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RESEARCH ARTICLE

Antioxidant phenylpropanoid glycosides from *Buddleja davidii*

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Abstract
Phytochemical investigations on the *n*-BuOH-soluble fraction of the whole plant of *Buddleja davidii* led to the isolation of the phenylpropanoid glycosides **1-10**. Their structures were determined by 1D and 2D NMR spectroscopic techniques. All the compounds showed potent antioxidative activity in three different tests, with IC_{50} values in the range 4.15-9.47 μM in the hydroxyl radical ($^{\bullet}OH$) inhibitory activity test, 40.32-81.15 μM in the total ROS (reactive oxygen species) inhibitory activity test, and 2.26-7.79 μM in the peroxynitrite ($ONOO^-$) scavenging activity test. Calceolarioside A (**1**) displayed the strongest scavenging potential with IC_{50} values of (4.15 ± 0.07, 40.32 ± 0.09, 2.26 ± 0.03μM) for $^{\bullet}OH$, total ROS and scavenging of $ONOO^-$, respectively.

Keywords: *Buddleja davidii; Buddlejaceae; phenylpropanoid glycosides; antioxidant; Buddleia*

Introduction

Antioxidants, which scavenge active oxygen species (free radicals), were found in a variety of foodstuffs and are commonly referred to as scavengers [1, 2]. Many oxidants are plant based and play an important role in protecting plants that are exposed to sunlight and live under severe oxygen stress. Antioxidants also play an important role in human health because the biologic defense mechanism cannot operate under severe oxygen stress. According to recent research, activated oxygen is thought to be major factor in aging, hardening of the arteries, diabetes, cancer and tissue in injury skin [3, 4]. Indeed approximately 90% of age related diseases are linked to activated oxygen.

The genus *Buddleja (*synonym *Buddleia)*, belonging to the family Buddlejaceae, comprises of 100 species mainly distributed in East Asia, America and South Africa. In Pakistan it is represented by four species [5]. The genus *Buddleja* was previously placed in the family Loganiaceae [6] while Hegnauer and Kooiman classify the families Buddlejaceae and Loganiaceae together in family Scrophlariaceae [7].

Various species of the genus *Buddleja* are used for the treatment of a variety of ailments such as ulcer, conjunctival congestion, clustered nebulae and skin disorders. Different parts of *B. asiatica* are used as antiinflamatory, abortifacient, antifungal and also for the treatment of skin diseases [8, 9]. The leaves and flowers of *B. globasa* are used for washing wounds and treatment of ulcer [9].The flowers of *B. officinalis* are used in Chinese medicine for the treatment of conjunctival congestion and clustered nebulae [9].

Previous studies on the genus *Buddleja* have resulted in the isolation of various compounds including glycosides of phenylpropanoids, triterpenes, iridoids and flavonoids, sterols, aryl esters, phenolic fatty acid esters, alkaloid, lignans, neolignans, diterpenes and sesquiterpenes [10, 11].

Buddleja davidii is deciduous shrub, up to 5 m tall with tap root system. Stem is erect and branched. Leaves are sub-sessile, 7-25 cm long, 1-7 cm broad. Stipules are interpetiolar and 2-lobed. Flowers are sub-sessile, bractcite, fragrant, forming a terminal 6-30 cm long inflorescence panicle. Capsules (fruits) are glabrous, up to 1 cm long. It is distributed in Pakistan, China, India and Afghanistan [5].

As a part of our ongoing search for antioxidative constituents from the genus *Buddleja* [12], we investigated the antioxidative activities of the solvent-partitioned fractions

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Table 1. Antioxidative activity of the solvent fractions.

Sample	IC_{50} [μg/mL][a]		
	•OH[b]	Total ROS[c]	$ONOO^-$[d]
$CHCl_3$	51.42 ± 0.05	>400	56.41 ± 0.09
EtOAc	27.17 ± 0.03	>300	31.09 ± 0.02
n-BuOH	7.39 ± 0.04	49.21 ± 0.07	9.10 ± 0.05
H_2O	62.25 ± 0.06	>400	69.29 ± 0.01
Trolox[e]	5.50 ± 0.07	35.07 ± 0.09	_
DL-Penicillamine[f]	_	_	1.03 ± 0.05

[a]Values of •OH, total ROS, and $ONOO^-$ are expressed as the mean ± standard error of triplicate experiments.
[b]Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c]Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d]Inhibitory activity of authentic peroxynitrite.
[e]Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control.

from the EtOH extract of the whole plant of *Buddleja davidii* (Table 1). The *n*-BuOH-soluble fraction of the plant showed stronger antioxidative activity than that of the other fractions 7.39±0.04 µg/mL, IC_{50} in hydroxyl radical inhibitory activity test) which prompted us to carryout phytochemical studies on this plant. As a result, from the *n*-BuOH-soluble fraction, we isolated and characterized ten known phenylpropanoid glycosides, Calceolarioside A (**1**) [9], Isomartynoside (**2**) [10], Martynoside (**3**) [8], 2``-Acetylmartynoside (**4**) [10], Jionoside D (**5**) [10], Plantainoside C (**6**) [10], Campneoside II (**7**) [9], Angoroside C (**8**) [10], Forsythoside B (**9**) [9], Poliumoside (**10**) [9] (Figure 1). Their structures were elucidated by 1D and 2D NMR spectroscopic analyses.

In the current study we have described the antioxidant activities of the phenylpropanoid glucosides (**1-10**), among which compounds (**1**, **7, 9** and **10**) were isolated for the first time from the title plant species.

Materials and methods

Extraction and isolation

The shade-dried whole plant (6 kg) were chopped and extracted with EtOH (3 X 20 L) at room temperature for 96 h. The ethanolic extract was evaporated *in vacuo* to give a dark greenish residue (250 g), which was partitioned between $CHCl_3$, EtOAc, *n*-BuOH and water to afford $CHCl_3$- soluble fraction (80 g), EtOAc-soluble fraction (45 g) and *n*-BuOH-soluble fraction (30 g), respectively. The *n*-BuOH soluble fraction was subjected to vacuum liquid chromatography (VLC) over silica gel with a step gradient solvent system of MeOH:$CHCl_3$ (1:9, 3:7, 5:5), and 100% MeOH as eluants to obtain four sub-fractions (1-4). Fractions 1 to 2 were combined and were subjected to silica gel column chromatography (CC) using MeOH:$CHCl_3$ (2:8, 3:7) followed by CC over Sephadex LH-20 with pure water to get semi pure compounds **1-5** which were finally purified on recycling HPLC (LC 908 W) [ODS-M80 semi preparative column, MeOH:H_2O (1:1), flow rate 3 ml/min), detection (UV and Refractive Index (RI) detectors). Fraction 3 and 4 were subjected to Sephadex LH-20 CC and then purified by recycling HPLC (MeOH:H_2O, 6:4) to obtain compounds **6**,**7** and **8-10**, respectively.

Evaluation of antioxidative activity

Measurement of the inhibition of total ROS generation

Rat kidney homogenates prepared from the kidneys of freshly killed male Wistar rats, weighing 130–180 g, were mixed with or without the suspension of extracts or compounds, which were dissolved in 10% EtOH (final concentration: 0.4%). The mixtures were then incubated with 12.5 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA, Molecular Probes Inc., Eugene, Oregon), which was dissolved in 100% EtOH (final concentration: 0.2%), at 37 ^{0}C for 30 min. A 50 mM phosphate buffer (Wako Pure Chemical Industries, Osaka, Japan) solution at pH 7.4 was also used. DCHF-DA is a stable compound, which is hydrolyzed by intracellular esterase to yield a reduced, nonfluorescent compound, 2′,7′-dichlorodihydrofluorescein (DCHF). The ROS produced by the homogenates oxidizes the DCHF to highly fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored using a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) with excitation and emission wavelengths of 460 and 530 nm, respectively [13]. Trolox being an effective standard oxidant was used as a positive control.

Measurement of the inhibition of hydroxyl radical generation

Extracts or compounds that were dissolved in 10% EtOH (final concentration: 0.4%) were added to 1 mM H_2O_2 and 0.2 mM $FeSO_4$ (Fisher Scientific, Fair Lawn, N.J.) and incubated at 37 ^{0}C for 5 min. Esterase-treated 2 M DCHF-DA (Molecular Probes Inc., Eugene, Oregon) in 100% EtOH was then added, and the changes in fluorescence were monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively, for 30 min [14]. Trolox being an effective standard oxidant was used as a positive control.

Measurement of $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of dihydrorhodamine 123 (DHR 123, Molecular Probes Inc., Eugene, Oregon) using a slight modification of the method reported by Kooy *et al* [15]. DHR 123

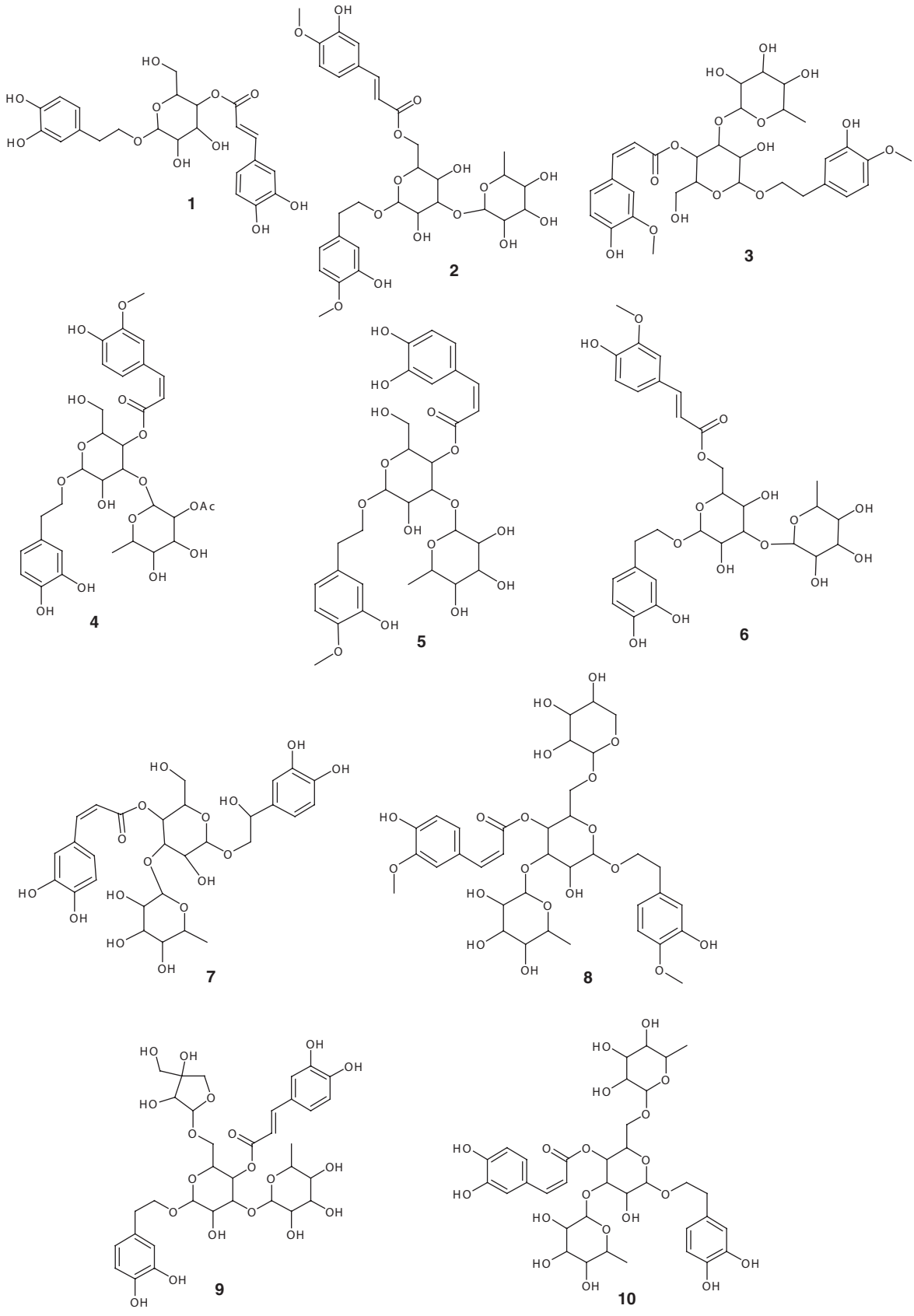

Figure 1. Structures of Compounds **1–10**.

Table 2. Antioxidative activity of Compounds 1–10.

	IC_{50} [μg/mL][a]		
Compound	•OH[b]	Total ROS[c]	ONOO⁻[d]
1	4.15 ± 0.07	40.32 ± 0.09	2.26 ± 0.03
2	8.10 ± 0.01	73.12 ± 0.02	5.31 ± 0.06
3	5.40 ± 0.04	47.51 ± 0.07	3.09 ± 0.09
4	5.09 ± 0.09	43.10 ± 0.06	2.69 ± 0.07
5	6.85 ± 0.02	62.29 ± 0.09	4.01 ± 0.03
6	5.90 ± 0.05	54.34 ± 0.04	3.18 ± 0.09
7	4.72 ± 0.01	43.71 ± 0.09	2.71 ± 0.07
8	9.47 ± 0.03	81.15 ± 0.07	7.79 ± 0.04
9	7.30 ± 0.07	69.93 ± 0.05	5.13 ± 0.06
10	7.54 ± 0.06	71.19 ± 0.08	5.29 ± 0.01
Trolox[e]	2.79 ± 0.05	30.09 ± 0.10	–
DL-Penicillamine[f]	–	–	1.03 ± 0.03

[a] Values of •OH, total ROS, and ONOO⁻ are expressed as the mean ± standard error of triplicate experiments.
[b] Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c] Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d] Inhibitory activity of authentic peroxynitrite.
[e] Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control.

(5 mM) in DMF, which was purged with N_2, was stored as a stock solution at 80°C. This solution was then placed on ice and kept in the dark prior to the study. The buffer consisted of 90 mM NaCl, 50 mM Na_3PO_4, 5 mM KCl at pH 7.4, and 100 M diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high-quality deionized H_2O and purged with N_2. The final concentration of DHR 123 was 5 M. The background and final fluorescent intensities were measured 5 min after treatment with and without the authentic ONOO⁻. DHR 123 was oxidized rapidly by the authentic ONOO⁻, and the final fluorescent intensity of the oxidized DHR 123 was measured using a FL 500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively. The results are expressed as the mean ± standard error (n = 3) for the final fluorescence intensity minus background fluorescence. The effects are expressed as the percent inhibition of DHR 123 oxidation, and the standard oxidant, DL-penicillamine was used as a positive control. The $IC_{50}s$ was defined as the concentrations of sample showing 50% scavenging activity and were calculated from a triplicate experiment in all of the three scavenging tests.

Results and discussion

Free radicals and ROS or RNS (reactive nitrogen species), including H_2O_2, $^{\bullet}O_2^-$, $^{\bullet}OH$, $NO^{\bullet}$, and $ONOO^-$, play an important role in the etiology of a variety of human degenerative diseases. These reactive species are formed in the body as a consequence of aerobic metabolism and damage all intracellular components, such as nucleic acids, proteins, and lipids. ROS are also implicated in both aging and various degenerative disorders [16].

We investigated the general antioxidative effects of the compounds to inhibit $^{\bullet}OH$ and total ROS and to scavenge authentic $ONOO^-$ for the crude fractions and isolated compounds **1**–**10**. The *n*-BuOH-soluble fraction of the plant showed stronger antioxidative activity than that of the other fractions in all the three scavenging tests (Table 1). The bioassay-directed isolation from this fraction provided compounds **1**-**10**. The compounds with phenolic hydroxyl groups in 1,4-orientation in their structures, showed strong antioxidative activity in those three tests (Table 1). Compounds **1** and **7**, with 3,4-dihydroxyphenylpropanoids and 3,4-dihydroxyphenylethanoids moieties, showed stronger activity with the IC_{50} values (4.15 ± 0.07, 40.32 ± 0.09, 2.26 ± 0.03 and 4.72 ± 0.01, 43.71 ± 0.09, 2.71 ± 0.07) for $^{\bullet}OH$, total ROS and to scavenge $ONOO^-$, respectively. In all the scavenging activity tests, Martynoside (**3**) and Jionoside D (**5**), with a 4-methoxyphenylethanoids moieties, showed lower activity than **1** and **7**. Isomartynoside (**2**), which has a 1,4-methoxyphenylpropanoid as well 1,4-methoxyphenylethanoid moieties in structure, further showed less strong activity (IC_{50} values 7.10 ± 0.01, 67.12 ± 0.02, 4.91 ± 0.06) than all the tested compounds except compounds **8**. Angoroside C (**8**) with a 4-methoxyphenylethanoids and three glycoside moieties was observed with lowest activities among the tested compounds with IC_{50} values 9.47 ± 0.03, 81.15 ± 0.07, 7.79 ± 0.04 for $^{\bullet}OH$, total ROS and to scavenge $ONOO^-$, respectively, but still significant activities in all tests.

The antioxidative activity was also examined in term of the chemical structures including those of functional radical and its orientation. Hydroxy and methoxy groups in the 1,4- and 1,3-orientation (glycoside ring) are mainly involved in the scavenging of phenyl propanoids/phenylethanoids (**1**–**10**). This was observed in particular for Calceolarioside A (**1**) and Campneoside II (**7**) containing the hydroxyl group in the 1,4-orientation. We also found that the scavenging activity tended to decrease for increasing number of glycosidation in the series of the compounds as was observed in compounds **1**, **7**, **9** and **10**. Furthermore, the development of scavenging activity was found low comparatively for compounds **3**, **5**, in which the methoxy group is in 1,4-orientation. Changing of hydroxyl group to methoxy group at 1,4-orientation also decrease the

scavenging activity. The *ortho* substitution with electron donor methoxy group in 1,4-oriennted phenyl hydroxyl compounds slightly increase the scavenging activity. This was observed in compounds **3** and **2**. Based on these results, a phenyl ring where the hydroxyl radical is in 1,4-orientation allows the oxygen atom to share a positive charge, thereby causing stabilization through delocalization. Because of the electron donating effect of the methoxy group in 1,3-orientation, it helps to stabilize positive charge and this is thought to influence the scavenging ability. The substitution radical of 1,2- or 1,4-orientation generally donate an electron to the aromatic ring to activate it, either by the resonance effect or inductive effect. This tendency was also found in the all scavenging tests against the phenylpropanoids (**1–10**). To the best of our knowledge this is a first report of such phenylpropanoids glycosides in these particular antioxidant activity tests.

The compounds **1–10** could lead to compounds for the treatment of oxidative stress-related human diseases. However, a further *in vivo* study would help in exploring the pharmacological properties of these compounds.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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