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Full Length Research Paper

Potential antioxidant phenolic metabolites from doum palm leaves

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The antioxidant activity of the aqueous ethanolic extract of doum leaves, *Hyphaene thebaica* L. (Palmae) was studied. Data obtained showed that the extract can inhibit reactive oxygen species attack on salicylic acid (IC_{50} = 1602 µg/ml) in a dose dependant manner using xanthine/hypoxanthine oxidase assay. Four major flavonoidal compounds were identified by LC/SEI as; Quercetin glucoside, Kaempferol rhamnoglucoside and Dimethyoxyquercetin rhamnoglucoside. While, further in-depth phytochemical investigation of this extract lead to the isolation and identification of fourteen compounds; their structures were elucidated based upon the interpretation of their spectral data (UV, ^{1}H, ^{13}C NMR and ESI/MS) as; 8-*C*- -D-glucopyranosyl-5, 7, 4`-trihydroxyflavone (vitexin) 1, 6-*C*- -D-glucopyranosyl-5, 7, 4`-trihydroxyflavone (iso-vitexin) 2, quercetin 3-*O*- - $^{4}C1$-D-glucopyranoside 3, gallic acid 4, quercetin 7-*O*- -$^{4}C_{1}$-D-glucoside 5, luteolin 7-*O*- -$^{4}C_{1}$-D-glucoside 6, tricin 5 *O*- -$^{4}C_{1}$-D-glucoside 7, 7, 3` dimethoxy quercetin 3-*O*-[6''-*O*- -*L*-rhamnopyranosyl]- -*D*-gluco-pyranoside (Rhamnazin 3-O-rutinoside) 8 kaempferol-3-*O*-[6''-*O*- -L-rhamnopyranosyl]- -D-glucopyranoside (nicotiflorin) 9, apigenin 10, luteolin 11, tricin 12, quercetin 13 and kaempferol 14.

Key words: Doum leaves, *Hyphaene thebaica* (Palmae), phenolics, hypoxanthine/xanthine oxidase assay.

INTRODUCTION

Doum palm, *Hyphaene thebaica* L. (Palmae), is growing wild throughout the dry regions of tropical Africa, the Middle East and Western India (Fanshawe et al., 1966 and Ledin, 1962). Roots of doum were used in treatment of Bilharziasis, while the resin of the tree has demonstrated, diuretic, diaphoretic properties and also recommended for tap worm as well as against animal bites (Boulos, 1983). The fruits of doum showed antimicrobial and antihypertensive activities, these activities were attributed to the presence of flavonoids [Sharaf et al., 1972; Irobi et al., 1999; Elegami et al., 2001).

Also, the aqueous extract of doum fruits showed an antioxidant activity; this is due to the substantial amount of their water-soluble phenolic contents (Cook et al., 1998). Five flavone glycosides were isolated and identified from doum fruits viz, luteolin 7-*O*- -glucuronoide, apigenin 7-*O*- -glucuronoide, luteolin *O*- -glycoside, luteolin 7-*O*-rutinoside and chrysoeriol 7-O-rutinoside (Amany, 1994). Several fatty acids were identified and isolated from the seeds of doum viz; caprylic, capric, lauric, myristic, palmitic, stearic, oleic and linoleic (Foschini et al., 1968), while oleic was found to constitute the major fatty acid contents in the edible part of doum (Amany, 1994). GC analysis of the sterol fraction on OV-17 column resulted in separation and identification of 6 sterols, of which beta-sitosterol, stigmasterol and campesterol were the major (Gaydou, 1980). Trace constituents were isolated from doum kernel as p, p' nitrophenylazobenzoyl derivatives and identified as estrone (Amin et al., 1973). The kernels were also found to contain crude protein and lipids (Maymone et al., 1950; Bonde et al., 1990).Although doum fruits were known to Ancient Egypt, considered sacred and the palm pictured on the tombs in different situations, nothing could be traced in literature concerning the biological activity or chemical composition of doum leaves. Therefore, the present study is the first one to deal with the biological and chemical composition of doum palm leaves.

EXPERIMENTAL

Reagents and materials

Hypoxanthine, xanthine oxidase and EDTA were obtained from Me-

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rk (Darmstadt, Germany); NH4SO4 and Phosphate buffer were obtained from Serva (Heidelberg, Germany); salicylic acid and $FeCl_3.6H_2O$ were obtained from Aldrich Chemie (Steinheim, Germany). Sephadex LH-20: Phatrmacia fine chemicals, Paper chromatography was carried out on sheets of unwashed Whatman No. 1 paper (Whatman Ltd. Maidstone, Kent, England), spotted with the material under investigation and then eluted by the respective developing systems; H_2O, HOAc 6%: Acetic : water (6 : 94), BAW: *n*-Butanol: acetic acid : water (4 : 1 : 5, top layer). For preparative paper chromatography, Whatman No. 3 MM paper was also used.

Preparation and extraction protocol

Leaves of doum *H. thebaica* (Palmae) were collected from Orman garden, Giza, Egypt (2004). It was authenticated by Prof. Dr Abdel Salam El Noyehy, Prof. of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimens were deposited at the herbarium of Pharmacognosy department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The plants were dried in shade and reduced to a fine powder. The dried leaves of doum 5.0 Kg were extracted by 70% ethanol on cold till exhaustion. The solvent was distilled of in rotary evaporator at 55°C till dryness. The extract was concentrated till constant weight in vacuum desiccators over anhydrous calcium chloride.

Hypoxanthine/xanthine oxidase assay

The antioxidant test of the aqueous ethanolic extract of doum leaves was assessed according to the method of Owen et al. (2000). The aqueous-ethanolic extract of doum leaves was tested in the range of 0 - 2000 µg/ml. The relevant concentration range in methanol was added to 15.0 ml plastic tubes in duplicates and the solvent was removed under a stream of nitrogen. The dried residue was suspended in phosphate buffer 1.0 ml, containing EDTA 500 µM, 300 µM hypoxanthine, $FeCl_3.6H_2O$ (50 µM with respect to elemental iron), salicylic acid 2 mM and 5.0 µl of a 1:5 dilution of xanthine oxidase in ammonium sulphate 3.20 mol/L was added to initiate the reaction. The tubes were incubated for 3 h until the completion of the reaction at 37°C. After incubation, 20 µl of the reaction mixture was analyzed by HPLC using the mobile phase and condition described under HPLC. The exact amount of diphenols 2, 3 DHBA and 2, 5 DHBA produced by OH radical (HO•) attack on salicylic acid is determined from standard curve of respective diphenols.

Analytical high performance liquid chromatography (HPLC)

HPLC analysis was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a C - 18, reversed-phase 5 µl column 25 cm x 4 mm I.D.; Latex, Eppelheim, Germany); UV detector was set at 325 nm for the detection of 2, 5-dihydroxybenzoic acid and 2, 3-dihydroxybenzoic acid produced by reactive oxygen species (ROS) attack on salicylic acid. Liquid chromatography electrospray-ionisation mass spectrometry (LC-ESI).

LC-ESI was conducted on an Agilent 1100 HPLC coupled to an Agilent LC/MSD (HP 1101) . Chromatographic separation of all samples was conducted using a C - 18, reversed phase 5 µm column (25 cm x 2 mm I.D. Latex, Eppelheim, Germany) using mobile pha-se consisting of 2% acetic acid in doubly distilled water (solvent A) and methanol (solvent B) and gradient with a flow rate of 0.5 ml / min. The analyses were conducted in the negative-ion mode under the following conditions: drying gas (nitrogen) flow = 101/min; nebulizer pressure = 30 psi, drying gas temperature = 350°C, capillary voltage = 2500 V; fragmentor voltage = 100 V; mass range 50 - 3000 D.

Isolation and purification of doum phenolics

Fresh leaves of doum 5 Kg were exhaustively extracted with aqueous alcohol ethanol 75% and 15 L. The extract was dried in vacuum at low temperature till dryness 220 g. 2-DPC of the extract revealed the presence of nine major components (several dark purple spots on paper chromatograms under UV light, which turned yellow when fumed with ammonia vapors and one intense blue spot) were detected. The extract 120 g was applied on Sephadex LH-20 column, using H_2O and H_2O / MeOH mixtures of decreasing polarities as solvent system. Five fractions (I – V) were eluted individually and then subjected to 2-DPC. Compounds (1, 63 mg; 2, 75 mg; 3, 56 mg and 4, 88 mg) were separated from fraction I by fractionation over polyamide column using MeOH/H_2O (decreasing polarity) for elution then preparative paper chromatography to the sub fractions using HOAc: H_2O (6%). Compounds 5, 16 mg; 6, 28 mg were isolated as pure compounds from fraction II by column made of Sephadex LH-20 and *n*-BuOH saturated with H_2O as developing system. Application of fraction III on Sephadex LH-20 column using *n*-BuOH saturated with H_2O for elution then preparative paper chromatography yielded 3 compounds (7, 28 mg; 8, 13.9 mg; 9, 16.9 mg) . Compounds (10, 7.0 mg; 11, 8.2 mg; 12, 9.6 mg; 13, 9.5 mg and 14, 10.1 mg) were isolated from fraction IV by fractionation on sphadex LH-20 column using *n*-BuOH saturated with H_2O for elution then preparative paper chromatography.

Ultraviolet spectrophotometric analysis

Chromatographically pure materials 1 mg each were dissolved in analytically pure methanol then subjected to UV spectroscopic investigation in 4 ml capacity quartz cells 1 cm thick using a Carl Zeiss spectrophotometer PMQ II. $AlCl_3$, $AlCl_3$/HCl, fused NaOAc / H_3BO_3 and NaOMe reagents were separately added to the methanolic solution of investigated material and UV measurements were then carried out.

Nuclear magnetic resonance spectroscopic analysis

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^1H- spectra run at 300 MHz and ^{13}C- spectra were run at 75.46 MHz in deutrated dimethylsulphoxide (DMSO- d_6). Che-mical shifts are quoted in and were related to that of the solvents. The mass spectra were recorded on a Shimadzu GCMS-QP-1000 EX mass spectrometer at 70 eV.

RESULTS

Antioxidant activity

The leaf extract inhibited the hydroxylation of salicylic acid by reactive oxygen species (ROS) in a dose-dependent manner (IC_{50} = 1602 µg/ml). The reduction of total oxidation products as a function of the volume of the extract added to the assay is shown in Figure 1.

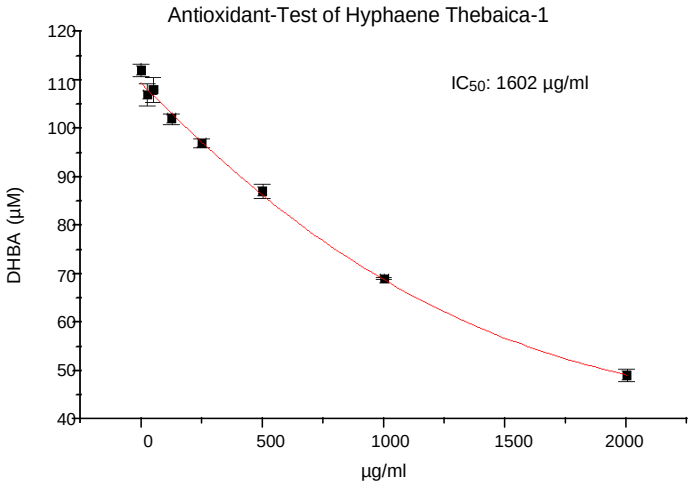

Figure 1. Inhibitory effect of the aqueous ethanolic extract of doum leaves on the production of dihydroxybenzoic acids (DHBA) from salicylic acid in the hypoxanthine / xanthine oxidase assay.

Table 1. Phenolic contents (mg/kg) of doum leaves.

Compound	Mg/kg
Gallic acid	25130
Quercetin glucoside	4721
Kaempferol rhamnoglucoside	10684
Dimethyoxyquercetin rhamnoglucoside	17461

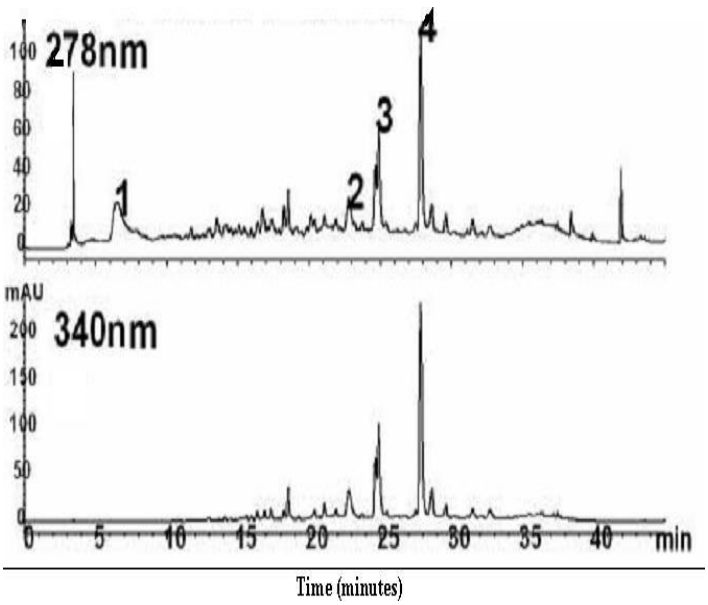

Figure 2. Analytical HPLC chromatogram monitored by UV absorption $_{278}$ and $_{340}$ for the aqueous alcoholic extract of doum leaves.1: Gallic acid, 2: Quercetin glucoside, 3: Kaempferol rhamnoglucoside, 4: Dimethyoxyquercetin rhamnoglucoside.

Profile of the phenolic compounds

LC-ESI identification as described in Table 1 and Figure 2 of the aqueous ethanolic extract of doum leaves, revealed the presence of four major components of which peaks 1-4 correspond to Gallic acid, Quercetin glucoside, Kaempferol, rhamnoglucoside, Dimethyoxyquercetin rhamnoglucoside respectively.

Identification of compounds 1 - 14

An in-depth phytochemical investigation of the aqueous ethanolic extract of doum leaves using column fractionation on Sephadex LH 20 and paper chromatography resulted in the isolation of 14 compounds: 8- *C*- -D-glucopyranosyl-5, 7, 4`-trihydroxyflavone (vitexin) 1 (Harborne et al., 1988; Numata et al., 1980; Agrawal, 1989). 6-*C*- -D-glucopyranosyl-5, 7, 4`-trihydroxyflavone (iso-vitexin) 2 (Harborne et al., 1988; Numata et al., 1980; Agrawal, 1989).

Quercetin 3-*O*- -$^{4}C_{1}$-D-glucopyranoside 3 (Eldahshan, 2002) , gallic acid 4 (Krenn et al., 2003) quercetin 7-*O*- -$^{4}C_{1}$-D-glucoside 5 (Jean et al., 2002) luteolin 7-*O*- -$^{4}C_{1}$-D-glucoside 6 (Singab, 1996), tricin 5-*O*- -$^{4}C_{1}$-D-glucoside 7 (Francis et al., 2000), 7, 3` dimethoxy quercetin 3-*O*-[6"-*O* - -L-rhamnopyranosyl]- -D-glucopyranoside (rhamnazin 3-O-rutinoside) 8 (Harput et al., 2004), kaempferol-3-*O*-[6"-*O*- -L-rhamnopyranosyl]- -D -glucopyranoside (nicotiflorin) 9 (Kazuma et al., 2003; Hunber et al., 1999; Agrawal et al., 1992) apigenin 10, luteolin 11, tricin 12 , quercetin 13 and kaempferol 14.

The structures of these compounds were unambiguously determined by their chromatographic behaviors as well as spectroscopic analysis via UV (Table 2), ESI/MS (Table 3), ^{1}H-NMR (Table 4) and ^{13}C–NMR (Table 5).

Conclusion

The result of this study showed that the aqueous ethanolic extract of doum leaves appeared to be a potent scavenger of reactive oxygen species. The extract inhibits (HO˙) attack on salicylic acid. The phenolic content of doum extract has been assessed by HPLC/ESI revealed the presence of four major compounds. An in-depth phytochemical investigation showed the presence of fourteen compounds. All of these compounds were isolated and identified for the first time in doum leaves.

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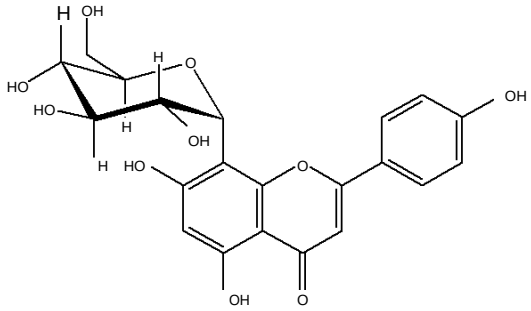

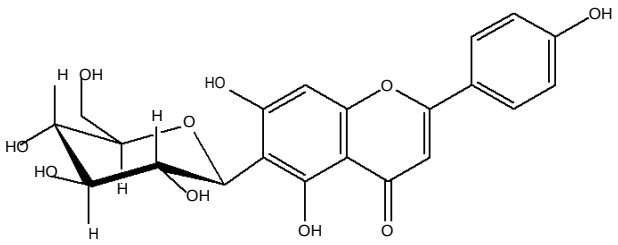

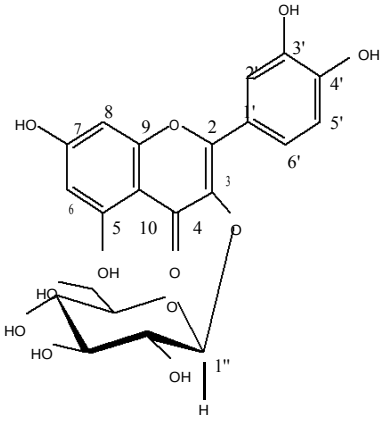

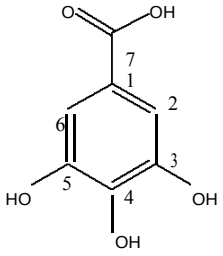

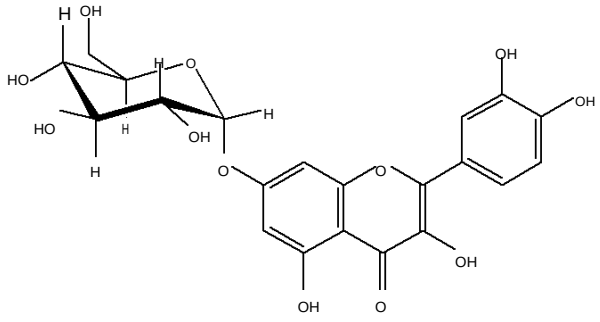

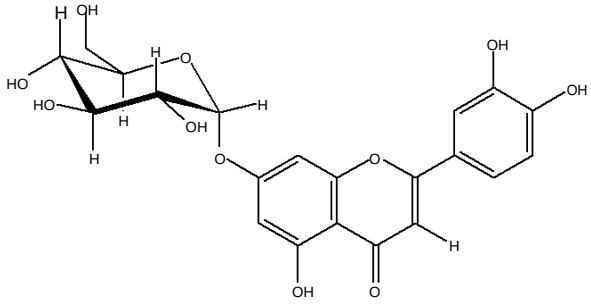

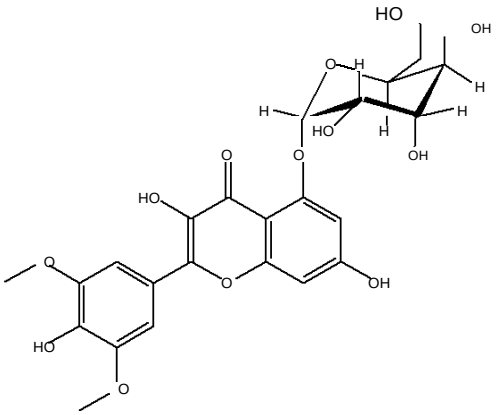

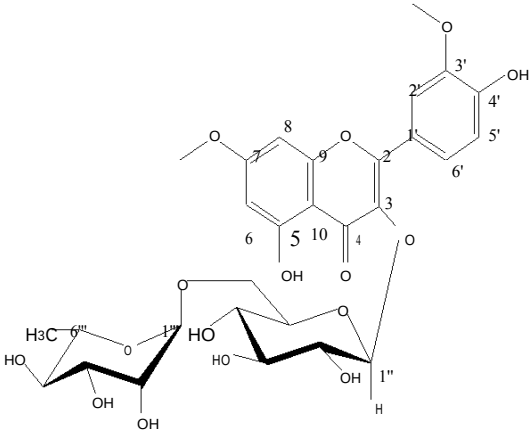

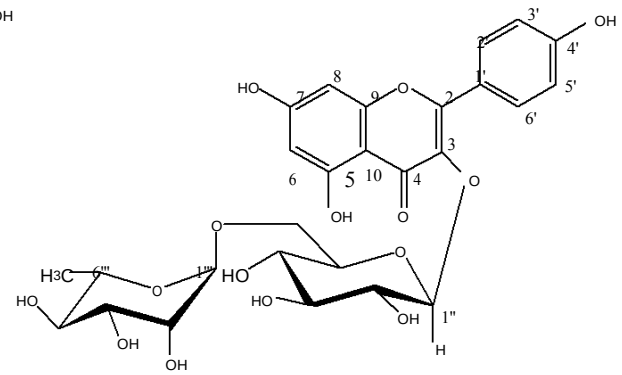

Table 2. UV-Spectral data for the phenolics of doum leaves.

	UV data; λ_{max}(nm)				
	MeOH(a)	**(a)+NaOAc:(b)**	**(b)+H_3BO_3**	**(a)+$AlCl_3$(c)**	**(a)+NaOMe**
Vitexin	334, 272	391, 305sh, 281	400sh, 339, 278	385, 340, 305, 278	391, 332sh, 281
Iso-vitexin	272, 332	281, 305sh, 391	278, 335, 400sh	278, 305, 340, 385	281, 333sh, 399
Isoquercetrin	258, 267*-56	256, 374-362	265*, 272-380, 420	263, 430	275, 470
Gallic acid	272				
Quercetin 7-*O*- -$^4C_1$-D-glucoside	255, 372	286, 378, 28(sh)	261, 289(sh), 386	259(sh), 273, 339, 58	241(sh), 291, 367, 457
Luteolin 7-*O*- -$^4C_1$-D-glucoside	255, 267*-346	259, 265*-360, 398	260,370	272, 300*-330, 430	264, 300-398
Tricin 5 *O*- -$^4C_1$-D-glucoside	244, 269, 299sh, 350	262, 275sh, 320, 412	270, 302sh,348, 420sh	245, 270sh, 300, 355	253, 272sh, 395
Rhamnazin 3-O-rutinoside	257, 358	259, 365	256,364	292, 370	262, 413
Nicotiflorin	267, 353	273, 355	271,355	272, 408	275, 310,402

Table 3. ESI / MS data for the phenolics of doum leaves.

Compound	**Vitexin**	**Iso-vitexin**	**Isoquercetrin**	**Quercetin7-*O*--$^4C_1$-D-ucoside**	**Luteolin7-*O*- -$^4C_1$-D-glucoside**	**Tricin 5 *O*- -$^4C_1$-D-glucoside**	**Gallic acid**	**nicotiflorin**	**Rhamnazin 3-O-rutinoside**
m/z [M-1]	431.37	431.37	461.37	463.37	447.37	507.42	169.11	593.51	637.57

Table 4. ^{1}H NMR data for the phenolics of doum leaves.

Pos.	**Vitexin**	**Iso-vitexin**	**Isoquercetrin**	**Quercetin 7-*O*- -$^4C_1$-D-glucoside**	**Luteolin 7-*O*- -$^4C_1$-D-glucoside**	**Tricin 5 *O*- $^4C_1$-D-glucoside**	**Rhamnazin 3-O-rutinoside**	**Nicotiflorin**
3	6.77, s	6.47, s				6.83 s		
6	6.21, s		6.2, d, *J* =2.5	6.44, d, *J* = 2.0	6.2, d, *J* = 2.5	6.13 d, *J* = 2.1	6.57,d, *J* = 1.8	6.17, s
8		4.7, d, *J*= 8	6.45, d, *J* =2.5	6.74, d, *J* = 2.0	6.45, d, *J* = 2.5	6.39 d, *J* = 2.1	6.64,d, *J* = 1.8	6.37, s
2`	7.93, d, *J* = 8	7.93, d, *J* = 8	7.57, m	7.74, d, *J* = 2.0	7.57, m	6.89 s		7.53, d, *J* = 7.5
3`	6.92, d, *J* = 8	6.92, d, *J* = 8						6.83, *d*, *J* = 7.5
5`	6.92, d, *J* = 8	6.92, d, *J* = 8	6.84, d, *J* =8	6.88, d, *J* = 7.6	6.84, d, *J* = 8.0		8.42,d, *J* = 1.8	6.83, *d*, *J* = 7.5
6`	7.93, d, *J* = 8	7.93, d, *J* = 8	7.55, m	7.65, dd, *J* = 7.6, 2.0	7.55, m	6.89 s	7.43,d, *J* = 8.5	7.53, d, *J* = 7.5
						3.73 s, H-3`, 5` of OMe	7.96,dd, *J* = 8.5 ,1.8,7-Ome	
							3.73, s, 3`-OMe	
				5.05, d, *J* = 7.2			6.31, d, *J* = 7.3	
1``	4.63, d, *J* = 8	4.7, d, *J* = 8.0	5.4, d, *J* =8.o		5.4, d, *J* = 8.0	5.36 d, *J* = 7.3	5.35, br s, H-1```(rhamnose)	5.31, *d*, *J* = 7.2
1```								4.39, *d*, *J* = 8.0
	3.1 - 3.9, m, Other sugar protons	3.00 - 3.90, m, Other sugar protons					1.48d, J = 6.1, H-6'''[CH_3]	1.16, *d*, *J* = 6.6 H-6'''[-CH_3]

Table 5. ^{13}C- NMR data for the phenolics of doum leaves.

	Vitexin	Iso-vitexin	Isoquercetrin	Gallic acid	Quercetin 7-*O*- -$^{4}C_{1}$-D-glucoside	Luteolin 7-*O*- -$^{4}C_{1}$-D-glucoside	Tricin 5 *O*- -$^{4}C_{1}$-D-glucoside	Rhamnazin 3-O-rutinoside	Nicotiflorin
1				120.6					
2	163.9	163.5	157.24	108.8	147.9	164.5	162.4	158.1	156.74
3	102.4	102.8	133.0	145.5	135.9	103.20	106.3	135.1	134.3
4	182.0	181.9	177.40	138.1	175.9	181.6	177.0	178.8	177.16
5	161.0	161.2	161.30	145.5	160.3	161.10	158.3	162.3	161.1
6	98.1	108.8	99.71	108.8	98.9	99.70	104.3	98.6	98.63
7	162.5	163.2	163.08	167.7	162.7	162.90	161.0	165.9	164.2
8	104.6	93.7	94.94		94.5	94.90	98.5	92.6	93.65
9	155.9	156.2	156.30		155.7	156.90	158.5	157.4	159.8
10	104.0	103.4	102.03		104.6	105.5	108.1	106.3	103.84
1`	121.5	121.1	120.03		121.9	121.60	120.4	121.9	120.76
2`	128.8	128.4	115.30		115.5	113.70	104.4	114.3	115.0
3`	115.7	116.0	142.0		145.0	145.9	148.1	149.5	130.77
4`	160.3	160.6	149.0		147.9	149.6	139.4	150.3	160.0
5`	115.7	116.0	77.50		115.4	116.1	148.1	116.4	130.77
6`	128.8	128.4	122.20		120.1	119.0	104.4	123.7	115.0
1``	73.3	73.1	100.23		100.3	100.4	104.0	103.8	101.2
2``	70.8	70.6	73.42		73.2	73.30	73.6	76.0	74.05
3``	78.8	78.9	76.77		76.5	76.60	75.6	77.5	76.23
4``	70.5	70.3	69.92		69.9	70.80	69.6	71.5	69.8
5``	81.7	81.4	116.32		77.2	77.30	77.5	78.5	75.61
6``	61.3	61.4	60.95		60.9	61.0	60.8	68.3	66.77
1```								102.6	100.65
2```								72.5	70.22
3```								72.6	70.47
4```								73.9	71.7
5```								69.7	68.13
6`````								18.5	17.6
							56.3, (C-3`, 5`-*O*-Me)	55.9(7-OMe) 56.1(3`-OMe)	

of Toxicology and Cancer Risk Factor, German Cancer Research Center (DKFZ), Heidelberg, Germany, for hosting the antioxidant activity and LC-ESI measurement.

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