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Der Pharmacia Lettre, 2014, 6 (1):152-156
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Phytochemical constituents of *Thymelaea microphylla* Coss. et Dur. from Algeria

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ABSTRACT

*Phytochemical investigation of chloroform and ethyl acetate soluble parts of the aqueous-EtOH extract of the aerial parts of Thymelaea microphylla Coss. et Dur. from Algeria led to the isolation of six compounds: vanillin **1**, (+)-syringaresinol **2**, daphnoretin **3**, (Z)- 8-hydroxylinalool **4**, chrysoeriol **5**, luteolin **6** and a mixture of trans-tiliroside **7** and cis- tiliroside **8** (97 and 3 % respectively). The structures were established by spectral analysis, mainly ESIMS, UV and 2D-NMR experiments and by comparison with the related known compounds.*

Keywords: Flavonoids; Lignans; Bis-coumarins; *Thymelaea microphylla*; Thymelaeaceae

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INTRODUCTION

The genus *Thymelaea* (Thymelaeaceae) consisting in about 31 species of shrubs and herbs [1], is present in Algeria with 8 species [2]. Many species of this genus which showed biological activities [3-6], have been object of some phytochemical investigations. These studies showed their wealth of bioactive secondary metabolites in particular flavonoids, diterpenes, sterols, triterpenoids and coumarins [7-18]. From the species of this genus *T. lythroides* which was known in the literature as purgative [19] is reported to be a medicinal plant used in Marocco to cure various deseases [17]. In Algeria *T. hirsuta* Endl. is recommended by herbalists in the treatment of human ailments (Leishmanicidal, vermifuge, eczema) in M'Sila region [20]. As a part of our on-going program of research on Algerian plants [21-28] we report our results on *Thymelaea microphylla* Coss. et Dur. an endemic species of North Africa [2]. Previous studies on this plant reported the isolation of oleanolic acid, □-sitosterol, □-sitosterol 3-*0*-□-glucopyranoside [29] and the essential oil composition and a phytoscreening of its aerial parts [30]. The purpose of the present study was the investigation of the chloroform and the ethyl acetate soluble parts of the aqueous-EtOH extract of the aerial parts of this species. In this paper, we report the isolation and the structure elucidation of eight known secondary metabolites **1-8** (Figure 1).

MATERIALS AND METHODS

Plant material

Thymelaea microphylla was collected during the flowering phase in April 2010 in the east of Algeria and was authenticated by Professors Nadra Khalfallah (Constantine 1 university) and Mohamed Kaabache (Setif 1university). A voucher specimen has been deposited at the Herbarium of the VARENBIOMOL research unit, University of Constantine 1.

Extraction and Isolation

Air-dried leaves and flowers (4980 g) of *Thymelaea microphylla* were macerated at room temperature with EtOH–H_2O (70:30, v/v) for 24 h, three times. After filtration, the filtrate was concentrated and dissolved in H_2O (1950 ml) under magnetic agitation. The resulting solution was filtered and successively extracted with $CHCl_3$, EtOAc and *n*-butanol.The organic phases were dried with Na_2SO_4 filtered and concentrated *in vacuo* at room temperature to obtain the following extracts: chloroform (8,20 g), EtOAc (14,94g) and *n*-butanol (56 g).

A part of the chloroform extract (6.3 g) was fractionated by CC on silica gel using petroleum ether, chloroform, acetone and methanol with increasing polarity to yield 26 fractions (F1–F26) obtained by combining the eluates on the basis of TLC analysis. Fraction F2 (751 mg) petroleum ether/$CHCl_3$ (55 :45) was rechromatographed on a silica gel flash column using hexane/ EtOAc (6:4) as eluent and then with increasing percentages of EtOAc to yield 9 sub-fractions. Sub-fraction 5 gave, after purification on preparative plates of silica gel (hexane/ EtOAc (6:4), vanillin **1** (5.8 mg) [31]. Fraction F7 (246 mg) petroleum ether/$CHCl_3$ (30 :70), was rechromatographed on a silica gel column using hexane/ EtOAc (5:5) as eluent and then with increasing percentages of EtOAc yielded 9 sub-fractions. Sub-fraction 7 was a pure compound: (+) syringaresinol **2** (13 mg) [32]. Fraction F10 (172 mg) petroleum ether/$CHCl_3$ (20:80) was a pure compound: daphnoretin **3** [16]. Fraction F13 (260 mg) petroleum ether/$CHCl_3$ (10:90) was rechromatographed on a silica gel flash column using $CHCl_3$/MeOH (9:1) as eluent and then with increasing percentages of MeOH yielded 6 sub-fractions. Sub-fraction 5 gave, after purification on preparative plates of silica gel (hexane/ EtOAc (5:5), (Z)-8-hydroxylinalool **4** (0.8 mg) [33]. Fraction F16 (91 mg) was washed with a mixture $CHCl_3$/MeOH to give yellow crystals of chrysoeriol **5** (6.3 mg) [34]. An aliquot of the ethyl acetate extract (1 g) was subjected to column chromatography of silica gel (70-400 mesh), eluted with CH_2Cl_2/MeOH with increasing polarity to give 17 fractions (F1 – F17). Fraction F6 (86 mg), CH_2Cl_2/MeOH (92 :08) was washed with a mixture of $CHCl_3$/MeOH to give luteolin **6** (7 mg) [35]. Fraction F8 (345 mg) CH_2Cl_2/MeOH (92 :08) was washed with a mixture of $CHCl_3$/EtOAc to give a major component as a yellow powder: trans-tiliroside (97 %) **7** [36] and cis-tiliroside (3%) **8** [37] (200 mg). The percentage of cis-tiliroside was observed to increase up to 30% by photoisomerization with UV lamp irradiating at 365 nm.

Structural identification

Nuclear Magnetic Resonance: NMR spectra (^{1}H-NMR, ^{13}C-NMR, COSY, HSQC and HMBC) for all the isolated compounds were recorded in hexadeuterated dimethylsulphoxide (99.9% CD_3SOCD_3), in deuterated chloroform (99.8%, CDC l_3) or in tetradeuterated methanol, (99.9%, CD_3OD) at 300 K on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90^0 proton pulse length of 8.7 µs at a transmission power of 0 db. Mass spectrometry : Liquid chromatography Electrospray- Mass Spectrometry (LC/ESI-MS) measurements were performed on reverse phase column (Agilent Zorbax eclipse XDB-C18 4.6 x150 mm x 3.5µm, 0.9 mL/min) with MeOH/H_2O gradient elution, mounted on a Hewlett-Packard HP1100 HPLC-UV Diode Array system mated with an Esquire LC Bruker-Daltonics ion trap mass spectrometer. Mass spectra were obtained with an ESI source in positive-ion and negative-ion modes. To analyze the mass and UV the following software has been used: Data Analysis (Version 3.0, Bruker Daltonik GmbH) and LC/MSD ChemStation (Agilent Technologies), respectively.

RESULTS AND DISCUSSION

Isolated and identified compounds

Compound 1 : yellow powder; ^{1}H-NMR (400 MHz, $CDCl_3$): δ(ppm) =9.82 (1H,*s* ,H-7), 7.42 (2H, *m* ,H-2,H-6), 7.05 (1H, *d*, *J* = 8.4 Hz, H-5), 3.96 (3H, *s*, OCH_3),; ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 190.32 (C,C-7),151.15 (C,C-4), 146.63 (C, C-3), 129.42 (C,C-1),108.27 (CH, C-2), 113.85 (CH, C-5), 126.98 (CH, C-6), 55.61(CH_3 ,OCH_3); (-) ESI-MS: *m/z* 151.1$[M-H]^-$, according to the formula $C_8H_8O_3$ for this molecule. All these data led to vanillin [31].This compound was isolated from the genus *Thymelaea* for the first time.

Compound 2 : brown powder; ^{1}H-NMR (400 MHz, DMSO-d_6 ; δ(ppm) = 6.58 (4H, *s*, H-2, H-6, H-2', H-6'), 5.49 (2H, *s*, 4-OH, 4'-OH),4.71 (2H, *d*, *J* =4.0 Hz, H-7, 7'),4.27 (2H, *m*,H-9a, 9'a), 3.91 (2H, *m*, , H-9b,9'b), 3.09 (2H, *m*, H-8,H-8'), 3.90 (12H, *s*, the four OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm) 147.16 (C, C-3, C-5,C- 3', C-5'), 134.33 (C, C-4, C-4'), 132.12 (C, C-1,C-1'), 102.72 (CH, C-2, C-6, C-2', C-6'), 86.07 (CH, C-7, C-7') ,71.80 (CH_2, C-9, C-9'), 56.40 (CH_3, 3-OCH_3, 5-OCH_3, 3'-OCH_3, 5'-OCH_3), 54.38 (CH, C-8, C-8') ; (+) ESI-MS :*m/z* 441.3 corresponding to $[M+Na]^+$, which led to the formula $C_{22}H_{26}O_8$for this molecule. All these data led to (+)-syringaresinol [32].This compound was isolated from the genus *Thymelaea* for the first time.

Compound 3 : white powder; ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 8.04 (1H, *d*, *J* =9.6 Hz, H-4'), 7.87 (1H, *s* , H-4), 7.71 (1H, *d*, *J* = 8.8, H-5'), 7.21 (1H, *s*, H-5), 7.18 (1H, *d* ,*J* =1.6 Hz , H-8'), 7.11 (1H, *dd*, *J* =8.8,1.6 Hz , H-6'), 6.86 (1H, *s*, H-8), 6.38 (1H, *d*, *J* = 9.6 Hz, H-3'), 3.82 (3H, *s*, 6-OCH_3);^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm) 159.90 (C, C-2'), 159.61 (C, C-7'), 156.89(C, C-2), 154.95 (C, C-8a'), 150.34 (C, C-7), 147.37 (C, C-8a),

145.62 (C, C-6), 143.99 (CH, C-4'), 135.67 (C, C-3), 130.79 (CH, C-4), 129.84 (CH, C-5'), 114.34 (C, C-4a'),113.83 (CH, C-3'), 113.39 (CH, C-6'), 110.10 (C, C-4a), 109.37 (CH, C-5),103.97 (C, C-8'), 102.71 (CH, C-8), 56.01 (CH_3, 6-OCH_3); (+) ESI-MS: *m/z* 375.1 corresponding to $[M+Na]^+$and *m/z* 353.2 corresponding to $[M+H]^+$, which led to the molecular formula $C_{19}H_{12}O_7$.This compound which was characterized as daphnoretin [16], was isolated from *T. microphylla* for the first time.

Compound 4 : brown powder; ^{1}H NMR (400 MHz, $CDCl_3$) : δ(ppm) 5.90 (1H,*d d*, *J* = 17.3, 10.8 Hz, H-7), 5.31(1H, *t*, *J* =7.0 Hz, H-3), 5.21 (1H, *dd*, *J* = 17.3, 1.1 Hz, H-8a), 5.07 (1H, *dd*, *J* = 10.8, 1.1 Hz, H-8b), 4.12 (2H, *m* , H_2-1), 2.12 (2H, *q*, *J* = 7.0 Hz, H_2-4), 1.79 (3H, *d*, *J* = 1.3 Hz,2-CH_3), 1.60 (2H, *t*, *J* = 7.0 Hz, H_2-5), 1.28 (3H, *s*,6-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) 145.1 (CH, C-7), 134.3 (C, C-2), 128.4 (CH, C-3), 111.8 (CH_2, C-8), 73.3 (C, C-6), 61.5 (CH_2, C-1), 42.1 (CH_2,C-5), 28.3 (CH_3, 6-CH_3), 22.4 (CH_2, C-4), 21.2 (CH_3, 2-CH_3). All these data led (Z)-2,6-dimethylocta-2,7-diene-1,6-diol or (Z)- 8-hydroxylinalool [33]. This compound was isolated from the family Thymelaeaceae for the first time.

Compound 5. Yellow powder , $C_{16}H_{12}O_5$, UV (MeOH, λ_{max}, nm): 240, 249sh, 271, 347; +NaOH: 263, 275sh, 329sh, 405; +$AlCl_3$: 260, 275, 296, 366sh, 390; +$AlCl_3$/HCl: 276, 293, 353, 386; +NaOAc: 271, 321, 397; + NaOAc/H_3BO_3: 270, 347; ^{1}HNMR (400 MHz, DMSO-d_6): δ(ppm), *J*/Hz): 6.89 (1H, *s*, H-3), 7.56 (1H, *dd*, *J*=9.5 ,2.3, H-6'), 7.55 (1H, *d*, *J*=2.3, H-2'), 6.93 (1H, *d*, *J*=9.5, H-5'), 6.51 (1H, *d*, *J*=2.3, H-8), 6.19 (1H, *d*, *J*=2.3, H-6), 3.90 (3H, *s*, 3'-OCH3); ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm), 181.7 (C, C-4), 164.1 (C, C-7), 163.6 (C, C-2), 161.4 (C, C-5), 157.3 (C, C-9), 150.7 (C, C-4'), 147.8 (C, C-3'), 121.5 (CH, C-6'), 120.3 (C, C-1'), 115.7 (CH,C-5'), 110.2 (CH, C-2'), 103.7 (C, C-10), 103.1 (CH, C-3), 98.8 (CH, C-6), 94.0 (CH, C-8), 55.9 (CH_3, 3'-OCH_3); (+) ESI-MS: *m/z* 301.2 corresponding to $[M+H]^+$, which led to the molecular formula $C_{16}H_{12}O_6$. This compound which was identified as chrysoeriol [34] was isolated for the first time from the family Thymelaeaceae.

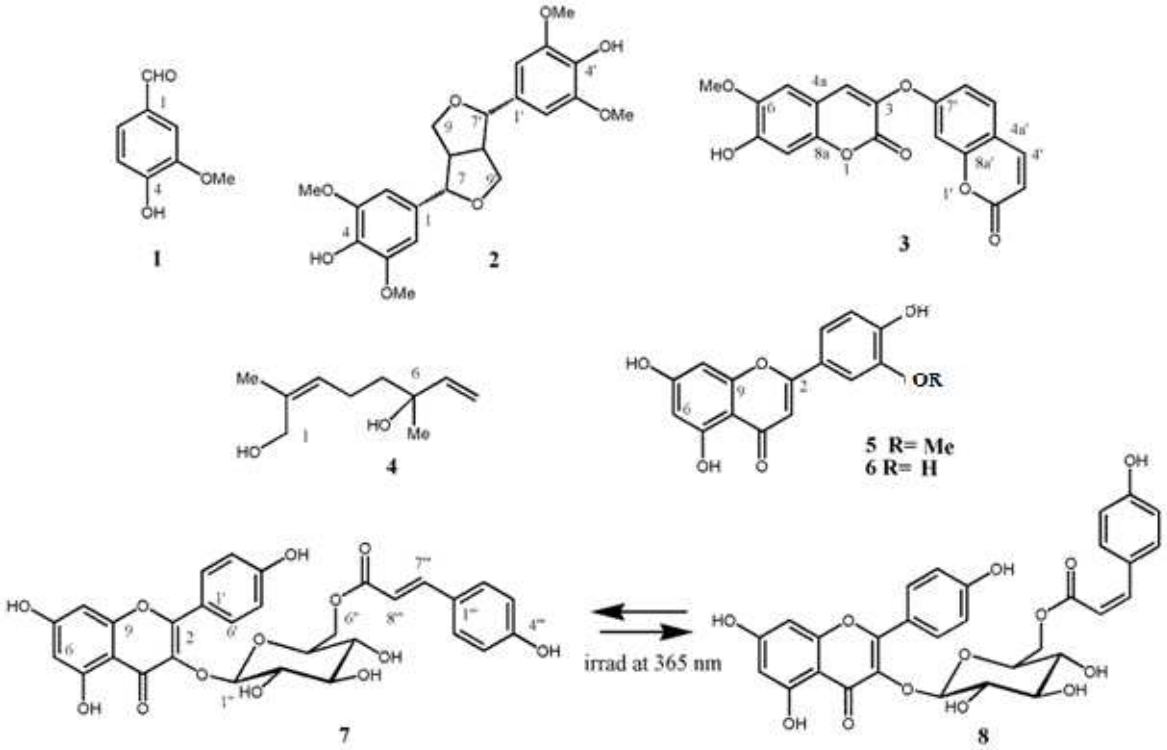

Figure 1: Structures of the compounds 1 - 8

Compound 6. Yellow powder , UV (MeOH, λ_{max}, nm):254, 266, 290sh, 349; +NaOH: 266, 326sh, 401; +$AlCl_3$: 273, 300sh, 329, 267, 425; +$AlCl_3$/HCl: 266, 275, 294sh, 355, 385; +NaOAc: 270, 326 sh, 384; + NaOAc/H_3BO_3: 259,301sh, 371, 430sh; ^{1}HNMR (400 MHz, CD_3OD, δ(ppm) : 7.41 (1H, *dd*, *J*=8.5, 2.0 Hz, H-6'),7.39 (1H, *d*, *J*=2.0 Hz, H-2'), 6.93 (1H, *d*, *J*=8.5 H-5'), 6.56 (1H, *s*, H-3), 6.46 (1H, *d*, *J*=2.0 Hz, H-8), 6.23 (1H, *d*, *J*=2.0 Hz , H-6). This compound which was identified as 3',4', 5,7-tetrahydroxyflavone (luteolin) [35] was isolated from the genus *Thymelaea* for the first time.

Compound 7 :yellow powder; ^{1}H NMR (400 MHz, CD_3OD) : δ(ppm) 7.99 (2H, *d*, *J* = 8.8 Hz, H-2'& H-6'), 7.40 (1H, *d*, *J* = 15.6 Hz, H-7'''), 7.31 (2H, *d*, *J* = 9.2 Hz, H-2'''& H-6'''), 6.80 (2H, *d*, *J* = 8.8 Hz, H-3'& H-5'), 6.80

(2H, *d*, *J* = 9.2 Hz, H-3'''& H-5'''), 6.31(1H, *d*, *J*=2.1 Hz, H-8), 6.14(1H, *d*, *J* = 2.1 Hz, H-6), 6.07 (1H, *d*, *J* = 15.6 Hz, H-8'''), 5.24 (1H, *d*, *J* =7.6Hz, H-1"), 4.30 (1H, *dd*, *J* = 12.6, 2.0 Hz, H-6"a), 4.19 (1H, *dd*, *J* = 12.8, 6.8 Hz, H-6"b), 3.50 (1H, *m*, H-3"), 3.48 (1H, *m*, H-2"), 3.47 (1H, *m*, H-5"), 3.35 (1H, *m*, H-4''), ^{13}C

NMR (100 MHz, CD_3OD): δ(ppm) 179.44 (C, C-4), 168.78 (C, C-9'''), 166.02 (C, C-9), 162.98 (C, C-5), 161.52 (C, C-4'), 161.18 (C, C-4'''),159.35 (C,C-7), 158.44 (C,C-2), 146.58 (CH, C- 7'''), 135.19 (C, C-3),132.19 (CH, C-2'& C-6'),131.17 (CH, C-2'''& C-6'''), 127.10 (C, C-1'''), 122.74 (C,C-1'), 116.03 (CH, C-3'''& C-5'''), 116.02 (CH, C-3'& C-5'), 114.75 (CH, C- 8'''), 105.59 (C, C-10), 103.66 (CH, C-1''), 100.08 (CH, C-6), 94.83 (CH, C-8),77.80 (CH, C-3''),75.50 (CH, C-2''),75.40 (CH, C-5''), 71.50 (CH, C-4''), 64.29 (CH_2, C-6"); (+) ESI-MS: *m/z* 617.2 $[M+Na]^+$; (-) ESI-MS*: m/z* 593.3 $[M-H]^-$.

These mass spectra led to the formula $C_{30}H_{26}O_{13}$ for this molecule. The combination of the results of all the spectroscopic analysis led to trans-tiliroside [36]. This compound was isolated from *T. microphylla* for the first time.

Compound 8 :yellow powder; ^{1}H NMR (400 MHz, CD_3OD) : δ(ppm) 7.96 (2H, *d*, *J* = 8.8 Hz, H-2'& H-6'), 7.51 (2H, *d*, *J* = 8.4 Hz, H-2'''& H-6'''), 6.80 (2H, *d*, *J* = 8.8 Hz, H-3'& H-5'), 6.70 (1H, *d*, *J* = 12.4 Hz, H-7'''), 6.68 (2H, *d*, *J* = 8.4 Hz, H-3'''& H-5'''), 6.19 (1H, *d*, *J* =2.1 Hz, H-8), 6.14 (1H, *d*, *J* = 2.1 Hz, H-6),5.50 (1H, *d*, *J* = 12.4 Hz, H-8''') 5.19 (1H, *d*, *J* =7.2 Hz , H-1"), 4.30 (1H, *dd*, *J* = 12.6, 2.0 Hz, H-6"a), 4.19 (1H, *dd*, *J* = 12.6, *J* = 6.8 Hz, H-6"b), 3.50 (1H, *m*, H-3"),3.48 (1H, *m*, H-2"), 3.47 (1H, *m*, H-5"),3.35 (1H, *m*, H-4''). This compound which was isolated as a mixture (3%) with the compound **7**, was characterized as cis-tiliroside [37]. This molecule was detected in the family Thymelaeaceae for the first time.

CONCLUSION

As a result of this investigation, from the aerial parts of *T. microphylla*, several compounds have been isolated and identified. These results were in accordance with those reported for *Thymelaea* species. Among the isolated compounds, we obtained two flavonoid aglycones, two acylated flavonoid glucosides, a bis-coumarin, a dihydroxylated monoterpene and a lignan. However, it is important to note that three of the isolated compounds (**4**, **5** and **8**) are new for Thymelaeaceae family, three (**1**, **2** and **6)** are new for the genus *Thymelaea* and two (**3** and **7**) are new for the studied species.

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