Pharmacogn. Mag.
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ORIGINAL ARTICLE

Bioassay-guided isolation and identification of antioxidant flavonoids from *Cyclotrichium origanifolium* (Labill.) Manden and Scheng

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Submitted: 07-06-2016 Revised: 22-07-2016 Published: 18-04-2017

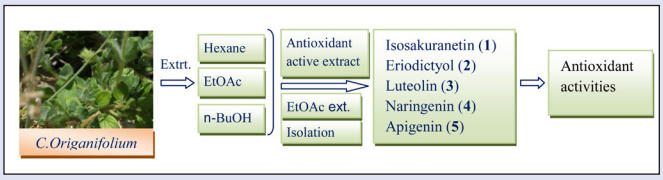
ABSTRACT

Background: Medicinal and aromatic plants play a significant role in drug discovery and development process. Flavonoids, revealing a wide spectrum of biological activities, extensively found in plants are important secondary metabolites. **Materials and Methods:** Aerial parts of *Cyclotrichium origanifolium* were collected, dried, and boiled in water then extracted with hexane, ethyl acetate, and *n*-butanol. Total phenolic content, DPPH• scavenging activity, reducing power (FRAP) activity, and ABTS•+ scavenging activity assays were applied for all extracts. The ethyl acetate extract revealing the most antioxidant activity as well as including the highest phenolic contents was subjected to chromatographic techniques (column chromatography, sephadex LH-20, semipreparative HPLC) to isolate the active compounds. The structure of isolated compounds were elucidated by spectroscopic methods (1D NMR, 2D NMR, and LC-TOF/MS). **Results:** Isosakuranetin (1), eriodictyol (2), luteolin (3), naringenin (4), and apigenin (5) were isolated and identified. All isolated flavonoids displayed the excellent antioxidant activity. **Conclusion:** The isolated flavonoids and also plant extract have potency to be a natural antioxidant.

Key words: antioxidant activity, *Cyclotrichium origanifolium*,, flavonoids, isolation, spectroscopy

SUMMARY

- Five flavonoids were isolated from *Cyclotrichium origanifolium*
- Isolated compounds revealed the good antioxidant activity
- *C. origanifolium* has a potency to be used in food industries



Abbreviations used: DPPH•: 1,1-diphenyl-2-picryl-hydrazyl free radical, ABTS•+: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), UV:Ultraviolet, DNA: Deoxyribonucleic acid, BHT: Butylated hydroxytoluene, BHA: Butylated hydroxyanisole, HPLC: High performance liquid chromatography

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DOI: 10.4103/0973-1296.204556

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INTRODUCTION

The *Cyclotichium* genus belonging to the Lamiaceae family is represented in Turkish flora by five species two of which are endemic. These species are *C. niveum*, *C. origanifolium*, *C. glabrescens*, *C. leucotrichum*, *C. stamineum* of which *C. niveum* and *C. origanifolium* are endemic.[1] *Cyclotrichium* species have been used for flavoring agent in food as well as herbal tea. *Cyclotichium* has been traditionally used as sedative, relaxant, carminative, and treatment of respiratory disorders.[2] Phytochemical investigation on *C. niveum* resulted in the isolation of isosakuranetin 7-*O*-rhamnoside, eriodictyol 7-*O*-glucoside, apigenin, apigenin 7-methyl ester, acacetin 7-*O*-rutinoside and triterpenoids vergatic acid, oleanolic acid, β-amyrin, and sitosterol.[3] Phytochemical studies including essential oils on *Cyclotichium* species were reported.[4-7]

Reactive oxygen species (ROS) including free radical such as superoxide anion ($O_2^{\bullet-}$), hydroxyl (OH•), peroxyl (ROO•), and nitric oxide (NO•) radicals are produced through oxidative process in eukaryotic cells. The human body maintains many protection mechanisms against oxidative stress, embracing antioxidant enzymes, and secondary metabolites. Exposure of environmental pollutant (cigarette smoke, pesticides, smog, UV radiation), the secondary metabolites production of mammalian become insufficient and then the excess of free radicals can damage to the cell membrane in chain reaction leading to degenerative diseases like Alzheimer disease, cardiovascular disease, ageing, cataracts, arteriosclerosis, diabetes mellitus, and DNA damage leading to carcinogenesis.[8] Recently, researches have focused on finding natural antioxidants for use in foods, cosmetics, and medicine to replace synthetic antioxidants due to their carcinogenicity.[9] The antioxidant phytochemicals particularly phenolic compounds exist in fruits, vegetables, and medicinal plants have gained the significant attention for having the potency to prevent the human diseases.[10]

Flavonoids, revealing significant and beneficial medicinal effects on human health, are one of the most common polyphenols. The extensive biological and pharmaceutical properties of flavonoids, such as antiinflammatory, antibacterial, immune-stimulating, antiviral, antioxidant, and anticancer, have been reported.[10-12] They are mostly found in fruits, vegetables, teas, and cocoa; therefore, an important quantity is consumed in our daily diet. It is generally accepted that

Cite this article as: Guzel A, Aksit H, Elmastas M, Erenler R. Bioassay-guided isolation and identification of antioxidant flavonoids from *Cyclotrichium origanifolium* (Labill.) manden and scheng. Phcog Mag 2017;13:316-20.

Table 1: Antioxidant assays of *C. origanifolium* extracts

Extracts	Total phenolic contents[a]	DPPH• scavenging activity[b]	$ABTS^{•+}$ Cation radical scavenging[b]	Reducing power[c]
Hexane	28.1±2.0	120.5±4.4	31.5±2.9	0.7±0.0
EtOAc	125.4±6	11.8±0.7	7.5±0.0	3.4±0.1
Butanol	76.9±4.0	19.4±0.1	19.4±0.9	2.4±0.0
Water	13.1±2.4	85.5±3.4	34.9±1.3	0.3±0.0
Trolox	-	3.5±0.2	9.5±0.8	-
BHA	-	3.3±0.2	12.5±0.3	-
BHT	-	8.5±1.0	9.0±0.5	-

[a]g gallic acid equivanet phenolic compounds/kg extract [b]IC_{50} values of of extracts and standards (µg /mL) [c]Mmol trolox equivalent activity/kg extract

flavonoids, as well as other polyphenols, play a significant role in protecting plants from both insect and mammalian herbivory.[13]

Although essential oil researches have been conducted on *C. origanifolium*,[14,15] no work has been reported on this species regarding the bioassay-guided isolation and identification of secondary metabolites and investigation of antioxidant activities. Herein, bioassay-guided isolation and identification of isosakuranetin (1), eriodictyol (2), luteolin (3), naringenin (4), and apigenin (5) were achieved and their antioxidant activities were presented.

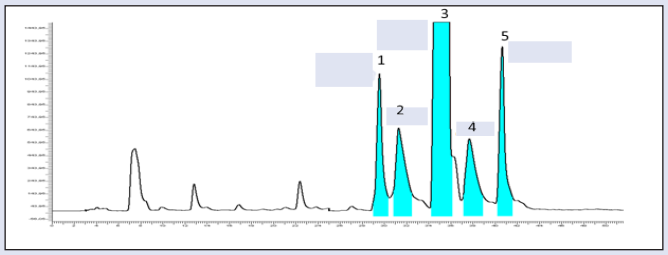

Figure 1: HPLC chromatogram of EtOAc extract

MATERIALS AND METHODS

General procedure

^{1}H and ^{13}C NMR analyses were executed on Bruker 400 MHz and 100 MHz spectrometer, respectively. UV measurements were executed on a Shimadzu UV-260 UV-Vis spectrometer. LC-TOF/MS analysis was performed on Agilent 6210 LC-TOF/MS. Silica gel 60 (70-230 mesh) and 60 F_{254} aluminum sheet 20 cm × 20 cm were used for column chromatography and thin-layer chromatography, respectively, purchased from E. Merck (Darmstadt, Germany). BHT, BHA, trolox, ammonium thiocyanate, α-tocopherol, polyoxyethylene sorbitan monolaurate (Tween-20), ferrous chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH·),3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (Ferrozine) were used for antioxidant assays and supplied from chemical company Sigma-Aldrich (Darmstadt, Germany).

Plant material

The plant material was collected from Hatay, Samandag, Turkey. After identification of plant by Prof. Dr. Ahmet Ilcim, Department of Biology, Mustafa Kemal University, a voucher specimen was deposited in the Herbarium of Biology Department (MKUH 1208).

Extraction and isolation

Aerial part of the plant materials were powdered by liquid nitrogen and then were boiled in water, afterwards extracted with hexane, ethyl acetate, and *n*-butanol successively. A portion of water solution was lyophilized to yield the water extract, besides the hexane extract, ethyl acetate extract, and *n*-butanol extract. Antioxidant activity tests were performed for these extract of which the EtOAc extract exhibited the most antioxidant activities [Table 1]. Therefore, chromatographic techniques were applied for EtOAc extract to isolate the bioactive compounds. The HPLC chromatogram of ethyl acetate extract showed that it consisted of five major products [Figure 1]. After Sephadex LH-20 (200 g) was kept in MeOH for 24 h, it was filled into the glass column (50 cm × 100 cm). EtOAc extract (4 g) was dissolved in MeOH (10 mL), then it was subjected to column chromatography with sephadex LH-20 as an adsorbent, eluted by MeOH with the flow rate as 0.1 mL/min. One hundred fractions (10 mL each) was collected. The fractions including the same compounds were combined and named as 0-30 (fatty acids), S1 (frs 31-51), S2 (frs 52-55), S3 (frs 56-60), S4 (frs 61-65), and S5 (frs 66-100). Semipreparative HPLC analysis was applied for S2 (frs 52-55) including the most secondary metabolites to isolate the bioactive flavonoids. Twenty milligram sample (S2) was dissolved in DMSO (1.0 mL) to obtain stock solution to be analysis. Two hundred microliter of this solution was injected to HPLC column, repeated 20 times to attain enough compounds for spectroscopic analysis (NMR) and antioxidant assays. Isosakuranetin (1), eriodictyol (2), luteolin (3), naringenin (4), and apigenin (5) were isolated, respectively.

DPPH· scavenging activity

DPPH free radical scavenging effect was executed according to the reported method.[16] To a DPPH solution (0.135 mM) in ethanol (1.0 mL) was added the samples solution at different concentrations (40, 80, and 160 µg/mL). The ethanol was added for final solution to be 4.0 mL. After the strongly mixing, the reaction mixture was incubated at room temperature in dark for 30 min. The absorbance measurement was executed at 517 nm on a spectrophotometer. Lower absorbance of the mixture revealed the higher free radical scavenging activity. The following equation was used to evaluate the scavenging ability of DPPH•.

DPPH• scavenging effect (%) = $[(A_c-A_s)/A_c] \times 100$

in which Ac is the control absorbance and As is the samples absorbance.

ABTS radical cation scavenging effect

The treatment of ABTS (2.0 mmol/L) in water with potassium persulfate (2.45 mmol/L) at room temperature in dark for 4 h yielded the ABTS cation radical. $ABTS^{•+}$ was diluted with sodium phosphate buffer (0.1 mol/L, pH 7.4) to measure the absorbance at 734 nm. The reactions of $ABTS^{•+}$ solution (1.0 mL) with samples solution in ethanol (3.0 mL) at different concentrations (5-40 µg/mL) were executed. The inhibition was calculated at 734 nm for each concentration. The scavenging activity of $ABTS^{•+}$ was calculated by the given equation:

$ABTS^{•+}$ scavenging effect (%) = $[(A_c-A_s)/A_c] \times 100$

in which A_c is the initial concentration of $ABTS^{•+}$ and A_s is the remaining concentration in the sample.[17]

Ferric ions (Fe3+) reducing antioxidant power assay (FRAP)

The reactions of extracts and compounds at different concentrations (5–40 µg/mL) in distilled water (1.0 mL) with sodium phosphate

Table 2: ^{1}H NMR spectral data of isolated compounds (400 MHz, DMSO-d6)

Position	Isosakuranetin	Eriodictyol	Luteolin	Naringenin	Apigenin
2	5.51 dd.(2.8 Hz, 12.6 Hz)	5.28 dd (3.1 Hz, 12.6 Hz)	-	5.30 dd (2.9 Hz, 13.0 Hz)	
3	3.28 dd (12.6 Hz, 17.0 Hz)	3.07 dd (12.6 Hz, 17.4 Hz)	6.52	3.08 dd (13.0 Hz, 17.1 Hz)	6.78 (s)
	2.71 dd (2,9 Hz, 17.0 Hz)	2.71dd (3.1 Hz, 17.4 Hz)		2.67 dd (2.9 Hz, 17.1 Hz)	
4	-	-	-	-	-
5	-	-	-	-	-
6	5.88 brs	5.86 d (2.0 Hz)	6.19 d (1.9 Hz)	5.90 d (2.2 Hz)	6.19 d (1.8 Hz)
7	-	-	-	-	-
8	5.89 brs	5.89 d (2.0 Hz)	6.42 d (1.9 Hz)	5.89 d (2.2 Hz)	6.49d (1.8 Hz)
9	-	-	-	-	-
10	-	-	-	-	-
1'	-	-	-	-	-
2'	7.45 d (8.6 Hz)	6.82 brs	7.35 brs	7.30 dd (1.5 Hz, 8.0 Hz)	7.92 d (8.8 Hz)
3'	6.98 Hz (8.6 Hz)	-	-	6.83 dd (1.5 Hz, 8.0 Hz)	6.93 d (8.8 Hz)
4'	-	-	-	-	-
5'	6.98 Hz (8.6 Hz)	6.94brs	6.92 d (8.04 Hz)	6.83 dd (1.5 Hz, 8.0 Hz)	6.93 d (8.8 Hz)
6'	7.45 d (8.6 Hz)	6.80 brs	7.37 d (8.04 Hz)	7.30 dd(1.5 Hz, 8.0 Hz)	7.96 d (8.8 Hz)
4'-OMe	3.77 s	-	-	-	-

Table 3: ^{13}C NMR spectral data of isolated compounds (100 MHz, DMSO *d6*)

Position	Isosakuranetin	Eriodictyol	Luteolin	Naringenin	Apigenin
2	78.6	78.9	165.1	79,0	164.6
3	42.5	43.4	102.3	42.5	103.2
4	196.4	195.9	182.3	196.2	182.2
5	163.3	163.4	161.6	164.1	161.8
6	96.4	96.1	98.9	95.8	99.3
7	163.9	164.1	164.8	163.4	164.2
8	95.6	95.3	93.8	94.9	94.4
9	167,9	168.7	157.9	167.3	157.7
10	102.0	101.5	103.7	101.8	104.1
1'	131.2	130.5	122.2	129.7	121.6
2'	128.7	113.3	112.8	127.6	128.9
3'	114.3	145.1	145.6	114.9	116.4
4'	159.9	145.4	149.6	157,5	161.6
5'	114.	118.8	115.4	114.9	116.4
6'	128.7	117.8	118.9	127.6	128.9
4'-OMe	55.7	-	-	-	-

buffer (2.5 mL, 0.2 M, pH 6.7) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%) were performed. The reaction mixture was incubated at 50°C for 20 min. Trichoroacetic acid (2.5 mL, 10%) was added to the mixture. Then distillated water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) were added to the 2.5 mL of this solution. Absorbance was measured at 700 nm in a spectrophotometer.[18] Absorbance increase of the mixture revealed an increase of reduction capability.

Determination of total phenolic compounds

Total soluble phenolic compounds in extracts were determined with Folin-Ciocalteu reagent using gallic acid as a standard.[19] Briefly, 1.0 mL sample solution in methanol (1.0 mg/mL) was mixed with distilled water (45 mL) in a volumetric flask and 1.0 mL of Folin-Ciocalteu reagent was added to the reaction mixture. Sodium carbonate (3 mL, 2%) was added after 5 min later, and then the mixture was kept for 2 h with shaking. The absorbance measurement was executed at 760 nm in a spectrophotometer. The concentrations of total phenolic compounds in the sample were determined as micrograms of gallic acid equivalent.

HPLC-TOF/MS Analysis

LC-TOF/MS (Agilent 6210) with Poroshell column (120 EC-C18, 3.0 mm × 50 mm, 2.7 µm) was used. The injection volume was applied as 10 µL. Eluent A-water with 0.1% formic acid and 5 mM ammonium formate and B-acetonitrile was used as a mobile phase. The gradient program was firmed as 0-1 min, 10% B, 1-10 min, 10% B, 10-13 min, 85% B, 13-15.2 min, 20% B, 15.2-16 min, 10% B. Total evaluation time was 16 min. TOF analysis was performed in negative ion mode and gas temperature was 325°C. Drying gas flow was 0.7 ml/min and fragmentation voltage was 175 V.

RESULTS AND DISCUSSION

Ethyl acetate extract revealing the most antioxidant effect as well containing high phenolic contents was subjected to chromatographic techniques to isolate isosakuranetin (1), eriodictyol (2), luteolin (3), naringenin (4), and apigenin (5) [Figure 2]. 1H NMR and 13C NMR spectral data of isolated compounds were given in Table 2 and Table 3, respectively. Isosakuranetin (1) is the first compound isolated from ethyl acetate extract by sephadex LH-20 column chromatography as well as semipreperative HPLC. Its molecular formula was determined as $C_{16}H_{13}O_5$ by LC-TOF/MS (*m/z* 285.0763 $[M-H]^-$) (calcd. 285.0741). 13C spectrum revealing the presence of one methyl, one methylene, seven methines, six quaternary carbons, and one carbonyl carbon accorded with the structure. In 1H NMR spectrum, the signal observed at δ 5.51 as doublet of doublet with *vicinal* coupling (J = 2.8 Hz, 12.6 Hz, H2), at δ 3.28 as doublet of doublet with *germinal and vicinal* coupling (J = 12.6 Hz, 17.0 Hz, H3α) and at δ 2.71 as doublet of doublet with *germinal* and *vicinal* coupling (J = 2.9 Hz, 17.0 Hz, H3β), at δ 5.88 (brs, H6), δ 5.89 (brs, H8) revealed the proposed structure. The observation of the cross

Table 4: Antioxidant effects of isolated compounds from *C. origanifolium*

Compounds	DPPH· scavenging activity[a]	ABTS·+ scavenging activity[a]	Reducing power
isosakuranetin	12.3 ± 0.3	13.3 ± 0.1	0.14 ± 0.01
eriodictyol	9.9 ± 0.1	9.2 ± 0.1	0.59 ± 0.01
luteolin	5.7 ± 0.2	4.4 ± 0.1	0.62 ± 0.01
naringenin	14.4 ± 0.3	8.8 ± 0.2	0.10 ± 0.01
apigenin	6.6 ± 0.2	5.3 ± 0.2	0.47 ± 0.02
Trolox	4.4 ± 0.2	15.3 ± 0.2	0.68 ± 0.02
BHA	6.2 ± 0.1	11.6 ± 0.1	1.11 ± 0.03
BHT	8.2 ± 0.1	12.1 ± 0.1	0.72 ± 0.03

[a]IC_{50} values of of compounds and standards (μg /mL)

peaks in HMBC spectrum from H2 to C-1', C-2', and C-6' revealed the attachment of phenyl group at C-2 position.[20] Eriodictyol (2), second compound isolated by chromatographic techniques, is a well-known bioactive compound existing in many medicinal plants. Its molecular formula was established as $C_{15}H_{11}O_6$ by LC-TOF/MS (*m/z* 287.0555 [M-H]-) (calcd. 287.0556). The 13C NMR spectrum along with DEPT experiments exhibited one methylene, six methines, seven quaternary carbons, and one carbonyl carbon. In the ^{1}H NMR spectrum, the signals appeared at δ 3.07 (dd, 12.6 Hz, 17.4 Hz) and δ 2.71 (dd, 3.1 Hz, 17.4 Hz) belonged to the diastereotopic protons bonded at C-3. The spectral data in the literature were also supported the proposed structure.[21] Another compound isolated as white solid was determined to be liteolin (3), $C_{15}H_9O_6$, representing 11 degrees of unsaturation. LC-TOF/MS analysis indicated the molecular ionic peak at *m/z* 285.0394 $[M-H]^-$ (calcd. 285.0399). The ^{13}C NMR spectrum as well as DEPT experiments revealing six methines, eight quaternary carbons, and one carbonyl carbon accorded with the structure. The comparison of spectral data with the literature confirmed the structure.[21] Narigenin (4), a well-known flavonoid possessing a broad spectrum of biological effects, was isolated as a fourth compound. Its molecular formula was determined as $C_{15}H_{11}O_5$ by LC-TOF/MS (*m/z* 271.0612 $[M-H]^-$) (calcd. 271.0606). The observation of one methylene, seven methines, six quaternary carbons, and one carbonyl carbon in 13C NMR spectrum suited with the structure. The characteristic signals appeared at δ 5.30 (dd, 2.9 Hz, 13.0 Hz, H2), δ 3.08 (dd, 13.0 Hz, 17.1 Hz, H3α), δ 2.67 (dd, 2.9 Hz, 17.1 Hz, H3β), δ 5.90 d (d, 2.2 Hz, H6), and δ 5.89 d (d, 2.2 Hz, H8) fitted with the structure.[22] The last compound isolated from *C. origanifolium* was apigenin (5). The comparison of spectral values with those reported in the literature confirmed the proposed structure.[23] Due to the bearing acidic protons, the isolated compounds revealed the excellent antioxidant activities in all assays [Table 4]. These flavonoids have capable of scavenging free radicals by donating of electrons or protons to the radicals and also have the ability of inhibition lipid peroxidation [Table 2]. The carbonyl group at C-4 and a double bond between C-2 and C-3 are also important features for high antioxidant activity in flavonoids.[24]

CONCLUSION

Due to the consisting of bioactive flavonoids of *C. origanifolium*, it has a potency to be used in drug discovery and development process as well as in food industry as natural antioxidant.

Financial support and sponsorship

This work was supported by Gaziosmanpasa University, Scientific Research Council (BAP, 2014-21).

Conflicts of interest

There are no conflicts of interest

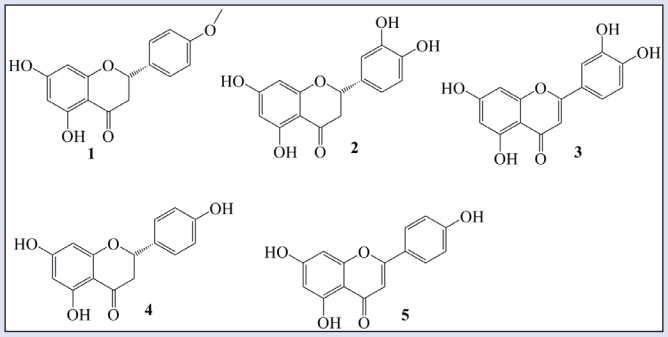

Figure 2: Isolated compounds from *C. origanifolium*

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