procedures.

Plant material

The aerial parts of *Vitex agnus-castus* Linn. (40 kg) were collected in September 1997 from Khost, near Quetta (Pakistan), and were air dried. The plant was identified by Dr. Rasool Bakhsh Tareen, Department of Botany, Baluchistan University, Quetta, Pakistan. An 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 *Vitex agnus-castus* plants (17 kg) were 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 aqueous extract was further fractionated using various solvents (9 L each of chloroform, ethyl acetate, and butanol) 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 8 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 the collected fractions using repeated column chromatography.

The EtOAc fraction (87.5 g) was further 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, 10 L) to afford 9 major fractions (EA-1 to EA-9). These fractions were subjected to repeated column chromatography (silica gel) using different gradient solvent systems to afford 6 compounds—**5** (28.4 mg, petroleum ether:EtOAc, 5.7:4.3), **6** (26.3 mg, $CHCl_3$), **7** (12.8 mg, petroleum ether:EtOAc, 8.8:1.2), **8** (7.2 mg, petroleum ether:EtOAc, 8.4:1.6), and **9** (13.5 mg, $CHCl_3$: CH_3OH, 9.2:0.8).

DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical scavenging antioxidant assay

Antioxidant activity was assayed using a non-physiological DPPH free radical scavenging assay. Different concentrations of test compounds were placed in the reaction mixture, ranging from 1000-10 μM, while the concentration of DPPH was kept constant at 300 μM. The reaction mixture containing 5 μL of test compound in DMSO (1 mM) and 95 μL of DPPH in ethanol (300 μM) was placed in a 96-well microtiter plate (Molecular Devices, SpectraMax 340, USA) and incubated at 37 °C for 30 min. Absorbance was measured at 515 nm. The percentage of radical scavenging activity of the test compounds was determined in comparison to a DMSO-treated control group. IC_{50} values represent the concentration of compounds required to scavenge 50% of DPPH free radicals, and were calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA). Propyl gallate (PG) was used as a positive control.[10,11]

Artemetin (1): Yellow crystalline compound, mp 162 °C. UV (MeOH) λ_{max} (log ε): 256 (4.18), 273 (4.22), 346 nm (4.73). IR ($CHCl_3$)ν_{max}: 3320-3150 (chelated OH), 1660 (aromatic C=O), 1582 cm^{-1} (aromatic C=C). EI-MS m/z (rel. int. %): 388 (100), 387 (81), 373 (89), 360 (2), 345 (29), 327 (18), 245 (7), 194 (18), 165 (46). HREI-MS m/z: 388.2130 ($C_{20}H_{20}O_8$, calcd 388.2132). ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) (data are shown in Tables 1 and 2, respectively).

Casticin (2): Yellow amorphous solid, mp 186 °C. UV (MeOH) λ_{max} (log ε): 257 (3.42), 270 (3.66), 348 nm (4.11). IR ($CHCl_3$)ν_{max}: 3410-3145 (chelated OH), 1662 (C=O), 1578 cm^{-1} (aromatic C=C). EI-MS m/z (rel. int. %): 374 (100), 373 (41), 359 (57), 346 (3), 331 (16), 273 (7). HREI-MS m/z: 374.1034 ($C_{19}H_{18}O_8$, calcd 374.1002). ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) (data are shown in Tables 1 and 2, respectively).

3-Hydroxy-5,6,7,4′-tetramethoxy flavone (3): Yellow amorphous solid, mp 138 °C. UV (MeOH) λ_{max} (log ε): 258 (3.12), 350 nm (4.26). IR ($CHCl_3$)ν_{max}: 3580 (OH), 1676 (aromatic C=O), 1580 cm^{-1} (aromatic C=C). EI-MS m/z (rel. int. %): 358 (100), 357 (30), 343 (48), 315 (12), 300 (8), 215 (5). HREI-MS m/z: 358.2341 ($C_{19}H_{18}O_7$, calcd 358.2328).^{1}H-NMR ($CDCl_3$, 400 MHz): δ 8.06 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.01 (2H, d, J= 9.0 Hz, H-3′ and H-5′), 6.49 (1H, s, H-8), 3.95 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.86 (3H, s, OCH_3).

Table 1. ^{1}H-NMR (400 MHz, $CDCl_3$)[a] chemical shift data for artemetin (**1**) and casticin (**2**) (δ in ppm and J in Hz).

Carbon	**1**	**2**
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	6.49 (s)	6.49 (s)
9	-	-
10	-	-
1′	-	-
2′	7.67 (d, $J_{2',6'} = 2.0$)	7.66 (d, $J_{2',6'} = 2.2$)
3′	-	-
4′	-	-
5′	6.98 (d, $J_{5',6'} = 8.6$)	6.95 (d, $J_{5',6'} = 8.6$)
6′	7.71 (dd, $J_{6',5'} = 8.6$; $J_{6',2'} = 2.0$)	7.71 (dd, $J_{6',5'} = 8.6$; $J_{6',2'} = 2.2$)
3-OCH_3	3.96 (s)[b])	3.95 (s)[b])
6-OCH_3	3.96 (s)[b])	3.98 (s)[b])
7-OCH_3	3.95 (s)[b])	3.91 (s)
3′-OCH_3	3.92 (s)	-
4′-OCH_3	3.85 (s)	3.87 (s)
3′-OH	-	5.70 (s)
5-OH	12.50 (s)	12.47 (s)

[a] Assignments based on HMQC.

[b] Signals may be interchanged.

Penduletin (4): Yellow amorphous solid, mp 218 °C. UV (MeOH) λ_{max} (log ε): 212 (3.02), 270 (3.82), 341 nm (4.16). IR ($CHCl_3$)ν_{max}: 3300-3150 (chelated OH), 1662 (C=O), 1596 cm^{-1} (aromatic C=C). EI-MS m/z (rel. int. %): 344 (100), 343 (27), 329 (56), 301 (8), 286 (4), 181 (8). HREI-MS m/z: 344.2821 ($C_{18}H_{16}O_7$, calcd 344.2811). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 12.58 (1H, s, 5-OH), 8.02 (2H, d, J = 8.0 Hz, H-2′ and 6′), 6.95 (2H, d, J = 8.0 Hz, H-3′ and 5′), 6.49 (1H, s, H-8), 3.94 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.85 (3H, s, OCH_3).

***p*-Hydroxybenzoic acid** (**5**): White amorphous solid, mp 214 °C. UV (MeOH) λ_{max} (log ε): 222 (3.26), 290 nm (3.87). IR ($CHCl_3$)ν_{max}: 3450-2800 (COOH), 1692 (C=O), 1600 cm^{-1} (C=C). EI-MS m/z (rel. int. %): 138 (95), 121 (100), 93 (52). HREI-MS m/z: 138.0323 ($C_7H_6O_3$, calcd 138.0321). ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.86 (2H, *dd*, J= 8.7 Hz, J= 1.9 Hz, H-2 and 6), 6.79 (2H, *dd*, J = 8.7 Hz, J = 1.9 Hz, H-3 and 5).

Table 2. ^{13}C-NMR (100 MHz, $CDCl_3$)[a,b] chemical shift data for artemetin (**1**) and casticin (**2**).

Carbon	**1**	**2**	Multiplicity
2	152.3	152.3	C
3	138.8	139.0	C
4	178.9	178.9	C
5	152.8	152.7	C
6	132.3	132.3	C
7	158.8	158.8	C
8	90.4	90.4	CH
9	155.9	155.7	C
10	106.6	106.6	C
1′	122.9	123.6	C
2′	111.3	110.4	CH
3′	148.8	145.6	C
4′	151.4	148.8	C
5′	110.9	114.4	CH
6′	122.2	121.5	CH
3-OCH_3	60.2	60.9	CH_3
6-OCH_3	60.9	60.1	CH_3
7-OCH_3	56.4	56.3	CH_3
3′-OCH_3	56.1	-	CH_3
4′-OCH_3	56.0	55.9	CH_3

[a]Multiplicities were determined by DEPT and BB experiments.

[b]Assignment based on HMQC and HMBC.

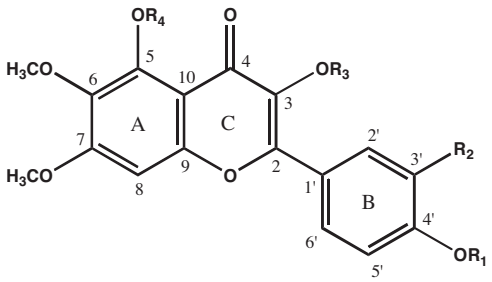
Methyl 3,4-dihydroxybenzoate (6): White amorphous solid, mp 135 °C. UV (MeOH) λ_{max} (log ε): 256 nm (3.74). IR ($CHCl_3$)ν_{max}: 3520 (OH), 1718 (C=O), 1608 cm^{-1} (C=C). EI-MS m/z (rel. int. %): 168 (30), 137 (100), 109 (30), 95 (28). HREI-MS m/z: 168.0448 ($C_8H_8O_4$, calcd 168.0442). ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.40 (1H, d, $J_{2,6}$ = 2.0 Hz, H-2), 7.39 (1H, dd, $J_{6,5}$ = 8.7 Hz, $J_{6,2}$ = 2.0 Hz, H-6), 6.78 (1H, d, $J_{5,6}$ = 8.7 Hz, H-5), 3.82 (3H, s, OCH_3).

Methyl isovanillate (7): White amorphous solid, mp 162 °C. UV (MeOH) λ_{max} (log ε): 296 nm (3.82). IR ($CHCl_3$)ν_{max}: 3540 (OH), 1712 (C=O), 1602 cm^{-1} (C=C). EI-MS m/z (rel. int. %): 182 (93), 151 (100), 123 (29), 95 (16). HREI-MS m/z: 182.0217 ($C_9H_{10}O_4$, calcd 182.0281). ^{1}H-NMR (CD_3OD, 400 MHz): δ 7.62 (1H, dd, $J_{6,5}$ = 8.3 Hz, $J_{6,2}$ = 1.9 Hz, H-6), 7.54 (1H, d, $J_{2,6}$ = 1.9 Hz, H-2), 6.93 (1H, d, $J_{5,6}$ = 8.3 Hz, H-5), 3.94 (3H, s, OCH_3), 3.88 (3H, s, OCH_3).

Vanillic acid (8): White amorphous material, mp 210 °C. UV (MeOH) λ_{max} (log ε): 282 nm (3.26). IR (KBr) ν_{max}: 3400-2800 (COOH and OH), 1688 (C=O), 1610 cm^{-1} (C=C). EI-MS m/z (rel. int. %): 168 (100), 151 (14), 123 (4), 97 (12). HREI-MS m/z: 168.1124 ($C_8H_8O_4$, calcd 168.1120). ^{1}H-NMR (CD_3OD,

400 MHz): δ 7.56 (1H, *dd*, $J_{2,6} = 1.8$ Hz, H-2), 7.50 (1H, *dd*, $J_{6,5} = 8.2$ Hz and $J_{6,2} = 1.8$ Hz, H-6), 6.77 (1H, *d*, $J_{5,6} = 8.2$ Hz, H-5), 3.88 (3H, *s*, OCH_3).

3,4-dihydroxybenzoic acid (9): White amorphous material, mp 198 °C. UV (MeOH) λ_{max} (log ε): 238 (3.46), 272 nm (3.84). IR ($CHCl_3$)ν_{max}: 3450-2600 (COOH and OH), 1690 (C=O), 1605 cm^{-1} (C=C). EI-MS m/z (rel. int. %): 154 (92), 137 (100), 109 (22). HREI-MS m/z: 154.2331 ($C_7H_6O_4$, calcd 154.2338). ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.41 (1H, *dd*, $J_{6,5} = 8.0$ Hz, $J_{6,2} = 2.0$ Hz, H-6), 7.40 (1H, *d*, $J_{2,6} = 2.0$ Hz, H-2), 6.78 (1H, *d*, $J_{5,6} = 8.0$ Hz, H-5). ^{13}C-NMR (CD_3OD, 100 MHz): δ 122.4 (C-1), 117.4 (C-2), 145.6 (C-3), 149.8 (C-4), 116.2 (C-5), 122.6 (C-6), 170.4 (CO).

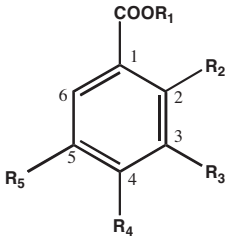


1. $R_1 = R_3 = CH_3, R_2 = OCH_3, R_4 = H$

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

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

7. $R_1 = CH_3, R_2 = R_5 = H, R_3 = OH, R_4 = OCH_3$

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

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

Figure. The structures of compoud **1-9** isolated from methanolic extract of *Vitex agnus-castus*.

Results and discussion

The present study on the methanolic extract of *Vitex agnus-castus* of Pakistani origin resulted in the isolation and characterization of compounds **1-9** (Figure). The structures of these compounds—artemetin (**1**),[12] casticin (**2**),[13] 3-hydroxy-5,6,7,4′-tetramethoxy flavone (**3**),[14] penduletin (**4**),[15] *p*-hydroxybenzoic acid (**5**),[16] methyl 3,4-dihydroxybenzoate (**6**),[17] methyl isovanillate (**7**),[17] vanillic acid (**8**),[16] and 3,4-dihydroxybenzoic acid

(**9**)[16]—were identified on the basis of spectroscopic methods and comparison with the data reported in the literature. Compound **7** was isolated for the first time from this plant.

The antioxidant activity of these compounds was evaluated using a DPPH radical scavenging assay. Propyl gallate was used as a positive control. It was observed that the derivatives of benzoic acid (**6** and **9),** which are 3,4-dihydroxy substituted, exhibited strong activity against the DPPH radical, while 3-methoxy-4-hydroxy-substituted compound **8** was a moderate inhibitor. Similarly, flavonoid **4,** which is 4-hydroxy substituted, exhibited moderate activity. Compound **2** showed weak radical scavenging activity. The remaining compounds—**1**, **3**, **5**, and **7**—did not show any activity. IC_{50} values were not determined due to insufficient quantities of the test compounds. The antioxidant activity of each compound is summarized in Table 3.

Table 3. DPPH radical scavenging activity of compounds **1-9** isolated from a methanolic extract of *Vitex agnus-castus*.

Compounds	DPPH Scavenging Activity (% Inhibition) at 1 mM Conc.
1	-
2	24.27
3	-
4	48.75
5	-
6	89.34
7	-
8	43.38
9	94.73
Propyl gallate*	94.0

*Standard radical scavenger as a positive control.

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