Received 03/25/06. Accepted 07/31/06

Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
16(3): 286-290, Jul./Set. 2006

Artigo

Chrozophorin: a new acylated flavone glucoside from *Chrozophora tinctoria* (Euphorbiaceae)

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RESUMO: "Crozoforina: uma nova flavona glicosilada e acetilada de *Chrozophora tinctoria* (Euphorbiaceae)". Análise das partes aéreas de *Chrozophora tinctoria* (Euphorbiaceae) através de HPLC preparativa com coluna de fase reversa produziu cinco glicosídeos de flavonóides, quercetina 3-*O*-rutinosídeo (**1**, rutina), acacetina 7-*O*-rutinosídeo (**2**), apigenina 7-*O*-β-D-[(6-*p*-cumaroil)]-glicopiranosídeo (**3**), apigenina 7-*O*-β-D-glicopiranosídeo (**4**) e apigenina 7-*O*-β-D-[-(3,4-diidroxibenzoil)]-glicopiranosídeo (chamado crozoforina, **5**), sendo o último um novo produto natural. As estruturas dessas substâncias foram inequivocamente elucidadas por análise de espectrofotometria de UV com o uso de reagentes de deslocamento, ESIMS, e técnicas de RMN 1D e 2D. A atividade de captura de radicais livres do extrato metanólico (RC_{50} = 2,24 x 10^{-1} mg/mL) bem como das substâncias isoladas (**1**-**5**) (RC_{50} = 4,38 x 10^{-3}, 2,26 x 10^{-2}, 7,69 x 10^{-4}, 8,71 x 10^{-3} e 3,19 x 10^{-4} mg/mL, respectivamente) foram analisados pelo método DPPH.

Unitermos: *Chrozophora tinctoria*, Euphorbiaceae, flavonóide, flavona, glicosídeo, quercetina, apigenina, rutina, crozoforina, DPPH.

ABSTRACT: Preparative reversed-phase HPLC analysis of the methanol extract of the aerial parts of *Chrozophora tinctoria* (Euphorbiaceae) yielded five flavonoid glycosides, quercetin 3-*O*-rutinoside (**1**, rutin), acacetin 7-*O*-rutinoside (**2**), apigenin 7-*O*-β-D-[(6-*p*-coumaroyl)]-glucopyranoside (**3**), apigenin 7-*O*- β-D-glucopyranoside (**4**) and apigenin 7-*O*-β-D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (named, chrozophorin, **5**), the last one being a new natural product. The structures of these compounds were elucidated unambiguously by UV spectroscopic analyses using shift reagents, ESIMS, and 1D and 2D NMR spectroscopic techniques. The free-radical scavenging activity of the methanol extract (RC_{50} = 2.24 x 10^{-1} mg/mL) as well as the isolated compounds (**1**-**5**) (RC_{50} = 4.38 x 10^{-3} 2.26 x 10^{-2}, 7.69 x 10^{-4}, 8.71 x 10^{-3} and 3.19 x 10^{-4} mg/mL, respectively) were assessed by the DPPH assay.

Keywords: *Chrozophora tinctoria*, Euphorbiaceae, flavonoid, flavone, glycoside, quercetin, apigenin, rutin, chrozophorin, DPPH.

INTRODUCTION

Chrozophora tinctoria (L.) A. Juss., an annual that is commonly known as 'dyer's-croton', 'giradol' or 'turnsole', belongs to the subtribe Chrozophorinae, tribe Chrozophoreae, subfamily Acalyphoideae and family Euphorbiaceae (GRIN Database, 2005). This species is native to a number of countries in Africa, (Algeria, Egypt, Libya, Morocco, Tunisia and Yemen) temperate and tropical Asia (Kuwait, Saudi Arabia, Afganistan, Iran, Iraq, Israel, Jordan, Lebanon, Syria, Turkey, Kazakhstan, Turkmenistan, India and Pakistan), and Europe (Ukraine, Albania, Bulgaria, Greece, Italy, Malta, France, former Yugoslavia, Portugal and Spain). *Chrozophora tinctoria* is well known for producing dye substances (Başlar; Mert, 1999) and flavonoids (Hashim et al., 1990). Alkaloids, coumarins, chromones (Mohamed, 2001), flavonoids (Hashim et al., 1990), xanthones (Agrawal; Singh, 1988), diterpenoids (Mohamed et al., 1994, 1995) and phenylpropanoid glycosides (Mohamed, 2001) have previously been reported from a few other species of the genus *Chrozophora*. While, in the Iranian traditional medicine, *C. tinctoria* is used to treat warts, this plant has been used as an emetic, cathartic, and for the treatment of fever elsewhere (Phytochemical and Ethnobotanical Databases, 2005). As a part of our continuing search for natural antioxidants (Delazar et al., 2005, 2006; Nahar et al., 2005; Nahar; Sarker, 2005a,b; Sarker et al., 2005a,b; Kumarasamy et al., 2004), we now report on the isolation, structure determination, and free radical

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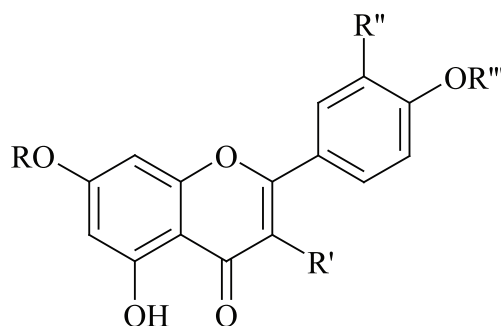
ISSN 0102-695X

Table 1. ^{1}H NMR (coupling constant *J* = Hz in parentheses) and ^{13}C NMR data, and COSY interaction of compound **5.**

Position	Chemical shift δ in ppm		^{1}H-^{1}H interaction obtained from COSY
	δ_H	δ_C	
2	-	162.2	-
3	6.56 s	102.3	-
4	-	182.9	-
5	-	158.4	-
6	6.11 br s	99.7	H-8
7	-	165.0	-
8	6.29 br s	94.1	H-6
9	-	161.6	-
10	-	106.2	-
1’	-	121.3	-
2’	7.53 d (8.4)	128.3	H-3, H-6’
3’	6.63 d (8.4)	115.8	H-2’, H-5’
4’	-	161.6	-
5’	6.63 d (8.4)	115.8	H-3’-H-6’
6’	7.53 d (8.4)	128.3	H-2’, H-5’
Glucose moiety			
1’’	5.17 d (7.0)	102.3	H-2’’
2’’	3.20-3.80*	73.0	-
3’’	3.20-3.80*	77.1	-
4’’	3.20-3.80*	70.8	-
5’’	3.20-3.80*	74.7	-
6’’	4.42 d (12.15)	64.3	H-5’’
Acyl moiety			
1’’’	-	121.8	
2’’’	7.91 d (1.9)	117.1	H-3’’’, H-6’’’
3’’’	-	144.9	H-2’’’
4’’’	-	150.1	-
5’’’	7.08 d (8)	115.8	-
6’’’	7.85 dd (1.9, 8)	121.8,	H-2’’’
7’’’	-	167.6	

*Overlapped peaks; Spectra obtained in MeOH-d_4



Compound number	R	R’	R’’	R’’’
1	H	Rutinosyloxy	OH	H
2	Rutinosyl	H	H	Me
3	(6-*O*-*p*-coumaroyl)-glucosyl	H	H	H
4	Glucosyl	H	H	H
5	(6-*O*-3,4-dihydroxybenzoyl)-glucosyl	H	H	H

scavenging activity of four known flavonoids (**1**-**4**) and a new acylated flavone glucoside, named chrozophorin (**5**), from the aerial parts of *C. tinctoria.*

MATERIAL AND METHODS

General

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV-vis spectrometer. NMR spectra were recorded in CD_3OD on a Bruker 200 MHz NMR Spectrometer (200 MHz for ^1H and 50 MHz for ^{13}C) using residual solvent peak as internal standard. ESIMS analyses were performed on a Finnigan MAT95 spectrometer. HPLC separation was performed in a Shimadzu HPLC system. A Shim-Pak ODS column 10 µm, 250 mm × 21.2 mm was used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractionation.

Plant material

The aerial parts of *Chrozophora tinctoria* were collected from Tabriz in eastern Azarbaijan province (situated in Iran) during May-June 2004, and the identity was confirmed by morphological characterisation in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences. Also, a voucher specimen (BSAD0001) representing this collection has been generated in the herbarium of the Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

Extraction, isolation and structure elucidation

The dried and ground aerial parts of *C. tinctoria* (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane and methanol (1.1 L each). The MeOH extract (2 g) was subjected to Sep-Pack fractionation using a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). The preparative reversed-phase HPLC analysis (Shim-Pak ODS column 10 µm, 250 mm × 21.2 mm; mobile phase: 0 to 50 min gradient 15 to 35% ACN in water; flow-rate: 20 mL/min, detection at 248 nm) of the 40% methanolic Sep-Pack fraction afforded quercetin 3-*O*-rutinoside (**1**, 4.7 mg, retention time: 14.1 min). Similar purification (Shim-Pak ODS column 10 µm, 250 mm × 21.2 mm; mobile phase: 0 to 50 min gradient 25 to 40% ACN in water; flow-rate: 20 mL/min, detection at 248 nm) of the 60% methanolic Sep-Pack fraction yielded acacetin 7-*O*-rutinoside (**2**, 4.5 mg, retention time: 12.2 min), apigenin 7-*O*-β-[(6-*O*-*p*-coumaroyl)]-glucoside (**3**, 3.8 mg, retention time: 18.9 min), apigenin 7-*O*-β-glucoside (**4**, 1.5 mg, retention time: 24.0 min) and apigenin 7-*O*-β-D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (chrozophorin) (**5**, 2.5 mg, retention time: 36.1 min). While compounds **1**-**4** were identified by direct comparison of its UV (in MeOH and using shift reagents), ESIMS, ^1H and ^{13}C NMR data with respective published data, the structure of compound **5** was elucidated by UV, ESIMS, ^1H-, ^{13}C- and COSY NMR data analyses.

*Apigenin 7-O-*β*-D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (Chrozophorin)* (**5**): Yellow amorphous solid. UV: λ_{max} (MeOH) nm: 270, 331; + NaOMe: 275, 379; + $AlCl_3$: 278, 300, 349, 387; + $AlCl_3$ + HCl: 279, 299, 344, 386; + NaOAc: 275, 290, 374; + NaOAc + H_3BO_3: 272, 323; ^1H- and ^{13}C NMR (Table 1). ESIMS: positive ion mode) *m/z* 591 $[M+Na]^+$ and negative ion mode) *m/z* 567 $[M-H]^-$

DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al. (1994) was adopted with suitable modifications to our particular circumstance (Kumarasamy et al., 2002). DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 µg/mL.

Qualitative assay: Test samples (MeOH extract, and **1**-**5**) were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were noted.

Quantitative assay: The MeOH extract, and test compounds **1**-**5** were dissolved in MeOH to obtain a concentration of 1.0 mg/mL. Dilutions were made to obtain concentrations of $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$ $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$, $5x10^{-10}$ mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive standards (quercetin).

RESULTS AND DISCUSSION

RP-HPLC analysis of the methanol extract of the aerial parts of *C. tinctoria* afforded four known flavonoid glycosides, quercetin 3-*O*-rutinoside (**1**), acacetin 7-*O*-rutinoside (**2**), apigenin 7-*O*-β-D-[(6-*p*-coumaroyl)]-glucopyranoside (**3**), and apigenin 7-*O*-β-D-glucopyranoside (**4**), and a novel acylated flavonoid glucoside, apigenin7-*O*-β-D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (named, chrozophorin, **5**). The known flavonoid glycosides were readily identified by direct comparison of the spectroscopic data with published data (Delazar et al., 2005; Mohamed, 2001; Markham; Geiger, 1993; Hashim et al., 1990; Agarwal; Raghunath,

1989; Mabry et al., 1970). The structure of the new compound was elucidated on the basis of comprehensive spectroscopic analyses (e.g. UV, ESIMS, and 1D and 2D NMR).

Compound **5** exhibited characteristic UV absorption maxima for a flavone skeleton (Mabry et al., 1970). The results from the UV analysis using various shift reagents identified the apigenin structure with an *O*-substitution, other than OH, at C-7 (Markham, 1982; Mabry et al., 1970). The ^{1}H NMR and ^{13}C NMR spectra of this compound (Table 1) also confirmed the presence of a flavone nucleus in this molecule (Markham; Geiger, 1993; Mabry et al., 1970). The ESIMS spectra of **1** revealed $[M+Na]^{+}$ (positive ion mode) ion peak at *m/z* 591, and $[M-H]^{-}$ (negative ion mode) ion peak at *m/z* 567, suggesting M_r = 568 and solving for $C_{28}H_{24}O_{13}$. The ^{1}H and ^{13}C NMR spectra (Table 1), in addition to the signals associated with the aglycone apigenin, showed signals for a glucose moiety and a 3,4-dihydroxybenzoyl moiety. The deshielded nature of the ^{1}H and ^{13}C NMR signals (δ_H 4.42 and δ_C 64.3) for C-6''confirmed the attachment of this 3,4-dihydroxybenzoyl moiety at C-6'' of the glucose unit. Apart from the signals associated with the 3,4-dihydroxybenzoyl moiety, all other ^{1}H and ^{13}C NMR signals were comparable to published data for apigenin 7-*O*-β-D-glucopyranoside isolated from various plant sources (El-Ansari et al., 1991; Agarwal; Raghunath, 1989). The ^{1}H-^{1}H COSY 45 spectrum of **5** (Table 1) displayed ^{1}H-^{1}H couplings and helped to assign key proton resonances. Thus compound **5** was identified as apigenin 7-*O*-β-D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (named, chrozophorin), which, to our knowledge, is a novel natural product.

Flavonoids **1**, **3** and **4** have previously been reported, in trace amounts, from the aerial parts of *C. tinctoria* (Hashim et al., 1990). This is the first report on the occurrence of acacetin 7-*O*-rutinoside (**2**) in this species, and flavonoid **5** is a novel natural product. It is noteworthy that among the flavonoids (**1**-**5**) isolated in this study, flavonoids **2**-**5** are apigenin derivatives with glycosylation at C-7. To our knowledge, none of these flavonoid glycosides has previously been reported from any other species of the genus *Chrozophora*. However, a few other flavonoids, including acacetin 5-*O*-rutinoside, which is closely related to **2**, has been reported from only a few other species of this genus (Mohamed, 2001; DNP CD-ROM, 2001; Hashim et al., 1990). From the published reports, it has appeared that most of the flavonoids found in this genus do not have oxygenation at C-3. While the family Euphorbiaceae is well known for producing various alkaloids, the distribution of flavonoids is rather limited to a few genera (ISI database, 2005; DNP CD-ROM, 2001).

The crude MeOH extract showed low levels of free radical scavenging activity (antioxidant activity) (RC_{50} = 2.24 x 10^{-1} mg/mL) in the DPPH assay (Kumarasamy et al., 2002; Takao et al., 1994). However, even this low level of activity was an indication of the presence of antioxidant compounds, which upon purification from the crude extract could show more significant activity. All flavonoids (**1**-**5**) showed prominent free radical scavenging activity. The RC_{50} values of the flavonoids **1**-**5** were found to be RC_{50} = 4.38 x 10^{-3} 2.26 x 10^{-2}, 7.69 x 10^{-4}, 8.71 x 10^{-3} and 3.19 x 10^{-4} mg/mL, respectively, compared to 2.88 x 10^{-5} mg/mL for quercetin, a well-known natural antioxidant. Clearly, the novel flavonoid **5** was the most active antioxidant among the compounds, and this high level of activity could be contributed by, in addition to the flavonoid structure, the 3,4-dihydroxybenzoyl moiety. Generally, the antioxidant activity of these flavonoid glycosides, like other natural phenolic antioxidants, is a consequence of the presence of the phenolic moieties in the structures (Kumarasamy et al., 2004). The antioxidant activity of phenolic natural products is predominantly owing to their redox properties, i.e. the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential.

ACKNOWLEDGEMENTS

We thank the EPSRC National Mass Spectrometry Service Centre (Department of Chemistry, University of Wales Swansea, Swansea, Wales, UK) for CIMS analyses.

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