Turkish J. Pharm. Sci. 4 (1) 1-14, 2007

ANTIOXIDANT ACTIVITY OF TWO FLAVONOL GLYCOSIDES FROM *CIRSIUM HYPOLEUCUM* DC. THROUGH BIOASSAY-GUIDED FRACTIONATION

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Abstract

The flowers, roots and stems of Cirsium species are used as a folk remedy in Anatolia. In order to evaluate antioxidant activity of Cirsium hypoleucum which is an endemic plant, superoxide radical (hypoxanthine-xanthine oxidase system) and free radical scavenging activities (DPPH) using in vitro electron spin resonance (ESR) spectrometry of the different extracts, fractions and compounds obtained by Bioassay Guided Fractionation (BAGF) technique were studied. The bioassay guided fractionation of methanol extract led to the isolation flavonoid 1 and 2 from n–butanol fr. The structures of these compounds were elucidated using UV, IR, MS, ^{1}H- and ^{13}C-NMR techniques as quercetin-3-O-rutinoside (rutin) (1) and kaempferol-3-O-rutinoside (nicotiflorin) (2). Flavonoid 1 showed more potent antioxidant activity than flavonoid 2.

Key Words: *Cirsium hypoleucum DC., Asteraceae (Compositae),; Electron Spin Resonance (ESR), Free radical scavenging activity, Superoxide radical scavenging activity*

Biyolojik Aktiviteyle Yönlendirilen Fraksiyonlama ile *Cirsium hypoleucum* DC.'dan Elde Edilen İki Flavonol Glikozitin Antioksidan Aktivitesi

Cirsium türlerine ait çiçekler, dallar ve kökler Anadolu'da halk ilacı olarak kullanılmaktadır. Endemik bir bitki olan Cirsium hypoleucum'un antioksidan aktivitesini değerlendirmek için, biyolojik aktivite ile yönlendirilen fraksiyonlama (BAYF) tekniği ile elde edilen ekstre, fraksiyon ve bileşiklerin süperoksit radikali (hipoksantin-ksantin oksidaz sistemi) ve serbest radikal süpürücü aktiviteleri (DPPH) in vitro elektron spin rezonans (ESR) tekniği ile incelenmiştir. Metanol ekstresinin biyolojik aktivite yönlendirmeli fraksiyonlanması neticesinde n-butanol fraksiyonundan flavonoit 1 ve 2 izole edilmiştir. Bu bileşiklerin yapıları UV, IR, MS, ^{1}H- ve ^{13}C-NMR teknikleri kullanılarak kersetin-3-O-rutinozit (rutin) (1) ve kemferol-3-O-rutinozit (nikotiflorin) (2) olarak tayin edilmiştir. Rutin, nikotiflorine göre daha güçlü bir antioksidan etki göstermiştir.

Anahtar Kelimeler: *Cirsium hypoleucum DC., Asteraceae (Compositae), Elektron Spin Rezonans (ESR), Serbest radikal süpürücü aktivite, Süperoksit radikal süpürücü aktivite*

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INTRODUCTION

The *Cirsium* species, known locally as "köy göçerten, çarık kesen, hamur kesen, su dikeni, körkenger, kazan kulpu, kangaldikeni, çakır dikeni, and eşek dikeni", are perennial herbs of the family Asteraceae (Compositae) (1-4). This genus is represented by 59 species in Flora of Turkey (5). In folk medicine, the decoction of the seeds and roots is used for healing hemorrhoids and the flowers are good remedy for peptic ulcer (1, 2). In addition, the stem is also used as treating ailment against cough and bronchitis in Anatolia (6). In *Cirsium* species, presence of flavonoids (luteolin-7-glucoside, -7-glucuronide, cirsiliol, hispudilin, cirsitakaoiside, cirsitakaogenin etc…), triterpenes, acyclic diterpenes (α-tocopherol, *trans*-phytol), polyolefins, fatty acids (linoleic, linolenic, palmitic, and stearic acids) and some widespread acetylenes have been reported (7-10).

Bohlmann and Abraham isolated an aplotaxane epoxide derivative (11, 12-epoxy-heptadeca-1, 8, 14-triene) from the roots of plant which, to the best of our knowledge, is the only report concerning the chemical content of *C. hypoleucum* (11).

In a previous work of our group, antimicrobial activities of various *C. hypoleucum* extracts (methanol, n-hexane, chloroform, ethyl acetate, n-butanol and remaining water extracts) were tested against various micro-organisms including *Herpes simplex* (HSV) and *Parainfluenza viruses* (PIV). The results showed that the remaining water fraction of the plant had a significant activity against DNA virus (HSV), same as the reference standard acyclovir (12).

In living systems, $O_2^{\cdot-}$ is synthesized by enzymatic (NADPH oxidase, or xanthine oxidase) and nonenzymatic (leak of electron from mitochondria) manners. Its overproduction would cause several diseases such as hypertension, edema, platelet aggregation, aging, inflammation, shock, stroke, gastrointestinal ulcer, pancreatitis, hepatitis, adult respiratory deficiency syndrome, epilepsy, dermatitis and sunburn (13, 14). Defence mechanisms against reactive oxygen species include radical scavenging enzymes and cellular antioxidants. Superoxide dismutase (SOD) is considered to play a very important role in protecting living cells against these reactive oxygen species. The enzyme catalyzes the dismutation of two superoxide radicals (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (15-16). In this study, we explain $O_2^{\cdot-}$ scavenging activities of extract, fractions and pure compounds with SOD-like activity. Moreover, we used the DPPH radical which is a very stable free radical used extensively in electron spin resonance studies (17), for the evaluation of the radical scavenging activity of *C. hypoleucum*. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability and this radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule (18).

Certain phytochemicals such as flavonoids, anthocyanins, catechins, carotenoids etc. in plants help to protect cells against oxidative damage caused by free radicals (19). The antioxidant activity of plants used for treating many ailments in traditional medicine, e.g. cough, bronchitis, ulcer and hepatitis, should therefore be assessed by using different antioxidant assays (20, 21).

The present study deals with the evaluation of superoxide radical (hypoxanthine-xanthine oxidase system) and free radical scavenging activities (DPPH assay) of the extracts, fractions and flavonoids obtained through bioassay-guided fractionation from *C.hypoleucum* by using Electron Spin Resonance (ESR) spin trapping method.

EXPERIMENTAL

General Experimental Procedures

IR spectra were determined on a Bruker Vector 22 IR (30 spectroscopic software version 2.0) spectrophotometer. UV spectra were recorded on a Beckman DU 650 spectrophotometer, using spectroscopic grade MeOH (Merck). NMR spectra were acquired on a Bruker ARX-400 instrument. ^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz, using TMS as internal Standard and MS were obtained on a JEOL JMSD-300 instrument. Chromatography column: Silica gel 60N (0.040-0.063 μm, Merck), Sephadex LH-20 (Pharmacia Fine Chemicals), ODS-A column (YMC Co. Ltd., 120-S75, Lot No. EBO7465). Precoated Kieselgel 60F_{254} (Merck, 1.13895.0001) prepared plates for TLC. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and xanthine oxidase (XO) were purchased from Sigma Chemical Co. (St. Louis, MO), hypoxanthine, diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Wako Pure Chemical Industry (Osaka, Japan). Methanol, n-hexane, chloroform, ethyl acetate and n-BuOH were purchased from Kanto Chemical Co. ESR spectrometer (JES-TE 300, JEOL Co Ltd., Tokyo, Japan) operated at X-band (9 GHz). Typical instrument conditions were: 5 mW microwave power, 1.0 gauss modulation amplitude, 0.1 s time constant, 60 s sweep time, and 100 Gauss scan range. Specta were stored on an IBM/PC computer with software (ESPRIT 432; JEOL Co Ltd., Tokyo, Japan) for analysis. Hyperfine coupling constants and spectral simulations were obtained with a computer program, Winsim (22). Acquisition parameters were; microwave power 5mW, frequency 9.42 GHz, sweep width 100 Gauss, time constant 30 ms, sampling time 60 s, gain 400.

Plant material

Cirsium hypoleucum DC. was collected from Ilgaz Mountain, Çankırı (June 1997). Identification of the voucher specimen was done by Prof.Dr.Mecit Vural (Department of Botany, Faculty of Science, Gazi University). Herbarium specimen were stored in the Herbarium of Gazi University, Faculty of Pharmacy (GUE 2291).

Extraction Method

The aerial parts of the plant (CH) (1200 g) were extracted at room temperature with methanol [MeOH] and combined extracts were evaporated to dryness under reduced pressure to obtain "MeOH extract" (158.18 g). The remaining plant material was then extracted with distilled water under same conditions and lyophilized to give "H_2O extract" (47 g). MeOH extract was then dissolved in 90% MeOH/distilled water and extracted with n-hexane. After removal of MeOH from the remaining MeOH extract diluted with distilled water and extracted successively with chloroform [$CHCl_3$], ethyl acetate [EtOAc] and n-butanol [BuOH] saturated with water (10 times each of 500 ml solvent). Each of combined fractions as well as remaining water fraction [R-H_2O] was evaporated to dryness under reduced pressure to give n-Hexane Fr. (yield 29.9 g), $CHCl_3$ Fr. (yield 15.6 g), EtOAc Fr. (yield 2.7 g), BuOH Fr. (yield 16 g) and R-H_2O Fr. (yield 78 g), successively (see Scheme 1 and 2).

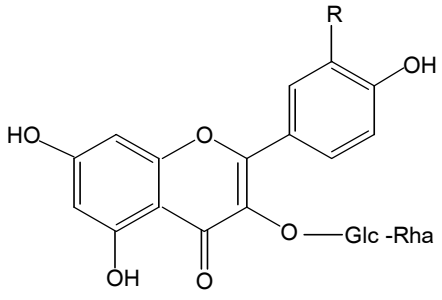
$CHCl_3$ fraction was subjected to a silica gel column and eluted with $CHCl_3$: MeOH (90:10→10:90) → MeOH, and the fractions were combined into eight main fractions according to TLC control.

n-BuOH fraction was applied to a silica gel column and eluted with $CHCl_3$:MeOH:H_2O (90:30:1, 80:20:2, 70:30:3, 50:50:5 and 30:70:7)→ MeOH, and the fractions were combined into ten main fractions according to TLC control (solvent system: $CHCl_3$:MeOH:H_2O 61:32:7). Spots were visualized by spraying 5% H_2SO_4 in EtOH after heating at 100°C. Fr. (23-44) and Fr. (45-54) showed very close activity and were further combined and chromatographed on a

Sephadex LH-20 column using MeOH as eluent. Combined Fr. (25-40) eluted from the Sephadex column were rechromatographed on a ODS-A column using solvent systems MeOH:H_2O gradient (45-95 % MeOH). And then Frs. (18-23) and (24-26) eluated from ODS-A column were chromatographed over silica gel column using $CHCl_3$:CH_3OH:H_2O (61:32:7) and $CHCl_3$:CH_3OH: H_2O (70:30:3) solvent systems to give **1** (25.1 mg) and **2** (15.1 mg).

Quercetin-3-O-rutinoside (Rutin) (1). $C_{27}H_{30}O_{16}$, M+ at *m/z* 611. UV (MeOH, λ_{max}, nm): 359; 257, Rf 0.57 (TLC $CHCl_3$:CH_3OH:H_2O, 61:32:7), IR (1% KBr): 3345 (OH stretching), 1658 (conjugated C=O stretching), 1535 (C=C stretching), 1090, 1045 (C-O-C). ^{1}H-NMR (400 MHz, CD_3OD) 6.08 (1H, brd, H-6), 6.25 (1H, brd, H-8), 7.61 (1H, brd, H-2'), 6.82 (1H, d, J= 8.6 Hz, H-5'), 7.59 (1H, d, J=8.6 Hz H-6'), 4.99 (1H, d, J=7.3 Hz, H-1'', anomeric proton of glucose), 4.47 (1H, brd, H-1''', anomeric proton of rhamnose), 1.09 (3H, d, J=6.0 Hz, CH_3), 3.25-3.81 (signal patterns of sugars unclear due to overlapping). ^{13}C-NMR (100 MHz, DMSO-d_6) 156.79 (C-2), 133.61 (C-3), 177.43 (C-4), 161.52 (C-5), 99.45 (C-6), 164.45 (C-7), 94.20 (C-8), 156.91 (C-9), 103.80 (C-10), 121.30 (C-1'), 116.43 (C-2'), 145.26 (C-3'), 149.85 (C-4'), 115.60 (C-5'), 122.02 (C-6'), 101.88 (C-1'', anomeric carbon of glucose), 74.49 (C-2''), 76.87 (C-3''), 70.95 (C-4''), 76.30 (C-5''), 67.40 (C-6''), 101.15 (C-1''', anomeric carbon of rhamnose), 70.40 (C-2'''), 70.75 (C-3'''), 72.26 (C-4'''), 68.62 (C-5'''), 18.13 (C-6''') (23-27).

Kaempferol-3-O-rutinoside (Nicotiflorin) (2). $C_{27}H_{30}O_{15}$, M+ at *m/z* 595. UV (MeOH, $\lambda_{max,}$ nm): 349.2; 266. Rf 0.66 (TLC $CHCl_3$:CH_3OH:H_2O, 61:32:7), IR (1% KBr): 3380 (OH stretching), 1650 (conjugated C=O stretching), 1555 (C=C stretching), 1085, 1057 (C-O-C). ^{1}H-NMR (400 MHz, CD_3OD) 6.18 (1H, d, J=2.2 Hz, H-6), 6.37 (1H, d, J=2.2 Hz, H-8), 8.05 (1H, d, J=9.0 Hz, H-2'), 6.87 (2H, d, J=9.0 Hz, H-3', H-5'), 8.05 (1H, d, J=9.0 Hz H-6'), 5.11 (1H, d, J=7.8 Hz, anomeric proton of glucose), 4.48 (1H, brd, anomeric proton of rhamnose), 1.10 (3H, d, J=6.1 Hz,), 3.24-3.80 (signal patterns of sugars unclear due to overlapping). ^{13}C-NMR (100 MHz, pyridine) 156.56 (C-2), C-3 overlapped, 177.11 (C-4), 161.18 (C-5), 98.33 (C-6), 164.27 (C-7), 93.16 (C-8), 156.20 (C-9), 103.71 (C-10), 120.38 (C-1'), 130.48 (C-2'), 114.61 (C-3'), 160.19 (C-4'), 114.61 (C-5'), 130.48 (C-6'), 103.02 (Anomeric carbon of glucose), 75.92 (C-2''), 74.51 (C-3''), 69.86 (C-4''), 77.07 (C-5''), 66.97 (C-6''), 101.07 (Anomeric carbon of rhamnose), 70.62 (C-2'''), 71.06 (C-3'''), 72.40 (C-4'''), 68.10 (C-5'''), 17.04 (C-6''') (23-27).



Rutin: R= OH, Nicotiflorin: R= H

Figure 1. The structures of rutin and nicotiflorin

Antioxidant Activity:

Antioxidant activity was measured using ESR in two manners:.

i. Radical Scavenging Activity using 1,1-Diphenyl-2-picrylhydrazyl (DPPH)

To determine the DPPH radical scavenging activity of each test sample, 1 mg of extract, fraction or compound was vigorously mixed with 1 ml of 2.5 mM DPPH in ethanol. 3 minutes later, the reaction mixture was to transferred to the ESR quartz flat cell which was, in turn, placed in the cavity of the ESR spectrometer (17). The DPPH radical was generated in ethanol solution and considered as a control. The DPPH radical scavenging activity was estimated as a ratio of individual DPPH signal peak height to that of control (Figure 2).

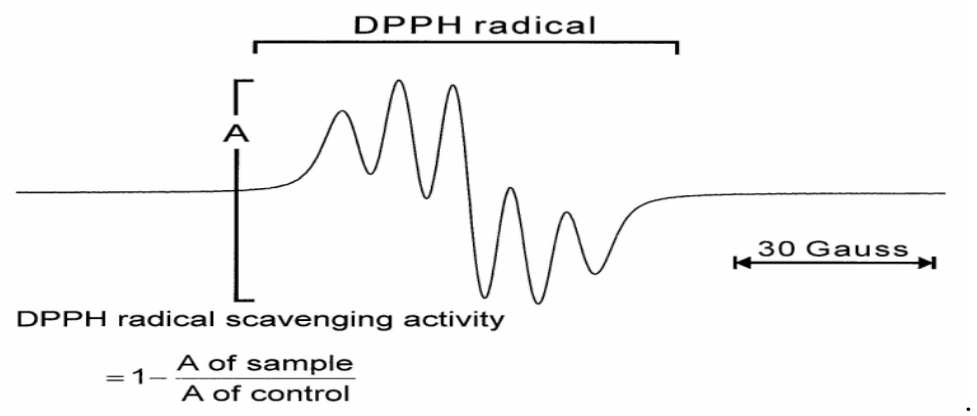


Figure 2. Typical ESR spectrum of DPPH radical

The DPPH radical scavenging activity of each sample was calculated by comparison of relative peak height for control (sample free) DPPH solution. DPPH radical reducing activity of each test sample was expressed as the percentage of DPPH residue (28).

ii. Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity of the test samples was measured by using ESR method according to Miyagawa et al. and Yoshikawa et al. (29, 30). In this experiment, activity was expressed as unit/g of SOD (SOD-like activity) by ESR method, as follows: 100 µl sample solution (4 µg sample/ml), 50 µl hypoxanthine (2 mM, diluted with buffer), 30 µl diethylenetriamine pentaacetic acid (DETAPAC) (5.5 mM, diluted with buffer) and 10 µl 5,5-dimethyl-1-pyroline-N-oxide (3 M, diluted in distilled water) were mixed in a test tube. The $O_2^{\cdot-}$ production was induced by the addition of 50 µl xanthine oxidase (0.272 Unit/ml, diluted with buffer, Grade I from butter milk) into the reaction mixture. One minute after the addition of XOD, aliquot of the solution was introduced into the ESR quartz flat cell and then put it into the ESR cavity. 0.1 M phosphate buffer (pH 7.4), which was used to prepare the sample solutions, was used as blank solution (control) and assayed simultaneously (Figure 3). The calibration curve for superoxide radical scavenging activity was obtained by using the native SOD (from erythrocyte) solution in various concentrations.

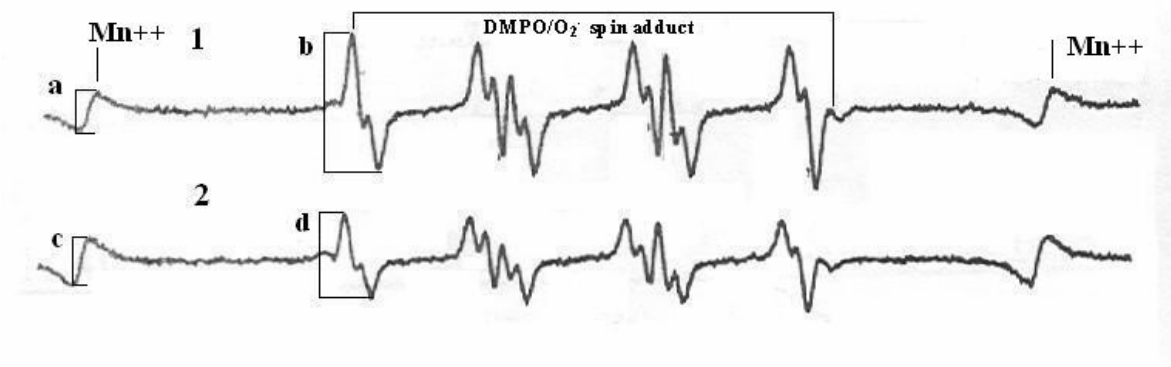


Figure 3. ESR spectra obtained **(1)** with 0.1 M phosphate buffer (pH 7.4) as control, **(2)** with test sample

The parameter for relative peak height (comparison between peak height of the first ESR signal of DMPO $O_2^{\cdot-}$ spin adduct and that of Mn^{2+}) was utilized to make a calibration curve, and the result was expressed in a signal (S) to noise (N) ratio, calculated by dividing the averaged signal intensity of the control (b/a) by dividing the averaged signal intensity of test sample (d/c) using the following equation :

SOD-like activity = (b/a) / (d/c)

where *a* is the noise height and *b*, the signal height of the control in cm and *c*, the noise height and *d*, the signal height of the test sample in cm.

RESULTS AND DISCUSSION

In DPPH test, the methanol extract (30 %) was found to be more prominent than water extract (97 %). On the other hand, according to the superoxide radical scavenging activity test results, MeOH extract (24017.8 Unit/g) was considered to be the more effective than water extract (7101.1 Unit/g). Therefore, MeOH extract wa s subjected to successive solvent

Table 1. Antioxidant Activity of *C.hypoleucum* extracts and fractions

Test Samples	**DPPH Residue (%)**	**SOD-like activity (Unit/g)**
Control	100	0.0
MeOH Extract	30	24018
Water Extract	97	7101
n-Hexane Fr.	60	330
$CHCl_3$ Fr.	14	34329
EtOAc Fr.	18	92201
n-BuOH Fr.	18	62804
R-H_2O Fr.	40	4453

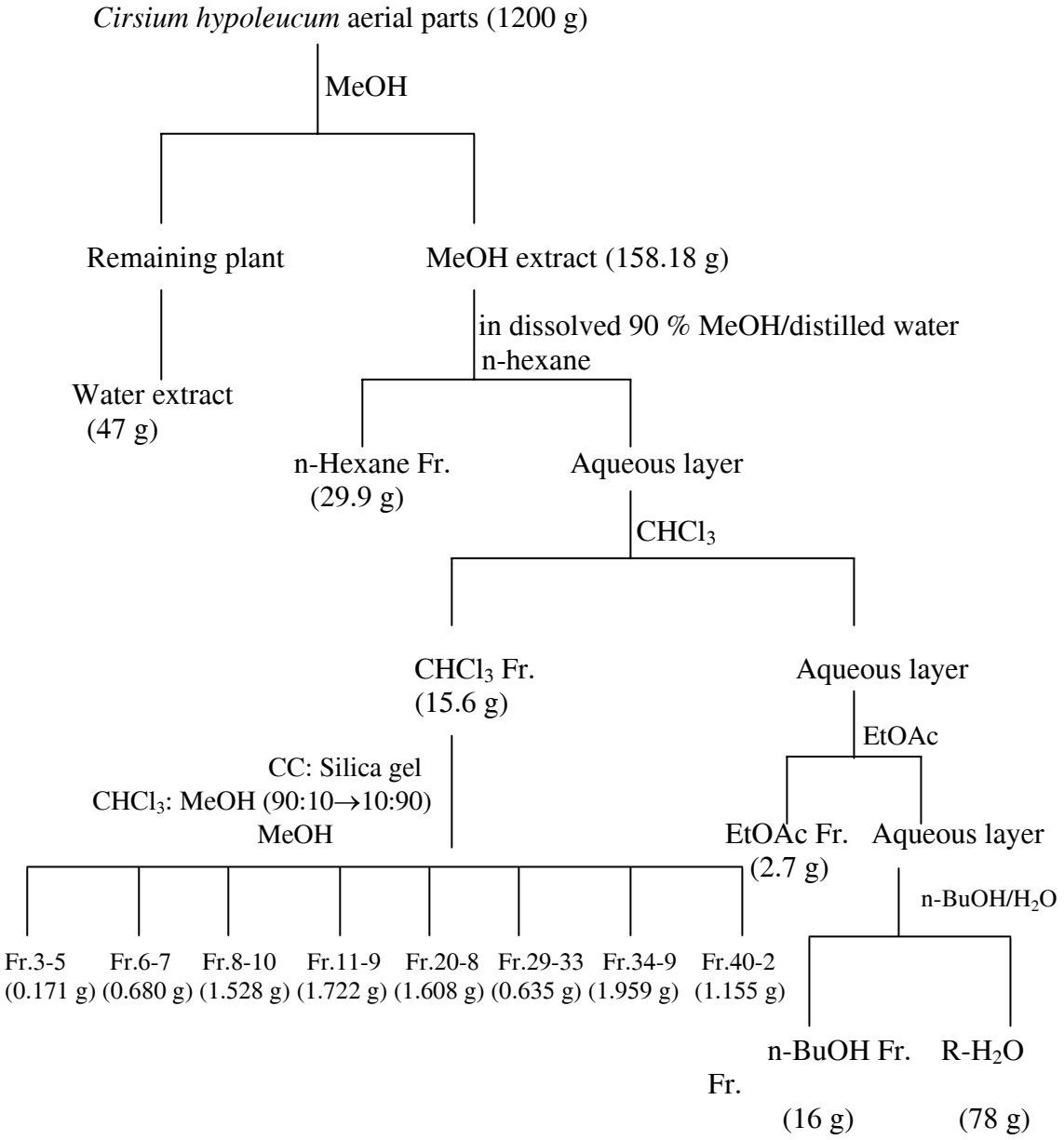


Figure 4. Extraction and fractionation of *Cirsium hypoleucum* aerial parts by solvent extraction and the subfractions of $CHCl_3$ fraction

extractions according to general bioassay-guided fractionation protocol and five fractions were obtained; Hexane Fr., $CHCl_3$ Fr., EtOAc Fr., n-BuOH Fr. and R-H_2O Fr. (31). Among these, n-hexane fr. consisting of liposoluble components of the plant hardly showed both free radical and superoxide radical scavenging activities whereas $CHCl_3$, EtOAc and n-BuOH frs. showed remarkable antioxidant activity, which could be a subject for further work. However, since the

amount of EtOAc fr. was not enough for further phytochemical separations, the studies were conducted on n-BuOH and $CHCl_3$ frs. (Table 1).

n-BuOH and $CHCl_3$ frs. were chromatographed first over silica gel column in order to obtain subfractions with different polarities. The subfractions of $CHCl_3$ fr. showed a very weak antioxidant activity (Figure 4). It is observed that the antioxidant effect of $CHCl_3$ fr. was decreased by the fractionation. Nevertheless, free radical scavenging effect of n-butanolic subfractions (23-44) and (45-54) was determined to be extremely high and showed very close activity. Therefore, the subfractions (23-44) and (45-54) were combined and further studies were conducted on this combined subfractions (Table 2).

Subsequently, the n-butanolic subfraction (23-55) was subjected to chromatographic separation on Sephadex LH-20 column and eluents were combined into eleven fractions. As seen in Figure 5, LH/Frs. (25-31), (32-35), and (36-40) exhibited the highest DPPH and superoxide anion scavenging activity. For this reason, these subfractions were combined and applied to ODS-A column. Frs. (18-23) and (24-26) eluated from ODS-A column showed significant both DPPH radical and superoxide radical scavenging activities (Table 3).

Table 2. Antioxidant activity of n-butanol and chloroform subfractions obtained from silica gel column chromatography

Test Samples	**DPPH Residue (%)**	**SOD-like activity (Unit/g)**
n-BuOH Fraction		
Control	100	0.0
Fr_{3-4}	93	15265
Fr_{5-8}	51	12684
Fr_{9-15}	53	45069
Fr_{16-22}	42	38745
Fr_{23-44}	14	34117
Fr_{45-54}	12	41713
Fr_{55-59}	65	21679
Fr_{60-69}	50	49132
Fr_{70-79}	100	10868
$CHCl_3$ Fraction		
Fr_{3-5}	78	11496
Fr_{6-7}	73	14604
Fr_{8-10}	66	9972
Fr_{11-19}	77	18704
Fr_{20-28}	78	28982
Fr_{29-33}	83	32127
Fr_{34-39}	70	17695
Fr_{40-42}	99	11030

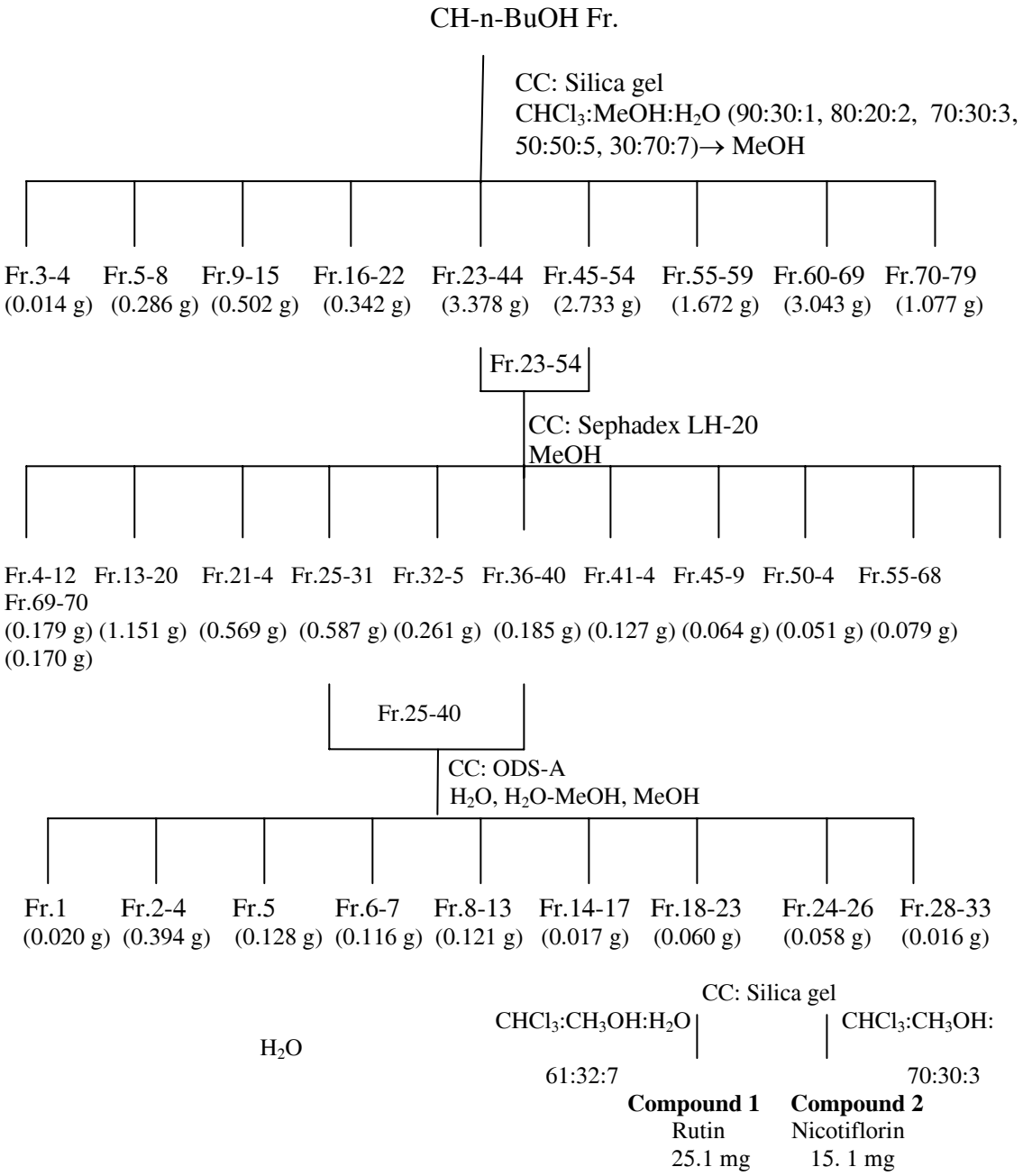


Figure 5. Isolation of compounds 1 and 2 from the n-BuOH fraction of *Cirsium hypoleucum* aerial parts by various chromatographical techniques.

Therefore, ODS-A/Frs. (18-23) and (24-26) were carried out over silica gel column using ($CHCl_3$:CH_3OH:H_2O 61:32:7) and $CHCl_3$:CH_3OH:H_2O (70:30:3) solvent systems, respectively and compound **1** and **2** were isolated from Frs. (18-23) and (24-26), respectively.

Table 3. Antioxidant activity of n-butanol subfractions obtained from Sephadex LH-20 column chromatography (SHFr.$_{23\text{-}54}$-Fr), first and second ODS-A column chromatography (ODSFr.$_{25\text{-}40}$-Fr)

Test Samples	DPPH Residue (%)	SOD-like activity (Unit/g)
SHFr.$_{23\text{-}54}$-Fr$_{4\text{-}12}$	62	10000
SHFr.$_{23\text{-}54}$-Fr$_{13\text{-}20}$	28	55000
SHFr.$_{23\text{-}54}$-Fr $_{21\text{-}24}$	25	69000
SHFr.$_{23\text{-}54}$-Fr $_{25\text{-}31}$	15	67000
SHFr.$_{23\text{-}54}$-Fr $_{32\text{-}35}$	14	72000
SHFr.$_{23\text{-}54}$-Fr $_{36\text{-}40}$	9	72000
SHFr.$_{23\text{-}54}$-Fr $_{41\text{-}44}$	42	50000
SHFr.$_{23\text{-}54}$-Fr $_{45\text{-}49}$	31	32000
SHFr.$_{23\text{-}54}$-Fr $_{50\text{-}54}$	13	40000
SHFr.$_{23\text{-}54}$-Fr $_{55\text{-}68}$	5	48000
SHFr.$_{23\text{-}54}$-Fr$_{69\text{-}70}$	17	43000
ODSFr.$_{25\text{-}40}$-Fr $_{1}$	41	28212
ODSFr.$_{25\text{-}40}$-Fr$_{2\text{-}4}$	52	7403
ODSFr.$_{25\text{-}40}$-Fr$_{5}$	64	9955
ODSFr.$_{25\text{-}40}$-Fr$_{6\text{-}7}$	43	3430
ODSFr.$_{25\text{-}40}$-Fr$_{8\text{-}13}$	48	6646
ODSFr.$_{25\text{-}40}$-Fr$_{14\text{-}17}$	35	8460
ODSFr.$_{25\text{-}40}$-Fr$_{18\text{-}23}$	22	19939
ODSFr.$_{25\text{-}40}$-Fr$_{24\text{-}26}$	32	18416
ODSFr.$_{25\text{-}40}$-Fr$_{28\text{-}33}$	74	9050

Table 4. Antioxidant activity of quercetin-3-*O*-rutinoside **(1)** and kaempferol-3-*O*-rutinoside**(2)** obtained from ODSFr.$_{25\text{-}40}$-Fr$_{18\text{-}23}$ and -Fr$_{24\text{-}26}$ by silica gel column chromatography

Test Samples	DPPH Residue (%)	SOD-like activity (Unit/g)
ODSFr.$_{25\text{-}40}$-Fr$_{18\text{-}23}$ Compound **1**	32	15476
ODSFr.$_{25\text{-}40}$-Fr$_{24\text{-}26}$ Compound **2**	48	11846

The structure of the compounds was elucidated through spectral techniques (UV, IR, ^{1}H-, ^{13}C-NMR and MS) as quercetin-3-O-rutinoside (rutin) **(1)** and kaempferol-3-O-rutinoside (nicotiflorin) **(2)** as well as by comparison of the spectral data with those of the previously reported data (23-27) (Figure 1). Rutin and nicotiflorin were successively tested both in assay systems and rutin showed higher antioxidant activity than nicotiflorin. None of these flavonoids had previously been reported in *C.hypoleucum* (Table 4).

Reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are considered to be important factors in the etiology of several pathological conditions such as cardiovascular diseases, diabetes, inflammation, cancer, ulcer etc. Antioxidants act as a major defence against radical-mediated toxicity by protecting the damages caused by free radicals (32-33). Therefore, antioxidant agents are beneficial in the prevention and treatment of these pathologies.

DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured ethanol solutions. DPPH method is an easy and rapid way to evaluate antioxidants (17).

Superoxide dismutase (SOD) specifically interacts with $O_2^{\cdot-}$ and then immediately decomposes into molecular oxygen and hydrogen peroxide. In this study, superoxide scavenging activity of the test samples was measured by using ESR method. Under the controlled conditions, DMPO in the radical-generating system trapped the superoxide anion radical ($.O_2^-$) generated by the HPX-XOD system to form the spin adducts, DMPO-$O_2^{\cdot-}$. By an addition of test sample, the signal intensity of the spin adduct was decreased (34).

In another study, it was reported that antioxidant activity of flavonoids is governed by the position and number of hydroxyl groups on the B-ring (35). It is known that the most effective radical scavengers are flavonoids with the 3',4'-dihydroxy substitution pattern on the B ring and/or hydroxyl group at the C-3 position. The presence of an *o*-dihydroxy structure on the B-ring confers a higher degree of stability on the flavonoid phenoxyl radicals by participating in electron delocalization and is an important feature for the antiradical potential (36). Indeed, in this study, nicotiflorin was slightly less active than rutine due to the presence of only one hydroxyl group at position 4' instead of *o*-dihydroxy structure on the B-ring. Both molecules contain free hydroxyl groups at C-3 and C-5 which is also important for producing an inhibitory effect on xanthine oxidase activity (37, 38).

In conclusion, this study demonstrated that MeOH extract of *C.hypoleucum* had potent free radical scavenging activities in both examined free radical systems. The significant decrease in antioxidant activity was observed after the fractionation of active n-butanol fr. Rutine and nicotiflorin exhibited weaker antioxidant activity than fractions containing isolated flavonoids. From these findings, it is concluded that rutine and nicotiflorin together with other compounds in n-BuOH fr., may contribute to the efficacy of this plant against peptic ulcer, cough and bronchitis. Further studies should be carried out in order to define the other active constituents.

ACKNOWLEDGEMENT

D.Deliorman Orhan, acknowledges receiving a grant from Fujii-Otsuka International fund to study in Japan. This research was supported by Gazi University Research Fund (02/2002-08) Ankara, Turkey.

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Received: 19.10.2006
Accepted: 18.01.2007