Antioxidant Effects of Flavonoids of *Anthriscus sylvestris* in Lard

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ABSTRACT: Ethanol/water (7:3) extracts of the plant species *Anthriscus sylvestris* possess antioxidant activity. Separation and identification of antioxidant components by thin-layer and column chromatography and spectral analysis demonstrated that quercetin and apigenin appeared to be the main flavonoid species in *Anthriscus sylvestris.* Rutin was one of the major quercetin glycosides. Structures of the isolated compounds were determined by infrared and ^{1}H nuclear magnetic resonance spectroscopies. Ethanolic extract (70%) of *A. sylvestris* showed concentration-dependent, strong antioxidant activity as determined by the Schaal Oven test of lard at 60°C. Rancimat analysis at 100°C showed that the antioxidant activity of 70% ethanolic extract of *A. sylvestris* was superior to quercetin, apigenin, or a tocopherol mixture.

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KEY WORDS: *Anthriscus sylvestris,* antioxidant activity, apigenin, flavonoids, quercetin, rutin, tocopherol.

The possible toxicity of synthetic antioxidants (1,2) has increased interest in preparing antioxidants from natural products by extraction, fractionation, and purification. Flavonoids are of particular interest among naturally occurring substances with potential antioxidant activity. In addition to essential oils, flavonoids are characteristic constituents of a great number of species of the Apiaceae (3). The Serbian plant species *Anthriscus sylvestris*, family Apiaceae, has been widely used in folk medicine as tonics, diuretics, and salad dressing (4,5). A preliminary study of its chemical composition was made on lignans of the root (6) and essential oils, fatty acids, and sterols of the flowers and leaves (7). Chemical analysis of the aerial parts of *A. sylvestris*, collected in the vicinity of Belgrade, have shown the presence of three flavonoid compounds—apigenin, quercetin, and rutin (8). It is known that flavonoids are not only soluble in water–alcoholic mixtures, but also have high solubility in fats and oils, which can promote their use as antioxidants in edible oils (9).

As natural sources of antioxidants are gaining importance for food technology (10), the present study was initiated to identify flavonoids in *A. sylvestris* with antioxidant activity and to evaluate their potential as antioxidants.

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MATERIALS AND METHODS

Preparation of extracts. Anthriscus sylvestris was collected at the time of full blossom in the vicinity of Mt. Avala, near Belgrade, Yugoslavia. A herbarium specimen was deposited at the Botany Department, Faculty of Agriculture (Belgrade). Air-dried aerial parts (2 kg) were extracted with 10 L of ethanol/water (7:3) for 48 h at 23°C. After removal of the ethanol, the remaining water solution of the plant material residue was extracted separately with chloroform and ethyl acetate. A brown oil remained (3.1/g) after elimination of the ethyl acetate. All extracts were concentrated on a rotary evaporator at 40°C.

Thin-layer chromatography (TLC). Silica gel G plates (20 × 20 cm, 0.25-mm thick; Merck, Darmstadt, Germany) were used. Solvents used to develop the TLC were *n*-butanol/acetic acid/water (BAW) (4:1:5, vol/vol/vol) for 70% ethanolic extract and chloroform/methanol (9:1, vol/vol) for ethyl acetate extract. A volume of 50 µL of the concentrated extracts were streaked on each precoated TLC silica gel plate, which had been activated for 30 min at 105°C. After development, the chromatograms were dried and examined by spraying with aqueous sulfuric acid (1:1, vol/vol), subsequent charring at 100°C, and spraying with α,α′-dipyridyl reagent (1% ethanol solution) (11). All spots were visualized after spraying under ultraviolet (UV) (360 nm) radiation after the TLC plates had dried.

Column chromatography (CC). CC was performed on silica gel 60, 0.063–0.020 mm (Merck). The brown oily ethyl acetate extract was chromatographed on the silica gel column, starting with 100% ethyl acetate as initial eluent. The first fraction eluted with 100% ethyl acetate contained one solid product, bright yellow crystals, which, after being recrystallized from ethanol, was identified as the flavone apigenin (*A*). In order of elution, we further isolated two pure solid fractions with a mixture of ethyl acetate/methanol (9:1) as eluent. The first one, as yellow crystals, recrystallized from ethanol, was the flavonol quercetin (*Q*). On further elution with a mixture of ethyl acetate/methanol (9:1), a powdery product was obtained and identified as rutin (*R*) upon recrystallization from ethanol/water (1:1). Further elutions with solutions of ethyl acetate/methanol (1:1 and 3:7, respectively) did not produce any additional flavonoid compounds. The final eluent of this separation was 100% methanol and did not produce any

compound. A total of nine fractions resulted from this elution pattern, but only three fractions crystallized. The elutions were monitored by TLC as described previously.

Spectroscopic procedures. Melting points (uncorrected) were taken on a Buchi and Micro-Heiztish-Boetius apparatus (VEB-Analitic, Dresden, Germany). Infrared (IR; ν max in cm^{-1}) spectra of the antioxidant compounds were recorded on a Perkin-Elmer instrument, Model 457 (Beaconsfield, Buckinghamshaire, England) (in KBr discs). ^1H nuclear magnetic resonance (NMR) spectra were taken on a Varian FT-80A (Mulgrave, Victoria, Australia) spectrometer in CD_3OD with tetramethylsilane as a reference marker. Chemical shifts are given in ppm as δ values, and the symbols *s, d, t,* and *m* stand for singlet, doublet, triplet, and multiplet, respectively. Specific rotation ($/\alpha/_D$) was determined in EtOH solution on a Polomat A (Karl Zeiss, Jena, Germany).

Antioxidant activity analysis. The antioxidant activity of each compound tested was based on its ability to prevent the formation of peroxides and secondary products of oxidation in prime steam lard samples. Antioxidant activities of ethanol/water (7:3) extract of *A. sylvestris* and of the flavonoids apigenin and quercetin were determined by the Schaal Oven test and the Rancimat method. For the Schaal Oven test (12), a glass tube (25 mL, 3-cm i.d.) with a flat bottom with 10 ± 0.01/g lard and 0.02 or 0.2% of each extract or antioxidant mixture was placed into an incubator kept at 60 ± −1°C in the dark, using a different tube for every new week during the test. A tocopherol mixture, consisting of 12% α-tocopherol, 1% β-tocopherol, 61% γ-tocopherol, and 26% δ-tocopherol (Coviox T-70; Chemishe Fabrik Grunau GmbH, Illertissen, Germany), was used as a positive control. Changes in peroxide value (PV) were determined by Official Method Cd 8-53 of the American Oil Chemists' Society (13). A sample size of 1 ± 0.001/g was used in each PV analysis. All Rancimat analyses were performed with a 617 Rancimat (METROHM AG, CH-9100; Metrohm, Herisau, Switzerland) at 100°C with air flow at 18–20 mL/min on 2.5 g of sample (14).

Statistical analysis of experimental data. All experiments were repeated in triplicate, and typical results are shown from one of the three independent experiments. Significance of treatments was estimated by the Student's *t*-test (15).

RESULTS AND DISCUSSION

Separation of the antioxidants from A. sylvestris. *Anthriscus sylvestris* was extracted with 70% ethanol, and after removal of the solvent, the residue was treated with chloroform and ethyl acetate separately. TLC was used for preliminary identification of the components or the class of compounds in these extracts. BAW gave the best separation for aqueous ethanolic extracts, and chloroform/methanol gave the best for ethyl acetate extracts. In the TLC analysis of the aqueous ethanolic extract, seven spots were detected, but the ethyl acetate extract showed only three distinct spots, after spraying with sulfuric acid and α,α′-dipyridyl reagent, respectively. Spots in which yellow color (with sulfuric acid) and purple blue color (with α,α′-dipyridyl) persisted were judged to have antioxidant activity, with the intensity of color related to the amount of phenolic compounds (11,16).

The results obtained from TLC of the aqueous ethanolic and ethyl acetate extracts indicated that flavonoid compounds were present. The ethyl acetate extract was further examined and separated by silica gel CC, which showed three fractions that contained flavonoid compounds. This extract was chromatographed, starting with 100% ethyl acetate as eluent. The first fraction contained only one substance, which was identified as flavone *A*. The second isolated yellow product, eluted with the mixture ethyl acetate/methanol (9:1), was identified as the flavonol *Q*. In the same eluent system, the quercetin rhamnoglucoside, *R*, was obtained.

Identification of antioxidative compounds. Apigenin was reported to melt at 345°C (16–18). All spectroscopic data, including the elemental analysis for $C_{15}H_{10}O_5$ of apigenin, are presented in Table 1. In the IR spectrum of *A*, the band at 1695 cm^{-1} belonged to the conjugated keto group, the band at 3450 cm^{-1} showed the presence of hydroxyl groups, and the other bands represented the absorptions of aryl ethers. The NMR spectrum indicated that positions C-7 and C-5 in the A-ring were substituted (doublets at 6.07 and 6.30 ppm). The doublet and quartet at 6.75 and 7.25 ppm were due to C-3′,5′ and C-2′,6′ protons of the B-ring and, finally, the singlet at 6.40 ppm was due to the proton at C-3 position of the C-ring. The second isolated pure compound, flavonol *Q*, was identified by spectroscopic methods (16–18) (Table 2). In the IR spectra of *Q*, the band at 3430 cm^{-1} corresponded to hydroxyl groups, the band at 1650 cm−1 belonged to the conjugated keto group, and the other bands of smaller intensity were due to aryl ethers. In the ^1H NMR spectra of *Q*, the proton order in the A-ring was identical to the structure of *A*. The doublet at 6.67 ppm indicated that the position C-3′ of the B-ring was occupied. The absence of a singlet at 6.3–6.4 ppm corresponded to the substituted C-3 position of the C-ring and finally proved the structure of the flavonol *Q*. The last isolated solid fraction, yellow powder, was identified as *R* on the basis of physical data. *R* melted at 195–197°C, and the specific rotation was $/\alpha/^{23} = +13.8$ (EtOH), and $R_f = 0.55$ was the value obtained in the BAW eluent system by TLC (20). The proposed structures of the isolated flavonoids *A, Q,* and *R* are presented in Scheme 1.

TABLE 1
Spectral Data of Apigenin (*A*) Isolated from *Anthriscus sylvestris*[a]

^1H NMR (CD_3OD: int. TMS) δ ppm		
6.07	(*d*, *J* = 2.5 Hz, 1*H*)	H-6
6.30	(*d*, *J* = 2.5 Hz 1*H*)	H-8
6.40	(*s*, ^1H)	H-3
6.75	(*m*, *J* = 9 Hz, 2*H*)	H-3′,5′
7.25	(*m*, *J* = 9 Hz, 2*H*)	H-2′,6′
m.p. 345°C	Anal.: Found: 67.16%C; 4.36%H;	
Lit. 345–350°C	Calcd.: 66.86%C; 3.83%H; $C_{15}H_{10}O_5$	

[a]Infrared (IR) (KBr): 3450, 1695, 1600, 1290, 1170, 1100, 1030 ν max (cm^{-1}); NMR, nuclear magnetic resonance; TMS, tetramethylsilane.

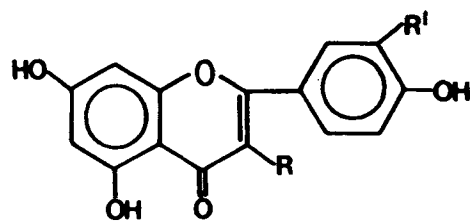
TABLE 2
Spectral Data of Quercetin (*Q*) Isolated from *Anthriscus sylvestris*[a]

^{1}H NMR (CD_3OD: int. TMS) δ ppm		
6.03	(*d*,*J* = 2.5 Hz, 1*H*)	H-6
6.25	(*d*,*J* = 2.5 Hz 1*H*)	H-8
6.67	(*d*,j = 8 Hhz, 1*H*)	H-5′
7.50	(*m*, *J* = 9 Hz, 2*H*)	H-2′,6′
m.p. 310–314°C	Anal.: Found: 59.96%C; 3.50%H;	
Lit. 314°C	Calcd.: 59.61%C; 3.33%H; $C_{15}H_{10}O_7$	

[a]Infrared (IR) (KBr): 3430, 1650, 1600, 1510, 1370, 1250, 1020 ν max (cm^{-1}). Abbreviations as in Table 1.

Antioxidant activity. The PV curves for the Schaal Oven test of prime steam lard with the 70% ethanolic extracts at 0.02 and 0.2%, as well as with tocopherol mixture, are shown in Figure 1. The lard control had an induction period (IP) of 12 h (0.5 d), and the tocopherol mixture at 0.02% level gave an IP value of 385 h (16 d). The same concentration of the 70% ethanolic extract with an IP of 192 h (8 d) showed significantly lower antioxidant effect than the tocopherol mixture ($P < 0.01$). In contrast, the 70% ethanolic extract, at 0.2%, had the longest induction time of 600 h (25 d), and showed significantly greater antioxidant activity than the tocopherol mixture ($P < 0.001$). The 70% ethanolic extract at 0.2% had approximately three times stronger antioxidant effect than at 0.02% level, indicating the presence of natural antioxidants in the extract whose antioxidant activity was concentration-dependent without any prooxidant effect at 0.02 or 0.2%.

The Rancimat method developed by Hadorn and Zurcher (19), based on the conductometric determination of volatile degradation secondary products of oxidation, was used to measure the antioxidant effect of the 70% ethanolic extracts at the 0.02 and 0.2% levels and of *A* and *Q* isolated from *A. sylvestris* (Fig. 2). According to Whittern *et al.* (20), *R* had no appreciable antioxidant activity and was not investigated further in this study. The IP value of the lard control, which contained no antioxidants, was 1.5 h. Ethanolic extract (70%)



A: R = R′ = H

Q: R = R′ = OH

R: R = Orut
R′ = OH

SCHEME 1

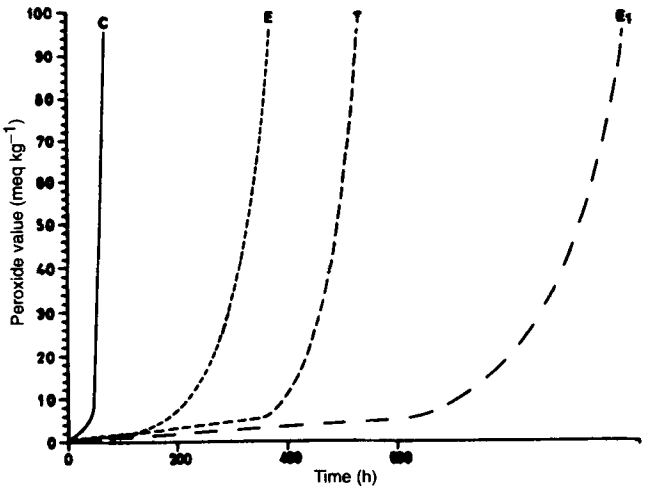


FIG. 1. Effect of antioxidants on rate of autoxidation of lard in the Schaal Oven test at 60°C: C, lard control; E, 70% ethanolic extract (0.02%); E_1, 70% ethanolic extract (0.2%); and T, tocopherol mixture (0.02%).

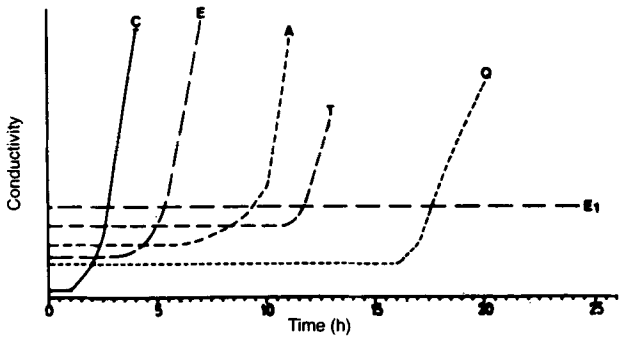


FIG. 2. Effect of antioxidants on rate of autoxidation of lard at 100°C by Rancimat method: C, lard control; E, 70% ethanolic extract (0.02%); A apigenin (0.02%); Q, quercetin (0.02%); T, tocopherol mixture, (0.02%); and E_1, 70% ethanolic extract (0.2%).

at the 0.02% level showed significantly higher antioxidative stability than the control with an IP of 4.5 h ($P < 0.05$). The antioxidant activity of a 0.02% commercial tocopherol mixture was stronger than the same concentration of ethanolic extract, with an IP of 9 h. However, 70% ethanolic extract at 0.2% showed greatest antioxidant activity ($P < 0.001$) compared to tocopherol. The pattern of the curve for this extract (E1) showed that practically no products of oxidation were formed because no volatile acids were detected after 25 h. No prooxidant effect at 0.2% was registered. The 15.5-h IP for *Q* was significantly longer than the 9-h IP for tocopoherol ($P < 0.01$). *A.* had an IP of 6.5 h ($P < 0.05$) compared with tocopherol. The results were in good agreement with the reported data of Kaufmann and El-Baya (21) for the antioxidant activity of hydroxyl flavones, which increased with increasing concentration without any prooxidant effect at high concentrations (21).

Antioxidant effects at 0.2% and 0.02% concentrations of 70% ethanolic extract differed depending on oxidation temperature. At 100°C, a fivefold difference between 0.2 and

0.02% was found, whereas only a threefold difference was noted at 60°C.

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