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Bioactive caffeic acid esters from *Glycyrrhiza glabra*

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Thin layer chromatography bioautography (using DPPH spray reagent) guided fractionation of *Glycyrrhiza glabra* led to the isolation of two caffeic acid derivative esters, viz. eicosanyl caffeate (**1**) and docosyl caffeate (**2**). The two compounds exhibited potent elastase inhibitory activity, with IC_{50} values of $0.99\,\mu g\,mL^{-1}$ and $1.4\,\mu g\,mL^{-1}$ for **1** and **2**, respectively. The compounds also showed moderate antioxidant activity in DPPH and ABTS scavenging assays. The results indicate a possible role of caffeic acid derivatives, in addition to flavonoids in the anti-ulcer properties of *G. glabra*.

Keywords: ABTS; antioxidant; docosyl caffeate; DPPH; eicosanyl caffeate; elastase; *Glycyrrhiza glabra*

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1. Introduction

The prevalence of gastric mucosal damage due to hyperacidity, ulcers, the use of nonsteroidal anti-inflammatory drugs, and alcohol consumption is rapidly increasing. Among other factors (like *Helicobacter pylori*), neutrophil-derived elastase and oxidative stress resulting from oxygen-derived free radicals like superoxide anion ($O_2^{\bullet}$) and hydroxyl (OH^-) radicals are considered to significantly contribute to such gastric damage (Liu et al., 1998; Repetto & Llesny Braz, 2002). It is therefore considered desirable that gastroprotective agents possess elastase inhibitory and antioxidant activity.

Glycyrrhiza glabra (Fabaceae), commonly known as liquorice, is a popular natural remedy useful in treating gastric damage (Borrelli & Izzo, 2000). *Glycyrrhiza glabra* has been reported to possess antioxidant activity in different *in vitro* assays and animal models (Bafna & Balaraman, 2005). The activity has mainly been ascribed to the flavonoid constituents of liquorice such as: isoflavans, viz. glabridin, hispaglabridins A and B, 4′-*O*-methylglabridin; chalcones, viz. isoliquiritgenin; and isoflavones, viz. formononetin (Belinky, Aviram, Fuhrman, Rosenbat, & Vaya, 1998; Fukai, Satoh, Nomura, & Sakagami, 2003a, 2003b; Haraguchi et al., 2000; Okada et al., 1989; Vaya, Belinky, & Aviram, 1997). During a preliminary study we evaluated the antioxidant potential of different extracts of *G. glabra* using a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay. Antioxidant activity was found to be concentrated in the flavonoid-rich ethyl acetate extract. Thin layer chromatography (TLC) of the latter with DPPH as the spraying

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reagent indicated a non-flavonoid band responding to the reagent, in addition to the numerous flavonoid spots. This article describes the isolation and characterisation of two long chain caffeoyl esters corresponding to the DPPH sensitive non-flavonoid region on the TLC. The IC_{50} values of the isolated constituents were determined in elastase inhibition, DPPH scavenging, and 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assays.

2. Experimental

2.1. *Material and methods*

NMR spectra (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) were obtained on a Bruker AV 500 spectrophotometer. The electron spray ionisation mass spectrum (ESI-MS) was acquired on a Bruker Daltronics Esquire 3000 plus ion trap mass spectrometer. A Thermo Finnigan Flash EA1112 elemental analyser was used for CHN analysis. The FTIR spectrum was measured on a IR Prestige-21 (Shimadzu) spectrophotometer.

2.2. *Plant material*

Roots of *G. glabra* were collected from the Agronomy Department, Natural Remedies Pvt. Ltd., Bangalore. A voucher (PP-541) was submitted to the Herbarium, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences.

2.3. *Extraction and isolation*

The powdered plant material (15 kg) was successively extracted with ethyl acetate (6 L × 3), methanol (6 L × 3) and water (6 L × 3) by refluxing. The three extracts were separately filtered and dried under vacuum to yield 0.4, 1.73, and 1.35 kg from ethyl acetate, methanol, and water, respectively. The ethyl acetate extract (300 g) was fractionated on a silica gel column using the following combinations of solvents: petroleum ether, ethyl acetate (95 : 5, 90 : 10, 80 : 20, 60 : 40, 25 : 75, 0 : 100); and ethyl acetate and methanol (95 : 5, 90 : 10, 75 : 25, 50 : 50, 0 : 100). The residue obtained from the petroleum ether : ethyl acetate (8 : 2) fraction was further subjected to preparative HPLC.

2.4. *Chromatography*

Thin layer chromatography was performed on layers of silica gel $60F_{254}$ (Merck, Germany) using chloroform : methanol (93 : 7) as the mobile phase, and then sprayed with a solution of (2 mg mL^{-1}) DPPH in methanol for the detection of antioxidant constituents appearing as white spots against purple background (TLC Bioautography). Flavonoids were detected on TLC by spraying with natural product reagent (1% solution of diphenyl boric acid 2-amino-ethyl ester (Sigma, USA) in methanol followed by 5% solution of PEG 4000 in ethanol).

The HPLC system consisted of a Shimadzu LC-8A Pump, a SPD-M10ADVP photodiode array detector (PAD) and a FRC-10A fraction collector. Analytical separations were carried out on a C_{18} column (250 × 4.6 mm; 5 μm, YMC Japan) using methanol as the mobile phase in isocratic mode at a flow rate of 1.2 mL min^{-1}. The retention times of **1** and **2**

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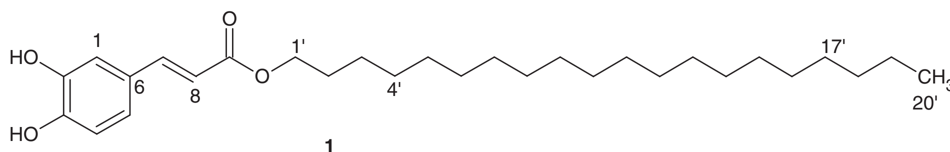
were 11.9 and 15.6 min, respectively. Preparative separations were carried out on a C_{18} semi-preparative column (250 × 20 mm; 5 μ, YMC Japan) using methanol as the mobile phase at a flow rate of 21.6 mL min^{-1}. Aliquots (150 mg) of purified fractions of **1** and **2** in methanol obtained from column chromatography were injected into HPLC. Effluent containing the solute corresponding to the observed peaks of **1** and **2** was collected in the fraction collector. The collected fractions were concentrated and dried under vacuum to get purified **1** (55 mg) and **2** (60 mg).

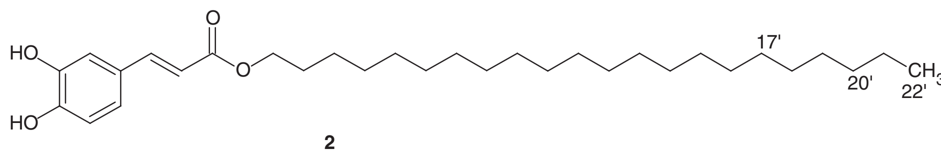
2.5. *Eicosanyl caffeate (1)*

Waxy crystalline solid. Mp 113°C; IR (KBr) cm^{-1}: 3466, 2823, 1682, 1599, and 1106; ^1H and ^{13}C NMR (Table 1); ESI-MS: 459.4 $[M-H]^+$; CHN Analysis: Found: C 76.35%, H 10.66% and O 12.99% (Required for $C_{29}H_{48}O_4$: C 75.61%, H 10.50% and O 13.89%).



2.6. *Docosyl caffeate (2)*

Waxy crystalline solid. Mp 110°C; IR (KBr) cm^{-1}: 3478, 2910, 1688, 1611, 1470, and 1111; ^1H and ^{13}C NMR spectra were exactly same as **1** except for an additional area corresponding to four hydrogens under the broad peak at δ 1.27 ppm in the ^1H NMR spectrum. ESI-MS: 487.4 $[M-H]^+$; CHN Analysis: Found: C 76.91%, H 10.91%, O 12.18% (Required for $C_{31}H_{52}O_4$: C 76.18%, H 10.72%, O 13.10%).



3. Elastase inhibition assay

In brief, 233 μL of each test solution/reference standard (various concentrations) in 100 mM Tris-HCl pH 8.0 and 7 μL of enzyme (porcine pancreatic elastase, 0.84 units mL^{-1}) solution was incubated at 37°C for 15 min. Following incubation, 20 μL of substrate (*n*-succinyl-ala-ala-ala-*p*-nitroanilide) solution was added and incubated at 37°C for 30 min. The absorbance was measured at 405 nm. A control reaction was carried out without the test sample. The percentage inhibition was calculated by the equation [absorbance (control) − absorbance (test)]/absorbance (control) × 100. The IC_{50} values were determined using log–probit analysis (Beith, Spiess, & Wermuth, 1974).

4. DPPH scavenging assay

A final reaction volume of 250 μL in methanol contained different concentrations of the tested samples/gallic acid (the final concentrations tested were 0.1–100 μg mL^{-1} for

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Table 1. ^{1}H and ^{13}C NMR data of **1**.[a]

Carbon	δ_H^b	δ_H^c	δ_C^c	HMBC[c]
1	7.19 (d, 1.9)	7.63 (s)	128.1	H-6, H-7, H-5, H-8
2	–	–	116.4	H-7
3	–	–	151.4	H-2, H-5
4	–	–	148.8	H-5, H-2, H-6
5	6.92 (d, 8.2)	7.19[d]	117.8	H-6
6	7.08 (dd, 1.9, 8.2)	7.19[d]	123.0	H-5, H-2, H-7
7	7.57 (d, 15.99)	8.02 (d, 15.9)	146.7	H-8, H-2, H-6
8	6.31 (d, 15.9)	6.65 (d, 15.9)	116.9	H-7
9	–	–	168.5	H-8, H-1′, H-7
1′	4.18 (t, 6.6)	4.28 (t, 6.7)	65.5	H-2′, H-8
2′	1.71 (m)	1.68 (m)	30.3	H-1′
3′	1.33 (brs)[e]	1.27 (brs)[e]	27.4	H-1′, H-2′
4′	1.33 (brs)[e]	1.27 (brs)[e]	30.6	H-2′
5′–16′	1.33 (brs)[e]	1.27 (brs)[e]	31.0	–
17′	1.33 (brs)[e]	1.27 (brs)[e]	30.9	–
18′	1.33 (brs)[e]	1.27 (brs)[e]	33.2	H-20′
19′	1.33 (brs)[e]	1.27 (brs)[e]	33.9	H-20′
20′	0.91 (t, 6.6)	0.86 (t, 6.7)	15.2	–
OH-3	8.29 (brs)	11.59 (brs)	–	–
OH-4	8.29 (brs)	12.07 (brs)	–	–

Notes: δ in ppm and J in parentheses in Hz. [a]Assignments were confirmed with HSQC and DEPT experiments. [b]In acetone – d_6. [c]In pyridine – d_5. [d]Merged with solvent signals. [e]Area under the peak corresponded to 34-H.

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extracts, 1.0–30 µg mL^{-1} for **1** and **2**, 0.5–2.5 µg mL^{-1} for gallic acid) and 10 µL of DPPH solution (1.3 mg mL^{-1} in methanol). The tubes were mixed thoroughly, incubated at 25°C for 15 min and the absorbance was measured at 510 nm. A control reaction was carried out without the test sample. Percentage inhibition was derived from the equation: [absorbance (control) − absorbance (test)]/absorbance (control × 100), and the IC_{50} values were calculated by log–probit analysis (Vani, Rajini, Sarkar, & Shishoo, 1997).

5. ABTS radical scavenging assay

In brief, 20 µL of each test solution/reference standard of various concentrations/phosphate buffer saline (PBS) and 230 µL of ABTS (0.238 mm) solution were mixed. The absorbance was measured immediately at 734 nm. The percentage inhibition was calculated by the equation [absorbance (control) − absorbance (test)]/absorbance (control) × 100]. The IC_{50} values were determined using log–probit analysis (Auddy et al., 2003).

6. Results and discussion

Roots of *G. glabra* were successively extracted with ethyl acetate, methanol, and water. The vacuum-dried extracts gave 2.8% w/w, 11.5% w/w, and 9.0% w/w yields, respectively, from the roots. The ethyl acetate extract had an IC_{50} value of 20.1 µg mL^{-1} in the DPPH assay, while the methanol and water extracts did not show any significant inhibition up to 100 µg mL^{-1} (Table 2). TLC bioautography of the ethyl acetate extract with DPPH as the

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Table 2. IC_{50} of fractions and isolates of *G. glabra* in DPPH scavenging and ABTS radical scavenging assays.

	DPPH		ABTS	
Sample	IC_{50}[a]	95% CI	IC_{50}[a]	95% CI
Ethyl acetate extract	20.1	13.2–32.5	ND	ND
Successive methanol extract	NA[b]	–	ND	ND
Successive water extract	NA[b]	–	ND	ND
1	8.8	6.8–12.6	20.3	17.9–23.5
2	13.2	11.4–15.6	23.1	19.6–27.6
Gallic acid (positive control)	0.8	0.6–0.9	1.4	1.2–1.6

Notes: CI – Confidence interval. ND – Not determined. [a]Expressed in $\mu g\,mL^{-1}$. [b]No significant inhibition observed up to $100\,\mu g\,mL^{-1}$.

spray reagent indicated a number of spots responding positively to the reagent. Most of the spots which responded to DPPH spray were found to be of flavonoid type, as also responded to the natural product reagent (see Section 2). Since the flavonoids of *G. glabra* have been widely studied from chemical and antioxidant activity point of view, we decided to isolate the non-flavonoid types of antioxidant constituents visible on TLC sprayed with DPPH reagent. The targeted compounds (at Rf band ~0.64) were isolated by column chromatography over silica gel with final purifications using preparative HPLC (see Section 2). Chromatographic behaviour of the targeted compounds was rather unusual, with retention times of 11.9 and 15.6 min in HPLC on C_{18} column with 100% methanol as the mobile phase, which indicated that the compounds are likely to be nonpolar.

The ^{1}H NMR of compound **1** indicated the presence of a caffeoyl moiety (3,4-dihydroxy-*trans* cinnamate) in the structure, as revealed by the presence of a two *trans* coupled protons on a double bond (δ 6.65 and δ 8.02 ppm, each doublet with $J = 15.9$ Hz), the two downfield aromatic hydroxyl protons (δ 11.59 and δ 12.07 ppm each broad singlet) and the characteristic aromatic protons in a 2,5,6 substitution pattern (Table 1). Further, a long carbon chain was present in the molecule (δ 1.27 brs, 34H), which appeared esterified to the carboxyl end of the caffeoyl moiety, as indicated by the downfield methylene protons (δ 4.28 ppm, t, $J = 6.7$ Hz, 2H) at the $H\text{-}1^{1}$ end, and the methyl protons (δ 0.86 ppm, $J = 6.7$ Hz, 3H) at $H\text{-}20^{1}$. These assignments were complemented in the ^{13}C NMR spectrum with the carboxyl carbon at δ 168.5 ppm, the hydroxyl bearing carbons from δ 151.4 ppm (C-3), δ 148.8 ppm (C-4), and δ65.5 ppm ($–CH_2O–$), and the long chain carbons at δ 15.2 to 33.2 ppm. ESI-MS gave $[M-H]^{+}$ at 459.4; based on the above data, the structure of **1** could be deduced as eicosanyl-3,4-dihydroxy-*trans*-cinnamate (eicosanyl caffeate). The NMR assignments were confirmed by DEPT, HSQC ($^{1}J_{CH}$), and HMBC ($^{2}J_{CH}$ and $^{3}J_{CH}$) experiments. The important HMBC correlations observed for **1** are given in Table 1.

The ^{1}H and ^{13}C NMR data of **2** were identical to those of **1** except for the additional four hydrogens integrated under the broad peak at δ 1.27 ppm in **2**. This gave an indication that structural difference between **1** and **2** lay only in the carbon chain length. The molecular weight of **2** was found to be 488, corresponding to two additional methylenes in the carbon chain of **1**. Based on the above observations, the structure of **2** was deduced as docosyl-3,4-dihyroxy-*trans*-cinnamate (docosyl caffeate). It may be noted that long chain alkyl esters of caffeic acid are not very common in the plant kingdom, and to the best of

Table 3. IC_{50} of isolates of *G. glabra* in elastase inhibition assay.

Sample	IC_{50}[a]	95% CI
1	0.99	0.8–1.1
2	1.4	1.2–1.5
Ursolic acid (positive control)	10.8	8.8–13.5

Notes: CI – Confidence interval. [a]Expressed in $\mu g\,mL^{-1}$.

our knowledge, this forms the first report of **1** and **2** in Fabaceae. For the first time, the complete assignments of ^{1}H and ^{13}C NMR data have been provided here for **1** and **2**, supported by HSQC, HMBC, and DEPT experiments.

The two compounds exhibited potent elastase inhibitory activity, with IC_{50} values of 0.99 and 1.4 $\mu g\,mL^{-1}$ for **1** and **2**, respectively (Table 3). Previous reports (Kisiel & Jakupowic, 1995; Kang & Kim, 1987; Gibbons, Mathew, & Gray, 1999) on these compounds provide neither the complete spectral details nor bioactivity information. In addition, as expected for caffeic acid structures, the compounds showed antioxidant activity in the DPPH scavenging assay, with IC_{50} values of 8.8 and 13.2 $\mu g\,mL^{-1}$, and in ABTS radical scavenging assay with IC_{50} values of 20.3 and 23.1 $\mu g\,mL^{-1}$ for **1** and **2**, respectively (Table 2). These results suggest a possible role played by caffeic acid esters in the gastroprotective activity of *G. glabra*, in addition to the already established contribution of liquorice flavonoids, which work as antioxidants.

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