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Original Article

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Studies on the Antioxidant and Antimicrobial Activity and Flavonoid Derivatives from Fruits of Trigonosciadium brachytaenium (Boiss.) Alava.

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Abstract

The objective of this study was to evaluate the potential use of the fruits of Trigonosciadium brachytaenium in the pharmaceutical and food industries. Flavonoids are present in food and medicinal plants and are thus consumed by humans. They are found in plants as glycosides. Their biological activities have an impact on human health so that they serve as target molecules to develop new drugs. From methanolic extract of fruits of Trigonosciadium brachytaenium (Boiss.) Alava. (Umbelliferae family), two flavonoid derivatives namely 5- Hydroxy- 3'-methoxy-4'- ethoxyflavone-7-O-(2''-(4'''-acetyl-rha)-rha)(1) and 5- Hydroxy- 4'-methoxy-8- ethoxyflavone-7-O-(2''-(2'''- acetyl-rha)-rha)(2) have been isolated by column chromatography (CC) and preparative TLC (PTLC). Those structures were elucidated by UV, 1H- and 13C- NMR, HMBC, EI-MS and IR spectra. The antioxidant activity of methanol extract was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method. The results indicate that methanol extract from aerial parts of T. brachytaenium possess considerable antioxidant activity. The highest radical scavenging activity was detected (IC50 = 47 μg/mL). This study reveals that the methanolic extract of this plant is attractive sources of flavonoid, especially the essential ones, as well as of effective natural antioxidants. The antimicrobial activity of the methanol extract of aerial part was determined against seven Gram-positive and Gram-negative bacteria (Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, S. epidermidis, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae), as well as three fungi (Candida albicans, Saccharomyces cerevisiae and Aspergillus niger). The bioassay showed that the extract exhibited good antimicrobial activity. These flavonoid compounds were isolated for the first time from T. brachytaenium.

Keywords: Trigonosciadium brachytaenium, Umbelliferae, Methanolic extract, flavonoid, antimicrobial activity, antioxidant activity

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INTRODUCTION

Flavonoids are ubiquitous polyphenolic metabolites in plants that have diverse beneficial biochemical and antioxidant effects [1]. Their dietary intake is quite high, compared to other dietary antioxidants like vitamins C and E. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been principally reported to have antioxidant activities [2]. It is reported that phenolics may prevent lipid peroxidation via hydrogen atom donation from the hydroxyl group(s) attached to the benzene ring [3]. However, the connection between the structure of phenolics and their antioxidant activity are still being actively investigated. In recent years, there has been an increased interest in phenolic compounds derived from fruits and vegetables for their possible health benefits. The anticarcinogenic, antimutagenic, and cardioprotective effects of phenolic compounds are reported to be generally associated with their antioxidant properties of eliminating free radicals and alleviating lipid peroxidation [4]. Our previous report on the methanolic extract of Tanacetum parthenium from North-west Iran showed that its flavonoids were Flavonol, Kaempferol, Fisetin and Naringenin [5]. In addition, identification of the flavonoids from Zosimia absinthifolia (Umbelliferae family) and Galium verum (Rubiaceae family) are found in the literature [6,7]. The genus Trigonosciadium is represented in Iranian flora by three species, among which T. brachytaenium is endemic [8]. In the course of phytochemical studies of the North- West medicinal plants from Iran in particular Trigonosciadium species, the Iranian Trigonosciadium brachytaenium (Umbelliferae) (GOLPARAK in Persian) was investigated. No phytochemical studies on T. brachytaenium have been reported. To the best of our knowledge, this is the first report on the flavonoids from aerial parts and those antioxidant and antibacterial activities of Trigonosciadium brachytaenium (Boiss.) Alava. from Iran.

EXPERIMENTAL

Plant material:

The fruits of Trigonosciadium brachytaenium was collected in June 2011 from Khalkhal area (Ardabil province) in northwest of Iran at an altitude of 1950m. A voucher specimen (No: 028) has been deposited at the Herbarium of the Agriculture Research Centre (A.R.C.) Ardabil, Iran.

Chemicals and Methods:

The IR spectra were determined on a Bruker Tensor 27 spectrometer. The 1H-NMR and 13C - NMR spectra were recorded on a Bruker AM 400 spectrometer. Column chromatography was performed over silica- gel (70-230 mesh, Merck,) using petroleum ether, AcOEt, methanol gradients as elution solvents. UV spectra were recorded on a Perkin- Elmer Lambda 12 spectrophotometer and Mass spectra were recorded on an AEI MS-50 spectrometer.

Extraction and isolation:

Dried and finely powdered T. brachytaenium aerial parts (300g) were exhaustively macerated with MeOH to yield 41 g of crude extract after evaporation of the solvent in vacuo. The concentrated total extract was extracted with petroleum ether, $CHCl_3$, EtOAc and n- BuOH, respectively. A part of the EtOAc portion (3g) was subjected to silica gel column chromatography (70-230 mesh, Merck), eluted with an equivalent petroleum ether, AcOEt, methanol stepwise gradients to obtain 23 fractions (15mL each). Fractions 6-11 after solvent evaporate were in turn chromatographed over silica gel with $CHCl_3$: MeOH mixture to provide 12 subfractions. Subfraction 8 (145 mg) was rechromatographed on silica gel into 13 fractions (13×15 mL.) using as eluents an 8.5:1.5, $CHCl_3$: MeOH mixture. The combined fractions 5 to 10(22 mg) were further purified on a preparative TLC to give compound 1 (17 mg).

A portion of the AcOEt (0.31 g of fractions 15- 21 after removal of solvent) was chromatographed over a small column (15cm × 1.5 cm) with AcOEt: MeOH (8.0: 2.0) as eluents. A total of 11 fractions were collected. The combined fractions 8 to 11(48 mg) according

to TLC analysis were further purified on a preparative TLC to give compound 2(23 mg). The flavonoids were readily identified as 5- Hydroxy- 3'-methoxy-4'- ethoxyflavone-7-O-(2''-(4'''-acetyl-rha)-rha)(luteolin derivative), and 5- Hydroxy- 4'-methoxy-8-ethoxyflavone-7- O-(2''-(2'''- acetyl- rha)-rha)(apigenin derivative) by comparing their physical and spectroscopic data with those reported in the literature [19-21].

Antioxidant activity tests:

The DPPH assay was carried out according to the modified method [22]. Briefly, 0.5 mL of DPPH in ethanol (0.1 mM) was added to 1 mL of extracts in different concentrations (0.1-1.6 mg/mL) and kept in the dark for 10 min. The absorbance of the resulting solution was recorded on a spectrometer at 520 nm against a blank of alcohol. Vitamin C was used as reference antioxidant. DPPH scavenging activity was expressed as IC50 values (μg extract/mL) for comparison. IC50 value of each sample defined as the concentration of sample required for the 50 % decrease in absorbance of the blank was calculated.

Antimicrobial activity:

The in vitro antibacterial and antifungal activities of the extract was evaluated by the disc diffusion method (DDM) using Mueller-Hinton agar for bacteria and Sabouraud Dextrose agar for fungi [23]. Discs containing 30 μL of the methanol extract were used and growth inhibition zones were measured after 24 h and 48 h of incubation at 37°C and 24°C for bacteria and fungi, respectively. Gentamicin and tetracycline for bacteria and nystatin for fungi were used as positive controls. The microorganisms used were: Bacillus subtilis ATCC 9372, Staphylococcus epidermidis ATCC 12228, Enterococcus faecalis ATCC 15753, Staphylococcus aureus ATCC 25923, Klebsiella pneumoniae ATCC 3583, Pseudomonas aeruginosa ATCC 27852, Escherichia coli ATCC 25922, Aspergillus niger ATCC 16404, Candida albicans ATCC 5027 and Saccharomyces cerevisiae ATCC 9763.

RESULTS AND DISCUSSION

The results obtained in the NMR analyses of the methanol extract of T. brachytaenium fruits are listed in Table 1. The 1H-NMR spectrum of compound 1 displayed the characteristic signals of the luteolin nucleus. Compound 1 was obtained in the form of yellow amorphous solid, mp 189-193°C. The molecular formula, C32H38O15 was obtained on the basis of the 13C-NMR and Mass spectra analysis. The identification of the compounds was supported by comparison with published data of related compounds [9-12]. Its UV absorptions in methanol were at λmax 337, 292 and 269 nm. Its IR absorptions showed the presence of hydroxyl (3355 cm-1), conjugated carbonyl (1672), and aromatic rings (1609, 1496 and 1439 cm-1). The combination of 1H- , 13C -NMR, and HMBC correlation spectral data of 1 indicated the presence of two methyl groups were observed on the spectrum which assignment to two groups of sugar at δH 3.41 to 3. 76(Table 1) and those two methyl groups [δH 0.98(3H, d, J=11.3Hz, Me-6'') and (δH 1.1(3H, d, J=11.2Hz, Me-6''')]. In this compound one methyl group was observed on the spectrum which assignment to –O-methyl [δH 3.92(3H, s)], with the corresponded δC 55.2 and an ethyl group which assignment to –O-ethyl [δH 4.13(2H, q), δH 1.25(3H, t)], with the corresponded δC 61.5 and 16.9 respectively. 1H-NMR spectrum of compound 1 showed three ABX type phenyl protons at δH 6.95 (1H, d, J = 8.2 Hz, H-5'), 7.79 (1H, dd, J = 8.2 & 1.9 Hz, H-6'), 7.91 (1H, d, J = 1.9 Hz, H-2')], two doublet signals at δH 6.49 (1H, d, J= 1.9Hz, H-6) and 6.82 (1H, d, J= 1.9Hz, H-8), one singlet signal at δH 2.0(3H,s, Me-acetyl). The 13C-NMR spectrum showed 32 signals corresponding to six -CH- and nine quaternary carbons in aromatic moiety [13]. 13C– NMR spectrum indicated the presence of two carbonyl carbons, which showed signal at δ175.9(C-4) and 187.2 ppm (CO-acetyl). The other signals of 13C- NMR spectrum showed at δ163.1 (C- 2), 111.2 (C- 3), 160.0 (C- 5), 113.4(C-6), 122.0(C-1'), 118.1(C- 2'), 137.2 (C-3'), 146.7(C- 4'), 105.1(C-5') and 122.5(C- 6') (Table 1).

Table 1: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra data and HMBC for compounds 1& 2 (CD3OD) Chemical shifts are in δ (ppm).

	Compound 1			Compound 2		
NO	δH	δC	HMBC	δH	δC	HMBC
1	-	-	-	-	-	-
2	-	163.1	-	-	162.5	-
3	6.23, s	111.2	C-1', C-10	s,7.99	114.8	C-1',C-10
4	-	175.9	-		165.1	-
5	-	160.0	-		157.1	-
6	6.49 (d, j=1.9)	113.4	C-8, C-10	s,6.43	115.2	C-8,C-10
7	-	122.4	-	-	113.1	-
8	6.82 (d, j=1.9)	120.1	C-10, C-6	-	150.0	-
9	-	157.0	-	-	135.0	-
10	-	112.8	-	-	148.1	-
1'	-	122.0	-	-	122.1	-
2'	7.91(d, j=1.9)	118.1	'C-2, C-4	(d, j=8.2)7.65	103.0	'C-2,C-6',C-4
3'	-	137.2	-	(d, j=8.2)6.92	101.5	'C-5',C-1
4'	-	146.7	-	-	131.9	-
5'	6.95(d, j=8.2)	105.1	'C-1', C-3	(d, j=8.2)6.92	101.5	'C-1',C-3
6'	7.79(dd, j=8.2, 1.9)	122.5	'C-2, C-2', C-4	(d, j=8.2)7.65	103.2	'C-2,C-2',C-4
1"	5.27, d	98.4	C-7	(d, j=3.4)5.2	98.2	C-7
1"'	5.46, d	98.8	-	(d, j=3.4)5.1	98.0	"C-2
2CH3(6",6"')	1.1, d; 1.2,d	14.9 ;13.1	-	1.1d,1,2d	15.4;15.1	-
-O-CH3	3.92, s	55.2	'C-3	s ,3.99	55.2	'C-4
-O-CH2- CH3	4.13, q	61.5	'C-4	q ,3.85	64.5	C-8
-O-CH2- CH3	1.25, t	16.9	-	t ,1.16	15.2	-
C=O (acetyl)	-	187.2	-	-	177.0	-
CH3 (acetyl)	2.0, s	19.8	-	s,1.96	18.8	-
H-rha (2 groups)	3.41- 3.76, m	77.2 55.3-	-	m ,(3.7 3.3-)	61.3-76.7	-

The portion of sugar signals were observed in δH 3.41- 3.76 that corresponding to ten -CH- carbons in δC 55.3- 77.2 ppm. Significant HMBC correlations were observed between H-1'' and C-7, and between H-1''' and C-1'', confirming the location of the sugar groups –rha and rha', respectively (fig. 2). The UV spectrum exhibited absorption maxima at 281 and 368 nm that are characteristic absorption bands of a flavanone skeleton. The compound was readily identified by comparing their physical and spectroscopic data with those reported in the literature [14- 16]. From these results, compound 1 was identified as

5- Hydroxy- 3'-methoxy-4'- ethoxyflavone-7-O-(2''-(4'''- acetyl-rha)-rha). Compound 2 was suggested to be a flavone based on the physico-chemical properties, chromatography performance and UV absorption maxima at 265 and 374 nm. The molecular formula, C32H38O15 was obtained on the basis of the 13C-NMR and Mass spectra analysis. The combination of 1H- , 13C-NMR, and HMBC spectral data of 2 indicated the presence of one methoxyl group [δH 3.99(3H, s), with the corresponding δC 55.2], one ethoxyl group [δH 3.85(2H, q), and 1.16(3H, t) with the corresponding δC 64.5 and 15.2, respectively]. 2 two proton doublets (A2B2 type protons) observed in its 1H- NMR spectrum at δ 7.65 (J=8.2 Hz) and δ 6.92(J=8.2 Hz) were clearly assignable to ring B proton at H- 2′, H- 6′ and H-3′, H-5′ respectively. One singlet signal observed at δ6.43 was assignable to H-6 proton with the corresponding δC 115.2. The appearance of two doublets and their coupling constants value were further in agreement with the methoxyl group at C-4′. A singlet appeared at δ 7.99 was assignable to H-3 proton of pyron ring [20].

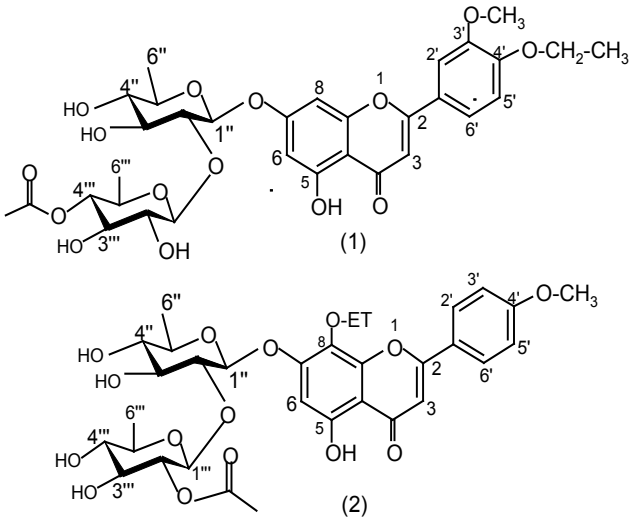

Figure 1: Structure of compounds 1 and 2.

The 1H- NMR spectra of the compound exhibited signals at δ 4.81 (J= 9.9 Hz) and 4.08 (J= 8.4 Hz) applicable for two sugar anomeric protons suggesting the presence of rhamnoside [17, 18]. The structure further supported by its 13C- NMR spectrum, which demonstrated a downfield signal at δ 165.1 clearly assignable to carbonyl carbon C- 4 of the pyron ring. Another signal observed at δ 114.8

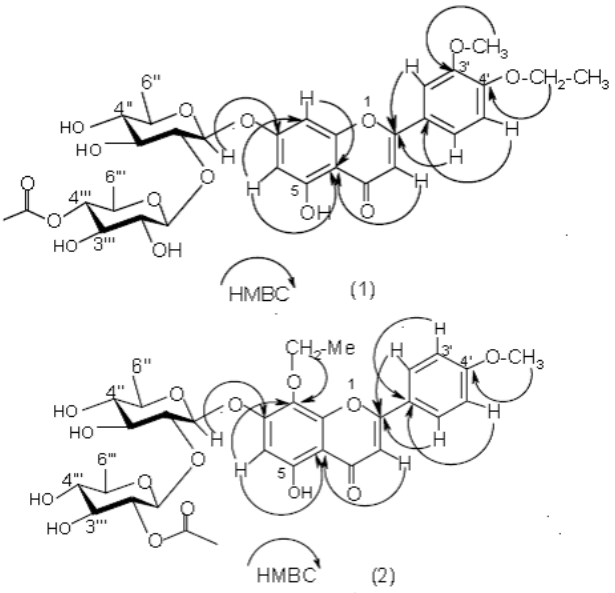

Figure 2: The selected HMBC correlation of compounds 1 and 2.

was indicative for C- 3. In the 13C- NMR spectra, a signal was observed at δ 150.0 indicating substitution at C- 8 position of the –O- ethyl group.

Further, a signal was observed at δ 76.7 suggested that a rhamnoside unit was attached to C-2′′. The three downfield signals appeared at δ157.1, δ 113.1 and δ 131.9 were assigned to C-5, C-7 and C-4′ carbon atoms bearing hydroxyl, -O-sugar and –O-methyl groups, respectively. In the HMBC spectrum, correlations were observed between δH 3.99 and δC 131.9 (C-4′), δH 3.85 and δC 150.0 (C-8), δH 5.2 and δC 113.1 (C-7), confirming the locations of the methoxyl, ethoxyl and sugar groups. Other key HMBC correlations are shown in Table 1. From the above described spectral evidence, compound 2 was identified conclusively as 5- Hydroxy- 4'-methoxy-8- ethoxyflavone-7-O-(2''-(2'''- acetyl-rha)-rha). This compound has been isolated for the first time in this genus.

The antioxidant activity of methanol extract obtained from T. brachytaenium was also reported for the first time. Antioxidant activity was tested according to the DPPH (2, 2-diphenyl-1-pycrylhydrazile) radical scavenging method. The extract obtained from T. brachytaenium scavenged the DPPH radical in a dose- dependent manner and the DPPH radical scavenging activity (IC50) are shown in Table 2. According to this data,

methanol extract of aerial part was the most efficient free radical scavenger by the lowest IC50 value of 47 μg/mL. The activity of the reference antioxidant (vitamin C) was higher than that of methanol extract.

Table 2: DPPH free radical scavenging activity of methanol extract of T. brachytaenium and standard antioxidant, vitamin C.

No	Sample(extract)	IC50 (μg/mL)
1	*methanol*	47
2	*Vitamin C(Ref.)*	27

The methanol extract from T. brachytaenium was tested against four Gram-positive and three Gram-negative bacteria, as well as three fungi. The results, presented in Table 3, show that the methanol extract exhibited a good biological activity against all tested fungi and bacteria except for a resistant Gram-negative bacteria, E. coli, as well as a fungi, Aspergillus niger.

The most sensitive microorganisms were Staphylococcus epidermidis Bacillus subtilis and, Saccharomyces cerevisiae with inhibition zones of 19.3, 16.1 and 15.9 mm, respectively. Other microorganisms were found to be less sensitive to the extracts with inhibition zones ranged from 8 to 14 mm (Table 3). It is conceivable that the antimicrobial property of the methanol extract from T. brachytaenium might be ascribed to its effective compounds such as flavonoids.

Table 3: Antimicrobial activity of the methanol extracts of Trigonosciadium brachytaenium.

	Zone of inhibition (mm) *			
microorganisms	Sample	Antibiotics		
	MeOH extract	Gentamicin	Nystatin	Tetra cycline
B. subtilis	16.1	NTb	*NT*	22.3
S. epidermidis	19.3	NT	*NT*	34.2
E. faecalis	8.0	NT	*NT*	9.5
S. aureus	14.2	NT	*NT*	21.7
K. pneumoniae	11.6	20.2	*NT*	NT
P. aeruginosa	NAa	11.6	*NT*	NT
E. coli	9.2	24.6	*NT*	NT
A. niger	10.8	NT	*16.4*	NT
C.albicans	13.1	NT	*18.8*	NT
S. cerevisiae	15.9	NT	*18.2*	NT

*a NA: Not Active; b NT: Not Tested. *Inhibition zone diameter (mm), including diameter of sterile disk 6 mm.*

In conclusion, the present study indicated that T. brachytaenium seeds are rich in flavonoids and exhibit strong antioxidant activity in the DPPH method tested and had a moderate antibacterial activity. The antioxidant activities correlated well with their content of flavonoid compounds. Although the antioxidant activity found in an in vitro experiment is only indicative for the potential health benefits, these results remain significant as the first step in screening antioxidant activity of T. brachytaenium fruits. It can be concluded that, T. brachytaenium fruits can be used as an accessible source of natural antioxidants with consequent health benefits.

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