Antioxidant Activity of Different Compounds from *Anthriscus cerefolium* L. (Hoffm.)

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Abstract

Characteristic constituents of chervil, *Anthriscus cerefolium* L. (Hoffm.) (Apiacae) were investigated for free radical scavenging effects. Different extraction and purification methods were used to separate essential oil and flavonoids from the leaves, and lignans from the root. *In vitro* test methods were used to determine whether the extracts had free radical scavenging and membrane protective activity. The identification of the chemical constituents was conducted by high pressure liquid chromatographic (HPLC) and gas chromatographic (GC) techniques. The following were concluded: flavonoids from the herb and lignans from the root showed strong free radical quenching activity, while the volatile oil obtained from the herb was less effective. The identification of the constituents of the extracts indicated that apiin is the main flavonoid, deoxypodophyllotoxin the major lignan, and methylcavicol the predominant constituent of the essential oil.

INTRODUCTION

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn on the attention of many experimental works.

Free radicals are produced either enzymatically or non-enzymatically in the organism and are eliminated by the action of certain vitamins and enzymes (Blázovics et al., 1995). The peroxidative chain-reaction of lipids, induced by accumulated oxygen free radicals might be important in the pathogenesis of certain liver, kidney, lung and age-related diseases (Blázovics et al., 1988, Gow-Chin et al., 1966). Epidemiological and laboratory experiments have shown that foodstuffs of natural origin contain complex components which are of protective value against these illnesses (Fehér et al., 1993, Kéry and Blázovics 1995). Keeping an eye on present information about natural free radicals, the close attachment between structure and effect, the reconsideration of herbal plants, and their outcome show a very promising area in the protection against free radical reactions. The mildly efficient herbal and spice plants - considering their chemical constituents and thus complex effects - can be important tools in the prevention. Numerous plant constituents have been proven to show free radical scavenging or antioxidant activity (Aruoma and Cuppett 1997). Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxycinnamic derivatives, catechins, etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Blázovics et al., 1994, Haraguchi at al., 1996). According to the newest research lignans also show considerable activity in this area (Hua, and Geng-Tao, 1992, Wagner et al., 1996).

The bases of such considerations were the reason why our attentions fell upon the not so well known and used herb and spice -chervil (*Anthriscus cerefolium* L. Hoffm.). Chervil has been used formally as a drug (herb cerefolii), but at present its principal use is

as a flavouring agent for culinary purposes. In folk medicine, however, the herb was used to alleviate circulation disorders (Bremness, 1989), while the root was and is even now employed as a hemanitic or a tonic in Japan and China (Mitsugi et al., 1982).

Characteristic constituents of the herb are flavonoids (apiin, luteolin-glycosides) (Tozaburo and Masao, 1979), and essential oil (methylchavicol = estragole, l-allyl-2,4-dimethoxybenzene) (Zwaving et al., 1970, Simándi et al., 1996). The root contains lignans (deoxypodophyllotoxin, anthriscinol methyl ether and angeloyloxymethly-2-butenoic acid) (Kozawa et al., 1978) (Fig. 1).

Since our previous studies underlined the free radical scavenging, antioxidant and membrane protecting activity of various water extracts prepared from different organs of *Anthriscus cerefolium* L. Hoffm., we intended to provide more information about the characteristic constituents which take part in the development of the therapeutical effect. To reach our goal, different solvent extractions, purifications and analytical methods were used. The three main groups of compounds where positive effects were estimated are the following: flavonoids, essential oil and lignans. Lignans were extracted from the root, flavonoids from the leaves; essential oil was produced by water-steam distillation. Flavonoids and lignans were purified by column chromatography. To prove the evidence for the non-specific free radical scavenging activity, a well-known test method - chemiluminescence- was used. The chemical characteristics of the samples were analysed using chromatography (GC, HPLC).

MATERIALS AND METHODS

Anthriscus cerefolium L. Hoffm. samples were collected before their full flowering period from the hills surrounding Budapest, Hungary. An herbarium specimen is deposited in the Department of Pharmacognosy, Semmelweis University, where chervil was identified. Before the plant material was dried in shade, the roots were removed from the herb. The dried root and herb parts were ground before the extractions.

Reagents. Luminol and microperoxidase were purchased from Sigma Chemical, the deoxypodophyllotoxin was a gift and apiin was ordered from Carl Roth GmbH. Sephadex LH 20 was obtained from Pharmacia. HPLC experiments were carried out using Farmitalia Carlo Erba HPLC clean solvents. The unlabeled chemicals and reagents were analytically clean from Reanal Rt. Budapest.

Extraction and Purification. The root (100 g) was extracted with methanol by the Soxhlet technique. Concentration of the extract was carried out under reduced pressure and yielded 6,5 g. An aliquot (6,0 g) was separated on a Sephadex LH 20 column (length: 75 cm; I.D: 3.5 cm) using methanol as solvent. Four fractions (AR1-4) were obtained: AR1 (0.9 g), AR2 (1.5 g), AR3 (2.9 g), and AR4 (1.2 g). 100 g of the herb was extracted successively with *n*-hexane, chloroform, ethyl acetate and methanol by the Soxhlet technique. Concentration of the extracts under reduced pressure gave 3.2 g of *n*-hexane, 7.4 g of chloroform, 4.3 g of ethyl acetate and 19.5 g of methanol extract. The methanol extract (15.0 g) was separated on a Sephadex LH 20 column (length: 75 cm; I.D: 3.5 cm) using methanol as a solvent. Five fractions (AH1-6) were obtained: AH1 (1.2 g), AH2 (1.0 g), AH3 (2.5 g), AH4 (3.2 g), AH5 (2.7 g), and AH6 (2.1 g). Water steam distillation was used to obtain the essential oil from the aerial part of chervil. 100 g drug was distilled for 4 hours according to the VIIth Hungarian Pharmacopoea and yielded 1.5 g essential oil. A methanol stock solution was prepared (0.2 g/mL), which was used in the chemiluminometric experiments.

Free Radical Scavenging Activity. The non-specific free radical scavenging activity was measured in a Lumat LB9501 luminometer using a chemiluminescence method (Blázovics and Fehér, 1995). During the experiment the $^{\bullet}OH$ radical, which is freed from hydrogen peroxide in alkali solution, reacts with luminol. This decomposes to inactive amino-phthallic acid while giving off light emission. The emitted light was monochromatic (425-nm wavelength) and its intensity was proportional to the concentration of the luminescence material. The samples and the luminol + Na_2CO_3 solution were injected into the measuring chamber manually with a Hamilton pipette

before the measuring process started. The H_2O_2 and microperoxidase solution was also injected, but automatically in volumes 300-300 µL. The measuring chamber was completely shut off from outside light. Experimentation time was 30 sec, and the procedure was carried out at room temperature. H_2O_2 concentration was 5.10^{-5} M, luminol concentration was 0.07 mM, Na_2CO_3 concentration was 1.18 mM and the microperoxidase concentration was 3×10^{-7} M. The emitted light signals were counted over the preselected time periods (30 sec) and were then integrated (chemiluminescence intensity). The changes in chemiluminescence intensity of the H_2O_2/·OH-luminol system at different concentrations of the samples were measured. The background (reference) luminescence was measured in a solution, which contained methanol in the same amount as the samples. The control solution represented the 100% light emission mark to which all the samples were compared. The free radical scavenging activity on the figures represents the percentage of the bound radicals compared to the background chemiluminescence.

High Pressure Liquid Chromatographic Parameters: The liquid chromatographic analysis was performed with a component Jasco PU 980 PUMP, a rheodyne 7125 injector with a 20 µL sample loop, an APEX ODS C-18 column (25 cm X 4.6 mm I. D.; particle size 5 µm, room temperature), and an UV 975 UV-VIS detector. The mobile phase was a mixture of two liquids distributed by the (A) pump: acetonitrile, and the (B) pump: 5% acetic acid (pH: 2.46) at a flow rate of 1 mL/min. For the lignan estimation a gradient elution was used beginning with (A): (B) 10:90 changing to (A): (B) 90:10 in 40 minutes. The UV detection was monitored at a wavelength of 265 nm.

A gradient elution was used for the flavonoid estimation, beginning with (A): (B) 10:90 changing to (A): (B) 30:70 in 40 minutes. The UV detection was monitored at a wavelength of 340 nm.

Identity of the principal constituents in the root and herb extracts was proved by peak addition of standard materials.

Gas Chromatographic Analysis: GC analysis was performed on a FISONS GC800 gas chromatograph equipped with a flame ionisation detector. Injector and detector temperatures were 200°C and 240°C respectively. The column was a 30 m X 0,32 mm, I.D; 0,25 µm, DB-1701 (OV-17). N2 was used as a carrier gas at pN_2 50kPa, V=6,8 cm^3/min. The column oven temperature was programmed from 60°C to 230°C at 8°C/min, and than held for a final 3 min at 230°C. Samples were injected at a split ratio of 1:10, in a concentration of 2µL/2cm^3 diluted in chloroform, in a volume of 0,4 µL. The injection technique was splitless 10 sec (bottom). The oil components were identified by comparing their retention times with those of authentic standards, essential oils of known composition and peak enrichment. Confirmation of identity was achieved by comparison of their mass spectra with those reported in the literature and reference compounds (Simándi et al., 1996). Estimation was carried out with a Chrom Card computer program.

Statistical Analysis: All the *in vitro* experimental results were mean ± S.D. of three parallel measurements. P-value <0.05 was regarded as significant.

RESULTS AND DISCUSSION

Different extracts of the roots and leaves of *Anthriscus cerefolium* L. Hoffm. were prepared and tested for free radical scavenging activity. The purified lignan extracts (AR1-4) and the stock methanol solution (AR) showed remarkable results in the chemiluminometric experiments. Sample AR3 was the most effective, scavenging almost 100% of the free radicals present at a concentration of 0.035 g/100 mL. The AR1 fraction did generate free radicals in the lower concentration range (0.035 – 0.12 g/mL). When the concentration of AR1 reached 0.168 g/100 mL, it scavenged 80% of the free radicals present. Only a slight difference could be detected between sample AR4 and AR3, which was not significant. Although sample AR2 generated free radicals at the concentration of 0.035 g/100 mL, it scavenged 100 % of the free radicals present at a concentration of 0.08 g/100 mL. The stock solution (AR) showed approximately the average scavenging activity of the purified samples (Fig. 2).

HPLC analysis of the lignan rich samples showed that the major compound is deoxypodophyllotoxin, which is present in all the samples, except AR1, where an unknown minor compound was present with a 28.69 min. retention time. Deoxypodophyllotoxin was the main compound in fractions AR2-4 (33.91-35.90 min.). AR2 and AR4 differed mainly in the quantity of deoxypodophylotoxin. AR3 contained an unidentified compound with a 45.33 min. retention time (Fig. 3).

The flavonoid rich samples, isolated from the herb, underlined our expectations. The first two (AH1-2) and the last (AH6) fractions from the Sephadex purification did not prove to have as good free radical scavenging effects as the middle fractions (AH3-5). At the concentration of 0.055 g/100 mL AH1,2 and AH6 generated free radicals, while at the concentration of 0.165 g/100 mL all samples quenched the free radicals present. The three middle fractions scavenged more than 80% of the radicals present in the lowest concentration (0.055 g/100 mL) and, at the concentration of 0.11 g/100 mL, no light emission was detected, showing that no free radicals were generated.

HPLC analysis of the samples showed that there are no major differences in the composition of the samples (Fig. 4). The main flavonoid of the herb was apiin with a retention time of 15.95±0.20 min. Deoxypodophyllotoxin was also found in all samples with a 39.52±0.60 min retention time. The highest relative amount of deoxypodophyllotoxin was measured in fraction AH6, whilst the lowest was traced in the AH5 fraction. The AH4 fraction, which was diluted 10 times before injection, contained the highest amount of an unknown compound (27.26±0.21). Presumably, this compound is apigenin-7-O-glucoside, but further investigations are in progress for identification.

The chemiluminometric evaluation of the essential oil obtained from the aerial part of chervil did not show very high free radical scavenging activity. Although increasing the concentration of the injected essential oil increased the scavenging capability, it was not significant. The highest effect was measured with a 0.04 g/mL sample concentration (21,2±5.6%) and it quenched only 20% of the present radicals. Gas chromatographic analysis of the essential oil showed three characteristic components: undecane (Rt.: 8.0 min.), methylchavicol (Rt.: 12.4 min.) and 1-allyl-2,4-dimethoxybenzene (Rt.:16.5 min.) (the structural isomer of methyl eugenol) (Fig. 5).

In our experiments, bioassay directed separation was used for studying the free radical scavenging activity of different group of compounds from *Anthriscus cerefolium* L. Hoffm. The active components of the herb and root extracts were different. In the herb they were mostly the flavonoids, while in the root they were the lignans. The main component of the flavonoids was apiin; on the other hand, of the lignans it was deoxypodophyllotoxin. Chemiluminometric analysis was a very useful method to screen the different solvent extracts and purified fractions to evaluate which compounds should be mixed together and which should be excluded to reach the highest free radical scavenging activity. We could conclude that the essential oil is not a valuable free radical scavenger in our experiments.

Bioassay directed purification may help to exclude the ineffective fractions giving us the possibility to reach the same effect with a lower concentration of the compounds. This can be important from a toxicological and financial point of view.

Antioxidant nutrient supplementation should be seriously considered as a preventive measure against diseases where reactive oxygen species have been related to different pathologies and the ageing process. Antioxidants and free radical scavengers may have a significant role in maintaining health when continuously taken as components of dietary foods, culinary herbs and crude drugs.

ACKNOWLEDGEMENTS

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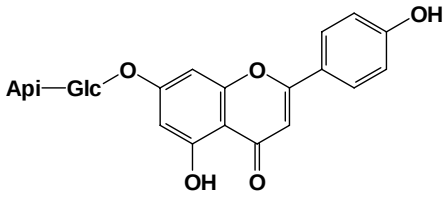
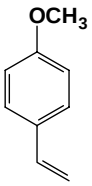
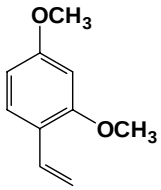
Figures



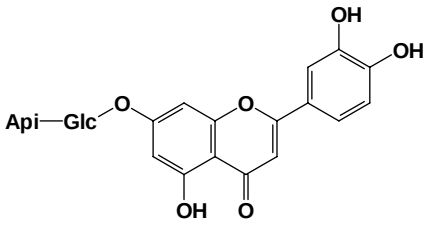
apiin



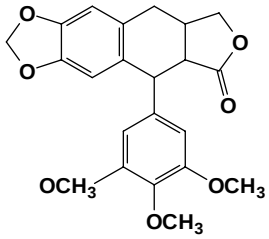
methylchavicol



1-allyl-2,4-dimethoxybenzene



luteolin-7-O-apiosylglucoside



deoxypodophyllotoxin

Fig. 1. Characteristic constituents of *Anthriscus cerefolium*

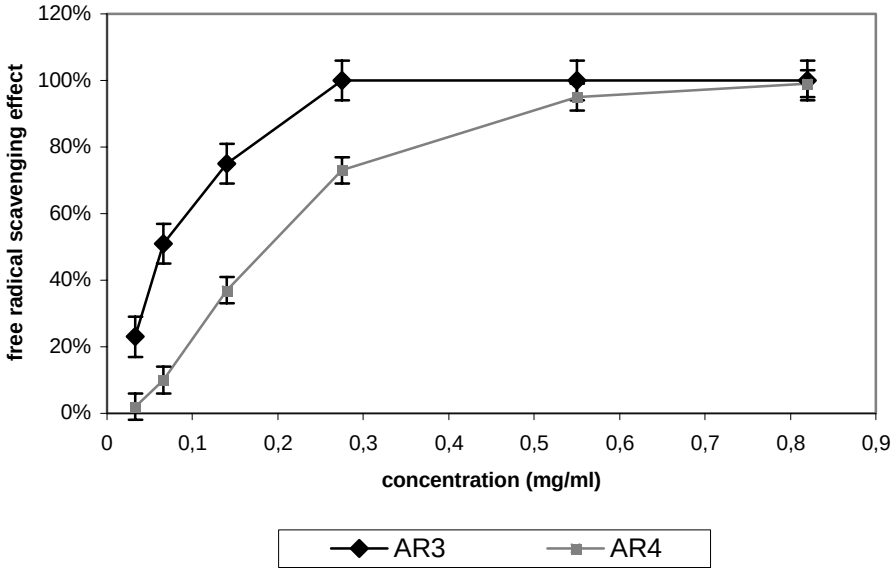


Fig.2. Free radical scavenging effect of the lignan-reach extracts from *Anthriscus cerefolium* root in $H_2O_2/^{\bullet}OH$-luminol system. Results are mean ± S.D. of five parallel measurements, $p<0.05$ when compared to control.

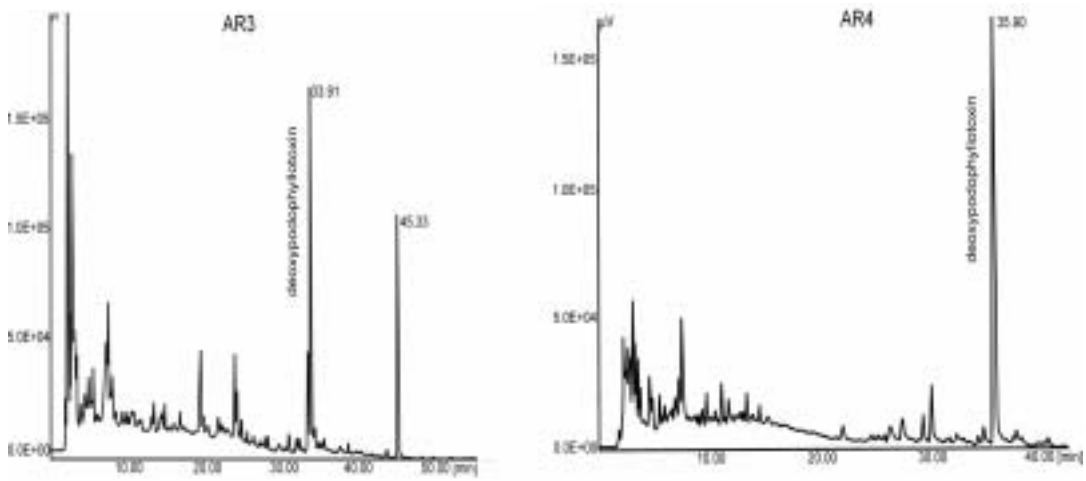


Fig. 3. HPLC analysis of the most effective methanol extracts from the root of *Anthriscus cerefolium*

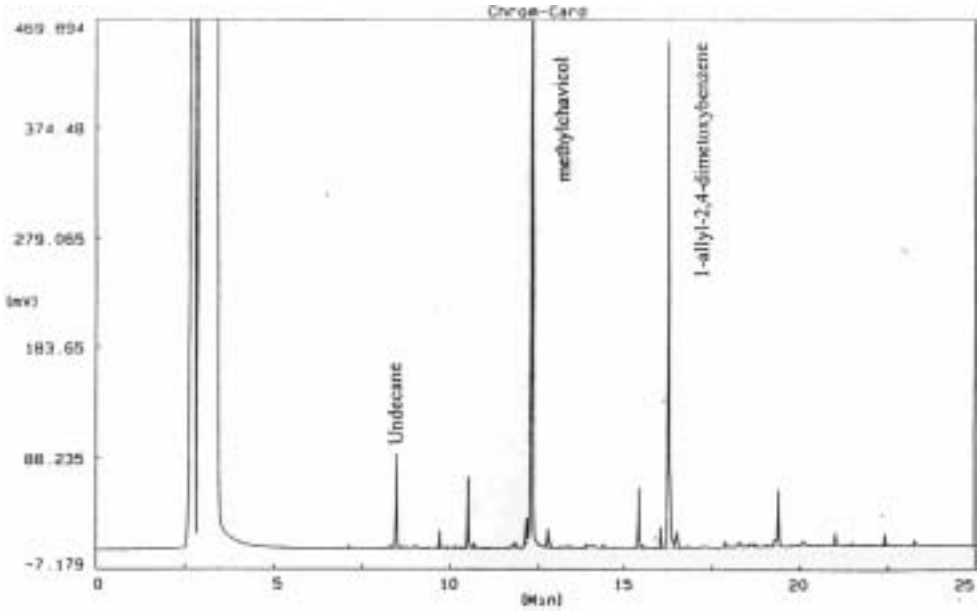
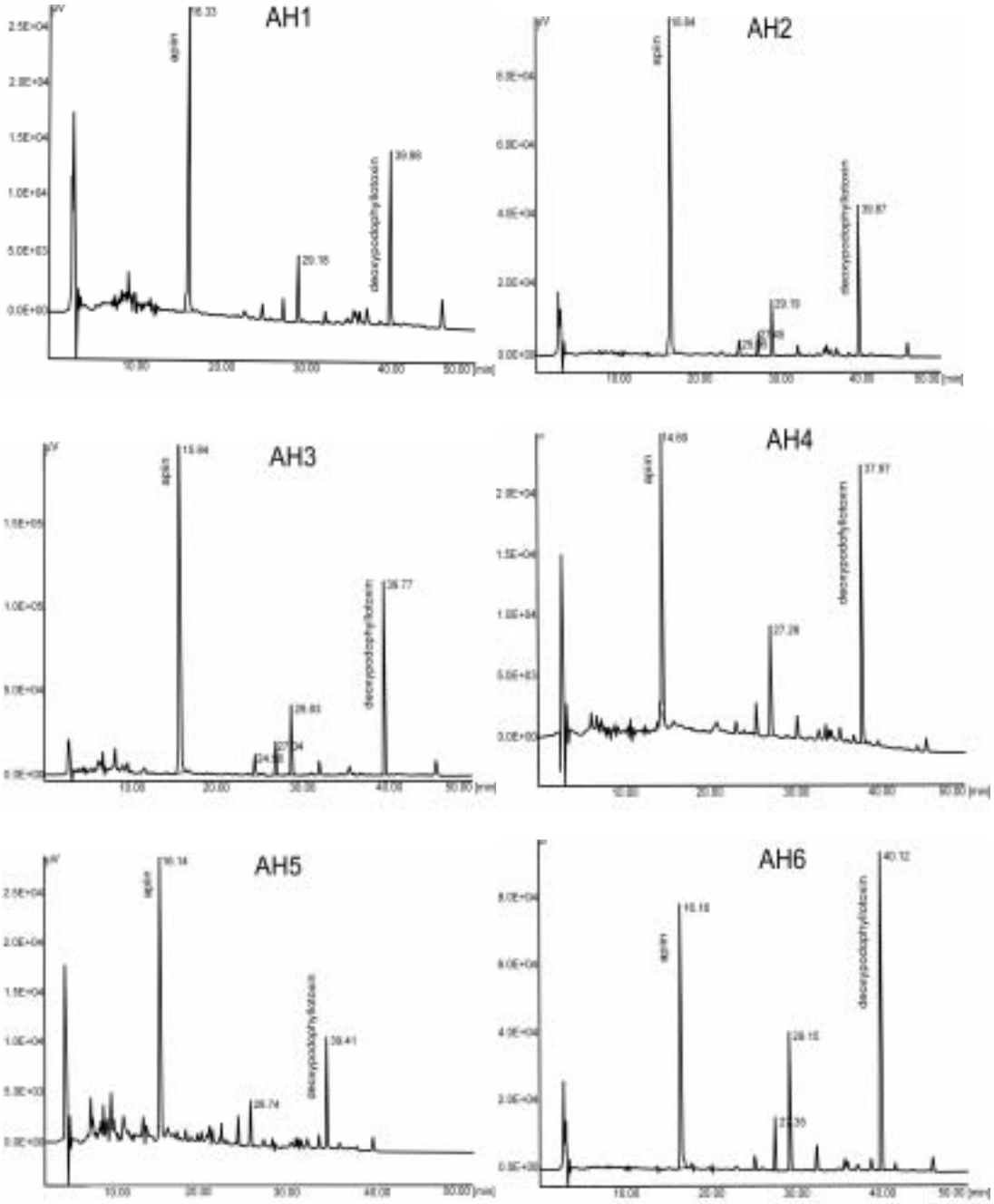


Fig. 5. Gas chromatographic analysis and free radical scavenging effect of the essential oil obtained from the aerial part of *Anthriscus cerefolium*



Sample concentration	10	20	30	40
*Free radical scavenging effect	12±4%	10±3%	5±4%	22±3%

*in H_2O_2/$^{\bullet}$OH luminol system. Results are mean ± S.D. of five measurements, p-value < 0.05 when compared to control.

Fig. 4. HPLC analysis of the crude methanol extracts from the herb of *Anthriscus cerefolium*