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Der Pharmacia Lettre, 2014, 6 (2):50-54
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Flavonoids from *Thymus numidicus* Poiret

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ABSTRACT

*Phytochemical investigation of the dichloromethane, ethyl acetate and n-BuOH soluble parts of the aqueous-EtOH extract of the aerial parts of Thymus numidicus Poiret led to the isolation of circilineol **1**, circimaritin **2**, apigenin **3**, luteolin **4**, apigenin 7-O-β-glucuropyranoside **5** and luteolin 7-O-β-glucopyranoside **6**. The structures were established by spectral analysis including HRESI-MS, UV and 2D NMR experiments (COSY, ROESY, HSQC and HMBC) and comparison with literature data. To the best of our knowledge compounds **1**, **2**, **5** and **6** were described for the first time from this endemic species. n-BuOH extract showed moderate scavenging effect against DPPH .*

Keywords: *Thymus numidicus* Poiret; Lamiaceae; Flavonoids; Antioxidant activity.

INTRODUCTION

The genus *Thymus* consists of about 350 species among which 13 grow in Algeria [1]. It is widely distributed in the Mediterranean area [2]. This very popular herbal plant known since the oldest civilizations is used as medicinal herbs, flavoring agents (condiment and spices) and herbal tea [3]. Decoctions of the aerial parts of *Thymus* species are used for antiseptic, antitussive, expectorant, antispasmodic, anthelminthic and anti inflammatory purposes [4-6]. A great number of scientific papers were devoted in studying analysis of the composition of *Thymus* species essential oils and their extracts [3].

Numerous works have been published on thyme flavonoids aglycone and glycosides which showed the presence of luteolin, apigenin, and their glycosides, vicenin-2, 6-hydroxy-luteolin and the existence of highly methylated 6-oxygenated flavones in most of the species [7-15]. As a continuation of our research on medicinal plants [16-21], we report the flavonoid constituents of the dichloromethane, ethyl acetate and *n*-butanol extracts of *Thymus numidicus* Poiret an endemic Algerian species. Only one previous study was devoted to the isolation of flavonoids from this species [22]. Our results report further flavonoid aglycones and glucosides which are described for the first time from this species .

MATERIALS AND METHODS

General Procedures

TLC: pre-coated aluminium foil silica gel 60F254 (Merck). Column chromatography (CC): silica gel 60 (Merck 230-400 mesh). UV Spectra (MeOH): Shimadzu (190–3200 nm, UV-3101PC) spectrophotometer. NMR Spectra: Bruker AMX-400 MHz and Avance DPX-250, 250 MHz spectrometers; chemical shifts (δ) are given in ppm using

TMS as internal standard and coupling constants (*J*) are given in Hz. Mass spectra**:** Q-TOF micro (waters) spectrometer.

Plant material

The aerial parts of *Thymus numidicus* were Collected in March 2012 in the area of Constantine (North of Algeria). A voucher specimen was deposited at the Herbarium of the reseach unity VARENBIOMOL of the University of Constantine.

Extraction and isolation

Air-dried aerial parts (920.5 g) of *T. numidicus* were macerated at room temperature with EtOH/H2O (70:30 v/v) for 24h, three times. After filtration, the filtrates were combined, concentrated and successively extracted with CH_2Cl_2, EtOAc and *n*-BuOH to give CH_2Cl_2 (1.6 g), EtOAc (4.6 g) and *n*-BuOH (18.1 g) extracts. During the concentration of the CH_2Cl_2 extract, a precipitate was formed. This precipitate was filtered and purified on a Sephadex LH-20 column to yield the pure compound **1** (35 mg). A part of the EtOAc extract (4.5 g) was fractionated by column chromatography (Si gel; $CHCl_3$/MeOH with increasing polarity) to give 16 fractions. Fraction 3 ($CHCl_3$/MeOH 99:1, 50 mg) was purified by preparative TLC (Si gel, $CHCl_3$/MeOH 95:5) to afford compound **2** (24 mg). Fraction 8 ($CHCl_3$/MeOH, 96: 4, 95 mg) was purified by preparative TLC (Si gel, $CHCl_3$/MeOH 95:5, two elutions) to afford compounds **3** (5 mg) and **4** (22 mg). A part of the *n*-BuOH extract (500 mg) was added to 5 ml of MeOH. During the solubilization, a precipitate was formed. This precipitate was purifed by paper chromatography (Watman III) eluted by AcOH (30%) to give compounds **5** (4 mg) and **6** (12 mg).

DPPH radical-scavenging activity assay

The hydrogen atoms or electron donation ability of the corresponding pure compounds was measured from the bleaching of a purple colored methanol solution of DPPH. This Spectrophotometric assay uses the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical as a reagent following the method described by Braca et al. [23]. Various concentrations (1, 2.5, 5, 10, 15, 20, 30, 50, 75 µg/ml) of the test samples in methanol were added to 3 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period in the dark and at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated using the following equation:

$$I\% = (Ac- As)/Ac \times 100$$

Where Ac is the absorbance of the control reaction, and As is the absorbance of the test compound. Tests were carried out in triplicate. Ascorbic acid and Trolox were used as controls.

RESULTS AND DISCUSSION

Isolated and identified compounds

Compound 1: yellow needles, HRESI-MS: $[M+ Na]^+$ *m/z* = 367.0792 calculated for $C_{18}H_{16}O_7Na$: 367.0794 corresponding to molecular formula $C_{18}H_{16}O_7$. UV (λ_{max}, MeOH, nm): 275, 340; +NaOH: 265, 403; +$AlCl_3$: 260, 290, 375; $AlCl_3$/HCl: 260, 290, 360, +NaOAc: 274, 345, 405, +NaOAc/H_3BO_3: 274, 345. ^{1}H NMR (400 MHz, CD_3OD, δ ppm, *J*/Hz): 7.54 (1H, dd, *J*= 8.0, 2.4, H-6'), 7.51 (1H, d, *J* =2.4, H-2'), 6.94 (1H, d, *J*= 8.0, H-5'), 6.83 (1H, s, H-8), 6.67 (1H, s, H-3), 3.97 (3H, s, 7-OMe), 3.96 (3H, s, 3'-OMe), 3.83 (3H, s, 6-OMe), ^{13}C NMR (100 MHz, CD_3OD , δ, ppm,): 184.2 (C-4, C=O), 166.5 (C-2), 160.6 (C-7), 154.9 (C-9), 153.7 (C-5), 152.3 (C-4'), 149.6 (C-3'), 133.8 (C-6), 123.6 (C-1'), 121.9 (C-6'), 116.8 (C-5'), 110.7 (C-2'), 106.7 (C-10), 104.1 (C-3), 92.4 (C-8), 61.2 (6-OMe), 57.0 (3'-OMe), 56.7 (7-OMe). These assignments were completed on the basis of the results of HSQC, HMBC and NOESY experiments. This Compound was characterized as 5,4'-dihydroxy-6,7,3'-trimethoxyflavone (Circilineol) [24].

Compound 2: $C_{17}H_{14}O_6$. UV (λ_{max}, MeOH, nm): 276, 333, +NaOH: 272, 386, +$AlCl_3$: 300, 360, $AlCl_3$/HCl: 300, 351, +NaOAc: 273, 342, 388, +NaOAc/H_3BO_3: 273, 337. ^{1}H NMR (400 MHz, CD_3OD, δ ppm , *J*/Hz): 7.78 (2H, d, *J*= 8.0, H-2', H-6'), 6.84 (2H, d, *J*= 8.0, H-3', H-5'), 6.71 (1H, s , H-8), 6.55 (1H, s, H-3), 3.88 (3H, s, 7-OMe), 3.73 (3H, s, 6-OMe). These assignments were completed on the basis of the results of ROESY experiment. This Compound was characterized as 5,4'-dihydroxy-6,7-dimethoxyflavone (Circimaritin) [25].

Compound 3: $C_{15}H_{10}O_5$, yellow needles, mp. 348-350 °C, Mass spectrum (EI, 70 eV, *m/z* (*I* rel %): 270 [M+•] (12.3), 271 [M+H]+ (47.7). UV (λ_{max}, MeOH, nm): 268, 338, +NaOH: 276, 325, 393, +$AlCl_3$: 278, 302, 349, 383, $AlCl_3$/HCl: 277, 300, 341, 374, +NaOAc: 274, 303, 362, +NaOAc/H_3BO_3: 268, 344. ^{1}H NMR(400 MHz, CD_3OD, δ ppm, *J*/Hz): 7.83 (2H, d, *J*=8.5, H-2', H6'), 6.91 (2H, d, *J*=8.5, H-3', H5'), 6.58 (1H, s, H-3), 6.44 (1H, d, *J*=2.0, H-8), 6.20 (1H, d, *J*=2.0; H-6). ^{13}C NMR (100 MHz, CD_3OD , δ ppm,): 183.9 (C-4, C=O), 166.5 (C-2), 166.3 (C-

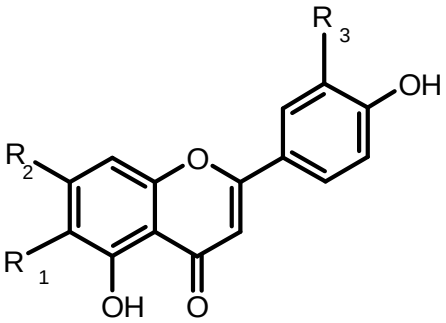
7), 163.2 (C-9), 162.8 (C-4'), 159.6 (C-5), 129.5 (C-2', C-6'), 123.4 (C-1'), 117.12 (C-3',C-5'), 105.3 (C-10), 103.9 (C-3), 100.3 (C-6), 95.2 (C-8). This compound was characterized as 5,7,4'-trihydroxyflavone (Apigenin) [26,27].

Compound 4: $C_{15}H_{10}O_6$, yellow needles, mp.329-331 °C, UV (λ_{max}, MeOH, nm): 267, 350, +NaOH: 267, 325, 402, +$AlCl_3$: 267, 358, 393, $AlCl_3$/HCl: 267, 354,383, NaOAc: 270, 322, 370. ^{1}H NMR (400 MHz, CD_3OD, δ, ppm, *J*/Hz): 12.97 (1H, br s, 5-OH), 7.42 (1H, dd, *J*= 8.1, 2.1, H-6'), 7.38 (1H, d, *J*=8.1, H-2'), 6.86 (1H, d, *J*=8.1, H-5'), 6.66 (1H, s, H-3), 6.43 (1H, d, *J*=2.1, H-8), 6.18 (1H, d, *J*=2.1, H-6). ^{13}C NMR (100 MHz, CD_3OD , δ ppm): 181.5 (C-4, C=O), 164.3 (C-7), 163.3 (C-2), 161.3 (C-5), 157.2 (C-9), 149.8 (C-4'), 145.7 (C-3'), 121.2 (C-1'), 118.8 (C-6'), 115.9 (C-5'), 113.2 (C-2'), 103.5 (C-10), 102.7 (C-3), 98.8 (C-6), 93.7 (C-8). The spectral data led to the structure of 5, 7, 3', 4'-tetrahydroxyflavone (Luteolin) [28] .

Compound 5: $C_{21}H_{18}O_{11}$, UV (λ_{max}, MeOH, nm): 266, 335, +NaOH: 270, 385, +$AlCl_3$: 274, 295, 345, 383, AlCl3/HCl: 276, 294, 340, 381, NaOAc: 266, 336, +NaOAc/H_3BO_3: 268, 338. ^{1}H NMR (400 MHz, CD_3OD, δ ppm, *J*/Hz): 12.97 (1H, br s , 5-OH), 8.03 (2H, d, *J*= 8.9, H-2', H-6'), 6.94 (2H, d, *J*=8.9, H-3', H-5'), 6.83 (1H, d, *J*=2.0, H-8), 6.83 (1H, s, H-3), 6.50 (1H, d, *J*=2.0, H-6), 5.17 (1H, d, *J*=6.8, H-1"), 3.64-3.91 (other sugar protons). ^{13}C NMR (100 MHz, CD_3OD , δ ppm): 181.9 (C-4, C=O), 164.25 (C-2), 162.7 (C-7), 161.4 (C-5), 161.05 (C-4'), 156.9 (C-9), 128.5 (C-2', C-6'), 120.9 (C-1'), 115.9 (C-3', C-5'), 105.3 (C-10), 103.0 (C-3), 99.3 (C-6), 94.6 (C-8). These spectral data led to 5,7,4'-trihydroxyflavone 7-*O*-glucuropyranoside, (Apigenin 7-*O*-β-glucuropyranoside) [29] .

Compound 6: $C_{21}H_{20}O_{11}$, UV (λ_{max}, MeOH, nm): 266, 345, +NaOH: 266, 395, +AlCl3: 273, 423, $AlCl_3$/HCl: 269, 295, 350, 383, NaOAc, 264, 353, 400, +NaOAc/H_3BO_3: 262, 374. ^{1}H NMR (400 MHz, CD_3OD, δ ppm, *J*/Hz): 7.43 (1H, dd, *J*= 8.1, 2.4, H-6'), 7.4 (1H, d, *J*=2.4, H-2'), 6.80 (1H, d, *J*=8.1, H-5'), 6.7 (1H, d, *J*=2.0, H-8), 6.69 (1H, s, H-3), ; 6.3 (1H, d, *J*=2.0, H-6), 5.04 (1H, d, *J*=6.8, H-1"), 3.12-3.56 (other sugar protons). The spectral data led to the structure of 5,7,3',4'-tetrahydroxyflavone 7-*O*-glucopyranoside (Luteolin 7-*O*-β-glucopyranoside) [30] .

The structures of all these compounds were elucidated by extensive UV, MS and NMR spectroscopic analysis as well as by comparing their spectroscopic data with those reported in the literature.



Compounds	R_1	R_2	R_3
1	OMe	OMe	OMe
2	OMe	OMe	H
3	H	OH	H
4	H	OH	OH
5	H	Ogluc	H
6	H	Oglu	OH

Figure 1: Structures of the isolated Compounds

DPPH radical scavenging activity

In the present study, the free radical scavenging activity of the *n*-BuOH extract of *Thymus numidicus* , Vitamin C and Trolox were evaluated through their ability to quench 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The *n*-BuOH extract has moderate DPPH radical scavenging activity (35,08±35,08 µg/ ml**)** in comparison with Trolox and Ascorbic acid (1,97±0,34 µg/ ml and 6,87±0,15 µg/ ml respectively). Figure 2 and tables 1 and 2 illustrated the decrease of DPPH radical due to the scavenging ability of the tested extract which correlates with a dose effect dependent.

Table 1: Scavenging effect of *n*- BuOH extract of *T. numidicus*, Trolox and Ascorbic acid

Concentration µg/ml	DPPH P%	Trolox	Acide ascorbique
5	5.67±4.95	32.20±1.44	60.84±0.82
10	14.49±6.49	83.94±0.17	67.84±0.62
20	33.89±4.18	84.56±0.17	84.87±0.96
50	76.91±2.36	84.40±0.07	97.08±1.30
100	79.23±0.28	84.17±0.01	
150	79.49±0,42	83.78±0.37	
200	78.96±0.59	83.71±0.09	

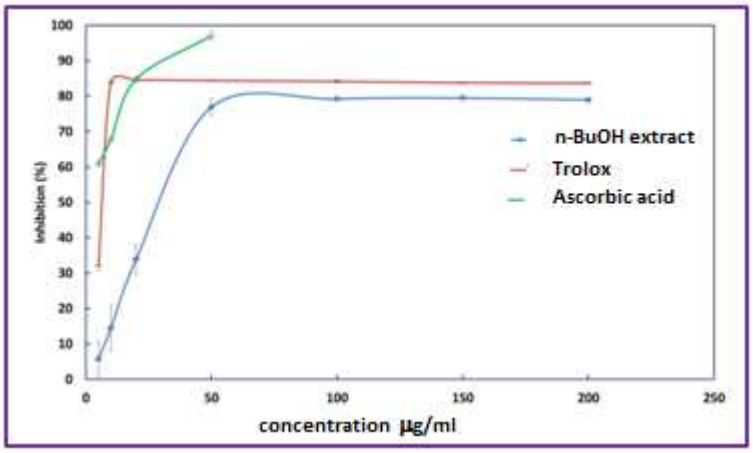


Figure 2 : Scavenging effect of *n*- BuOH extract of *T. numidicus* , Trolox and Ascorbic acid

Table 2 : IC_{50} of *n*- BuOH extract of *T. numidicus* , Trolox and Ascorbic acid

IC_{50}		Means ± SD (µg/ ml)
DPPH	33.34	35.08±35.08
	31.89	
	40.00	
Trolox	2,21	1.97±0.34
	1.73	
Ascorbic acid	7.03	6.87±0.15
	6.74	
	6.83	

CONCLUSION

Six flavonoids namely Cirsilineol **1,** Circimaritin **2,** Apigenin **3,** Luteolin **4,** Apigenin 7-*O*-β-glucuropyranoside **5** and Luteolin 7-*O*-β-glucopyranoside **6** have been isolated from the dichloromethane, ethyl acetate and *n*-buOH extracts of *Thymus numidicus*. Compounds **1**,**2**, **5**, **6** were described for the first time from this species. All the isolated compounds can be considered as chemotaxonomic markers of the genus *Thymus*. The *n*-BuOH extract showed moderate scavenging effect against DPPH in comparison with Trolox and Ascorbic acid used as controls.

Acknowledgments
We are grateful to ATRSS and MESRS (DGRSDT) for financial support.

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