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ORIGINAL CONTRIBUTION

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Antioxidant activity of extracts from the leaves of Smallanthus sonchifolius

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Summary *Background & Aims* Yacon (*Smallanthus sonchifolius,* Asteraceae) is a native Andean plant, cultivated for its tubers throughout South America. The leaves are used in folk medicine as a medicinal tea for hypoglycemia. This paper describes the antioxidant activity of various extracts from *S. sonchifolius* leaves for their content of phenolic components. *Methods* The dried leaves were extracted in several ways. Two fractions were selected for their high content of phenolic compounds and analyzed by RP-HPLC. The antioxidant activity of these fractions was tested in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and xanthine/XOD superoxide radical scavenging assays, as inhibition of lipoperoxidation of subcellular membranes and as protective activity against oxidative injury of rat hepatocytes in primary cultures. *Results and conclusions* The presence of protocatechuic (2.5 and 0.12 mg/g), chlorogenic (9.9 and 1.7 mg/g), caffeic (14.7 and 0.09 mg/g) and ferulic (traces) acids were determined in the two fractions. Both fractions showed potent antioxidant activity in DPPH (IC_{50} = 16.1 ± 3.4 and 24.3 ± 2.7 μg/ml) and xanthine/XOD superoxide radical scavenging (42.0 ± 20.3 and 34.3 ± 11.4 SOD equivalents (U/mg)) tests, they inhibited the lipoperoxidation of rat liver subcellular membranes and they protected rat hepatocytes against oxidative injury. Our results may predetermine the use of *S. sonchifolius* leaves in human diet as a potential remedy in the prevention of chronic diseases caused by radicals, e. g., arteriosclerosis.

Key words Smallanthus sonchifolius – phenolic acids – in vitro antioxidant activity – cytoprotective effect – rat hepatocyte

Introduction

As a part of our program to develop new nutraceuticals from South American flora, the leaves of *Smallanthus sonchifolius* (Asteraceae), vernacular name yacon, were investigated phytochemically and biologically. This plant may be agriculturally cultivated in European climate. In Japan yacon tubers have become popular as a dietary supplement for people suffering from diabetes mellitus. The only studies reported to date on *S. sonchifolius* include isolation of four kaurenoids and four sesquiterpene lactones from the leaves and investigated the hypoglycemic activity of a water extract in rats [1].

In this paper, we describe antioxidant activity of various extracts of *S. sonchifolius* leaves in relation to phenolic content. Yacon leaves were extracted in several ways. Two fractions were selected for their high phenolic compounds content and analyzed by RP-HPLC. *In vitro* antioxidant activity of these fractions was assessed

by DPPH and xanthine/XOD superoxide radical scavenging tests, as inhibition of subcellular membranes lipoperoxidation and as protection against oxidative injury induced in rat hepatocytes. The antioxidant activity was compared with chlorogenic and caffeic acids as controls.

Materials and methods

Plant material

Smallanthus sonchifolius, originally purchased from Ecuador, was cultivated in Havlickuv Brod, Czech Republic. Voucher specimens are deposited in our collection at the Institute of Medical Chemistry and Biochemistry, Olomouc, Czech Republic. The leaves were collected in October 2000 at harvest time of the tubers and dried at ambient temperature.

The dried drug was extracted in several ways:

1. In a Soxhlet extractor with MeOH, chlorophyll removal with petroleum ether, the aqueous layer was then acidified and extracted by ethyl acetate (SOX).
2. Percolation at room temperature (25 °C) in different solvents:
   a) *Successive extraction* of dried leaves performed by cool percolation with organic solvents: hexane (OF1), ether (OF2), ethyl acetate (OF3) and ethanol (OF4).
   b) *Cool percolation with methanol/water [3:7]:*
      – Extract → water/ethyl acetate → OF5, aqueous fraction OF6.
      – Extract → 0.01 M NaOH/ethyl acetate → OF7, the aqueous fraction was then neutralized and extracted with ethyl acetate → OF8.
      – Extract → 0.01 M H_3PO_4/ethyl acetate → **OF9**, the aqueous fraction was then neutralized and extracted with ethyl acetate → OF10.
   c) *Cool percolation with methanol/water (9:1):*
      – Extract – water/hexane → OF11, the aqueous fraction was then extracted with:
        i) Dichloromethane → OF12, OF13
        ii) 0.01 M NaOH/dichloromethane → OF14 + aqueous layer → neutralized/dichloromethane → OF15
        iii) 0.01 M H_3PO_4/dichloromethane → OF16 + aqueous layer → neutralized/dichloromethane → OF17.
   d) *Cool percolation with acetone/water (9:1):*
      – Extract – water/hexane → OF18, the aqueous fraction was then extracted with dichloromethane → OF19; OF20.
3. Supercritical fluid extraction (I–VIII).
4. High-pressure solvent extraction (EtOH, IX–XII).

Animals

Male Wistar rats weighing 200–250 g were conditioned in standard boxes for 15 days before the experiments. They were fed a standard laboratory diet, provided with water *ad libitum* and kept on a 12/12 h light-dark cycle.

Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH, 90 %), xanthine (99 %), xanthine oxidase (5 U), superoxide dismutase (30 000 U), 2-thiobarbituric acid (TBA, 98 %), *tert*-butylhydroperoxide (*t*BH, 70 % in water), nitro blue tetrazolium chloride (NBT), trypan blue, dimethylsulfoxide (DMSO) for cell cultures, Williams' medium E, fetal calf serum and additives were purchased from Sigma-Aldrich Ltd., Czech Republic. Collagenase was from Sevac, Czech Republic. Other chemicals and solvents were of analytical grade from Lachema, Czech Republic.

Phenolic content analysis

The fractions were analyzed by thin-layer chromatography (TLC, mobile phase: $HCOOC_2H_5$-HCOOH-H_2O-toluene (50:5:5:2.5), detection: NP/PEG) and high precision thin-layer chromatography (HP-TLC mobile phase: hexane-$CH_3COOC_2H_5$-HCOOH [20:20:1], detection: 366 nm), fractions SOX and OF9 were also analyzed by RP-HPLC (mobile phase: A: CH_3CN-H_2O-CH_3COOH (50:49.5:0.5), B: H_2O-CH_3COOH (99.5:0.5), v/v, linear gradient 0–60 min 5–75 % A in B, column: Luna (C18) 5 µm, 250x2 mm i. d., 0.2 ml/min, detection: DAD total ion current 190–500 nm and ESI MS).

Total phenolics in both fractions were determined using Folin-Ciocalteu reagent [2]. A total of 50 µl of the tested fraction in distilled water was mixed with 1000 µl of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and maintained at room temperature for 5 min; 1000 µl of sodium bicarbonate (75 g/l) was added to the mixture. After 90 min at 30 °C, absorbance was measured at 725 nm. Results were expressed as gallic acid equivalents.

DPPH scavenging

A total of 375 µl of a methanolic solution of the tested sample (6.25, 12.5, 25, 50, 75 and 100 µg/ml) was mixed with 750 µl of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH, 20 mg/l). After 30 min, absorbance at 517 nm was measured and IC_{50} values were obtained from the inhibition curves [3].

Superoxide scavenging and/or xanthine oxidase inhibition

A 0.1 ml sample of aqueous superoxide dismutase (SOD) standard solutions (5, 10, 25, 50, 100 U/ml) and a phenolic acid/extract solution in DMSO (1 mg/ml) were separately added to a 1.0 ml mixture of 0.4 mM xanthine and 0.24 mM nitro blue tetrazolium chloride (NBT) in 0.1 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA. A 1.0 ml sample of xanthine oxidase (0.05 U/ml), diluted in the same phosphate buffer, was added and the resulting mixture incubated in a water bath at 37 °C for 20 min. The reaction was terminated by adding 1.0 ml of 69 mM sodium dodecylsulphate solution (SDS) and the absorbance of reduced NBT was measured at 560 nm. Superoxide scavenging activity was calculated as superoxide dismutase equivalents (SOD U/mg) from SOD standard curve [4].

Protectivity against lipoperoxidation induced by tBH

Rat liver microsomes and mitochondria were prepared by fractional centrifugation of rat liver homogenate in 3 mM Tris buffer containing 250 mM sucrose and 0.1 mM EDTA (pH 7.4). The mitochondrial and microsomal fractions were characterized by their protein content. For inhibition of lipoperoxidation, a mixture containing 2 mg/ml of mitochondria or 1 mg/ml of microsomal fraction, 5–120 µg/ml of extracts or phenolic acids and 1 mM *tert*-butylhydroperoxide (*t*BH) were incubated in a water bath at 37 °C for 60 min. The incubation was then terminated by adding 2 ml of a mixture of thiobarbituric (26 mM) and trichloroacetic (918 mM) acid (TBA-TCA), the samples were subsequently incubated at 90 °C for 30 min. After cooling, the samples were centrifuged (10 min, 1000 g, 20 °C) and the absorbance of the supernatant was measured at 535 nm. IC_{50} values were obtained from inhibition curves [5, 6].

Rat hepatocyte primary cultures

Rat hepatocytes were isolated by two-step collagenase perfusion of rat liver [7]. The cell viability was determined by measuring trypan blue exclusion. Yields of 3–5 x 10^8 cells/liver with a viability greater than 80 % were routinely obtained. The hepatocytes were then dispersed in sterile conditions in Williams' medium E supplemented by penicillin (10 IU/ml), streptomycin (0.1 mg/ml), dexamethasone (1 µM), insulin (0.1 µM) and glutamine (2 mM) and then cultivated in collagen-coated 6-well dishes in a humidified atmosphere of 5 % CO_2 at 37 °C. For the culture stabilization, 10 % fetal calf serum was added to the cultivation medium for the first 4 hours. The tested samples (final concentrations 1–1000 µg/ml) were added to the incubation medium in DMSO (max 0.5 %).

For cytotoxicity studies, the hepatocyte monolayers were incubated with the tested samples 4, 24 and 48 hours and viability of the cells was assessed using the MTT test [8].

To study cytoprotective effects against *t*BH-induced damage, the primary cultures were intoxicated by *t*BH for 1.5 h (final concentration 0.5 mM) after preincubation with the tested samples (16–20 h). Quality of the culture was controlled by the following parameters: cell viability (MTT test, [8]), level of released LDH [9] and of lipoperoxidation products (thiobarbituric acid reacting substances, TBARS, [5]) in the medium.

Statistics

Data were analyzed with one-way ANOVA using the StatView Statistical Package. Differences were considered statistically significant when *P was < 0.05 and **P < 0.01.

Results

Phenolic content analysis

The fractions richest in phenolics were the ethyl acetate fraction resulting from the Soxhlet procedure (SOX) and the fraction OF9 from percolation at room temperature (TLC results in Fig. 1). Total phenolic content was 20.16 ± 2.51 and 36.58 ± 7.47 %, respectively. The pres-

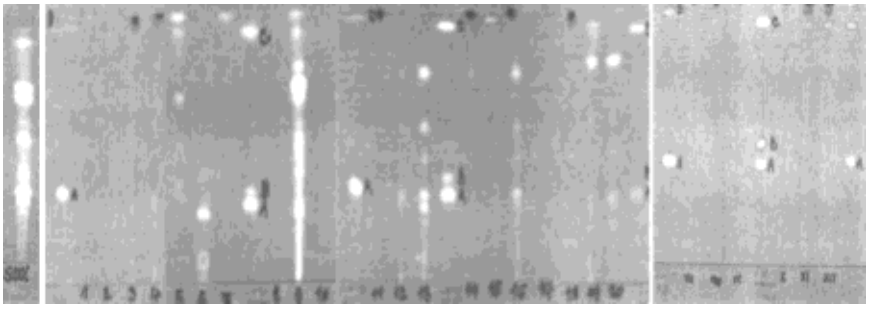

Fig. 1 HP-TLC of various extracts and fractions from *S. sonchifolius* leaves. Mobile phase: $HCOOC_2H_5$-HCOOH-H_2O-toluene (50:5:5:2.5), detection: NP/PEG, standards: A – chlorogenic acid, B – hyperoside, C – caffeic acid, D – ferulic acid

ence of protocatechuic, chlorogenic, caffeic and ferulic acids in the fractions were proven by comparison with standards (data not shown). The HP-TLC results were then confirmed by HPLC analysis; ferulic acid was present only in traces (Fig. 2, Table 1).

Table 1 HPLC quantification of phenolic acids in extracts from *S. sonchifolius* leaves (mg/g of extract)

Extract	Protocatechuic	Chlorogenic	Caffeic acid
SOX	2.5	9.9	14.7
OF9	0.12	1.7	0.09

Antioxidant activity

The results of antioxidant activities of extracts from *S. sonchifolius* leaves determined by different non-cellular tests are shown in Table 2. Since the extracts show moderate cytotoxicity at 1000 µg/ml after 48 h incubation (Fig. 3), their cytoprotectivity was further studied up to a concentration of 100 µg/ml. The cytoprotective effects of extracts on primary cultures of rat hepatocytes intoxicated by *tert*-butylhydroperoxide (*t*BH, 0.5 mM, 1.5 h)

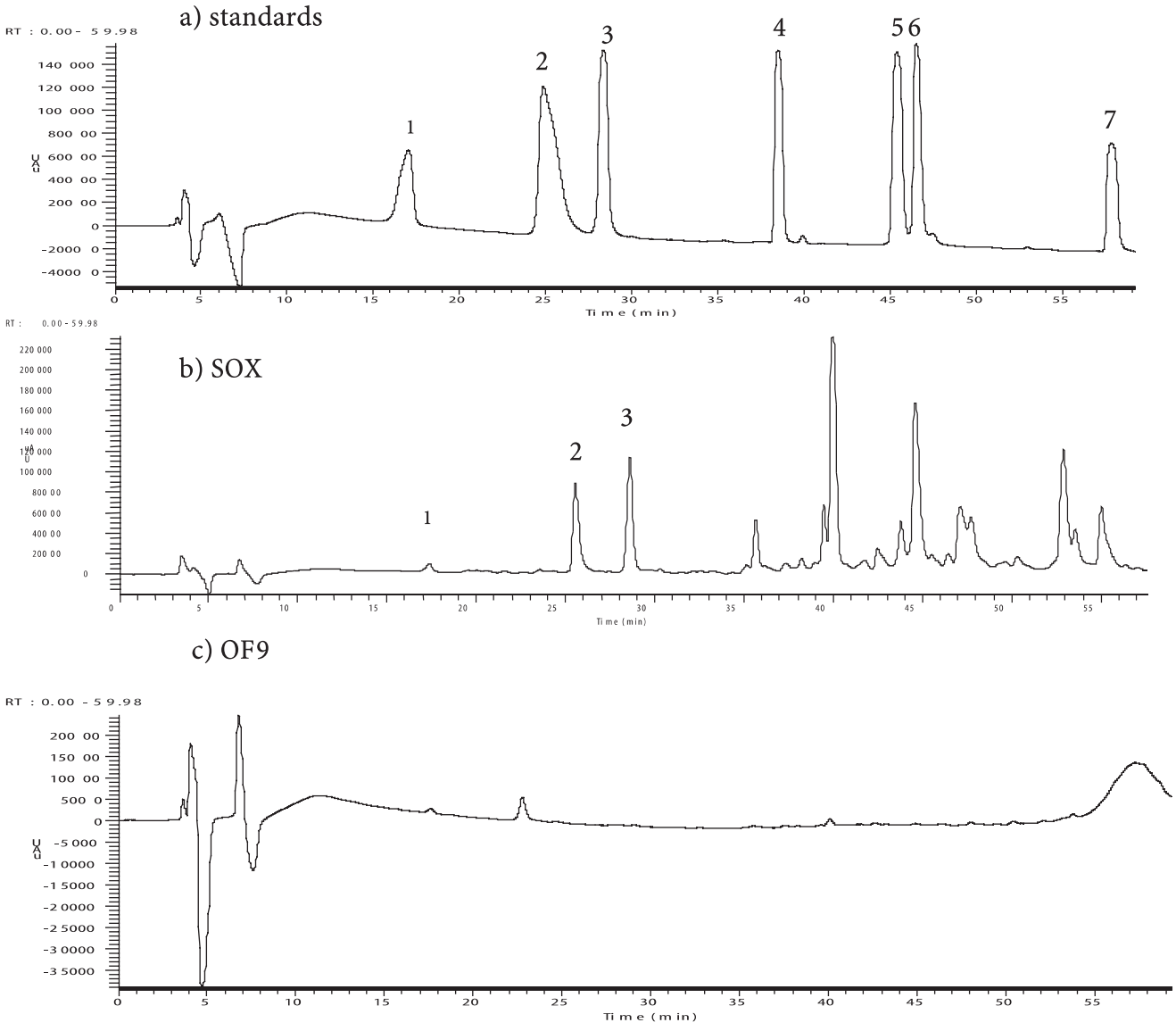

Fig. 2 HPLC analysis of extracts from *S. sonchifolius* leaves. Mobile phase: A: CH_3CN-H_2O-CH_3COOH (50:49.5:0.5), B: H_2O-CH_3COOH (99.5:0.5), v/v, linear gradient 0–60 min 5–75 % A in B, column: Luna (C18) 5 µm, 250x2 mm i. d., 0.2 ml/min, detection: DAD total ion current 190–500 nm and ESI MS, standards: 1 – protocatechuic, 2 – chlorogenic, 3 – caffeic, 4 – ferulic, 5 – rosmarinic, 6 – o-hydroxycinnamic, 7 – cinnamic acid

Table 2 Antioxidant activity of extracts from *S. sonchifolius* leaves

Method	DPPH	Xanthine/XOD	Inhibition of lipoperoxidation	
Sample	IC_{50} (μg/ml)	SOD equivalents (U/mg)	microsomes	mitochondria
			IC_{50} (μg/ml)	
SOX	16.1±3.4	42.0±20.3	23.8±7.8	22.2±9.3
OF9	24.3±2.7	34.3±11.4	32.8±2.3	30.2±9.4
Caffeic acid	0.86±0.05	417.3±4.3	9.86±1.92	37.4±8.8
Chlorogenic acid	2.46±0.17	131.2±12.0	64.1±16.6	102.9±16.4

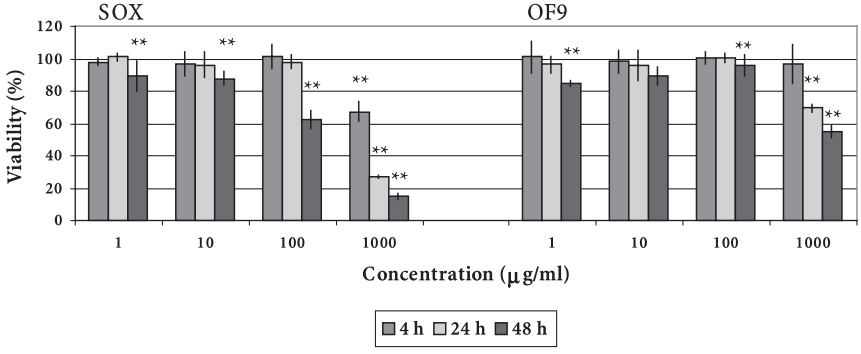

Fig. 3 Effect of extracts from *S. sonchifolius* leaves on viability of primary cultures of rat hepatocytes (MTT test). Each value is the mean of 3 individual determinations ± SEM. ** $P < 0.01$ relative to control

is shown in Fig. 4. In all the tests performed, the activity order of the tested samples was caffeic acid > chlorogenic acid > OF9 > SOX.

Discussion

Oxidative stress is an important factor in the development of many chronic diseases, mainly cardiovascular disorders such as arteriosclerosis. Antioxidants are compounds that can delay or inhibit the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and scavenging free radicals, quenching singlet and triplet oxygen or decomposing peroxides [10]. Due to the lack of antioxidants in daily diet, interest has greatly increased recently in finding naturally occurring antioxidants for use in foods and dietary supplements.

Our results suggest that *S. sonchifolius* leaves are a promising source of natural antioxidants, mainly phenolic acids. The fraction OF9, even if it contains according to HPLC analysis 14-fold less phenolic acids, displayed only 1.5-fold less antioxidant activity than the SOX fraction. The discrepancy between the antioxidant activity, total phenolic content and quantification of phenolic acid in the two tested fractions may be due to a variety of factors. The fraction OF9 has been prepared in a more economic way. Extraction with only 30 % MeOH permitted us to omit one step from the extraction procedure (removal of chlorophyll). Mild temperature conditions probably prevented hydrolysis of phenolic glycosides and polymers. This would play an important role in total phenolic content and antioxidant activity but would not appear in the HPLC analysis.

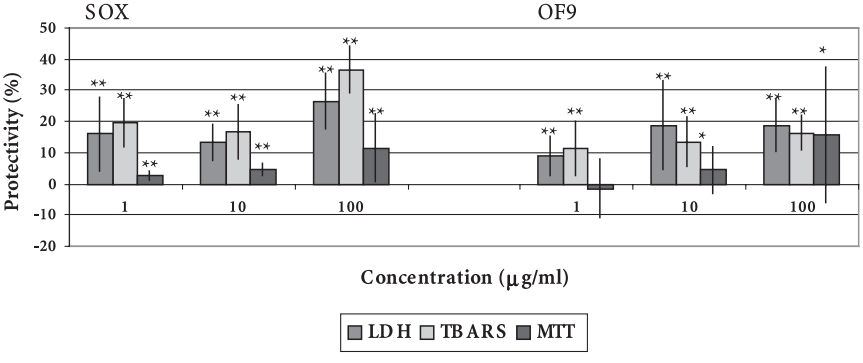

Fig. 4 Protective effect of extracts from *S. sonchifolius* leaves on primary cultures of rat hepatocytes intoxicated by *t*BH (0.5 mM, 1.5 h). Each value is the mean of 3 individual determinations ± SEM. * $P < 0.05$ relative to control, ** $P < 0.01$ relative to control.

The antioxidant activity of polyphenols is generally ascribed to their hydroxyl groups, but it is not the only factor in determining the potency of their activities. In the case of chlorogenic acid (caffeic acid ester with quinic acid), esterification decreased antioxidant activity [11]. This is in accordance with our results, in all the models utilized; chlorogenic acid was a less potent antioxidant than caffeic acid.

The relationship between phenolic content and antioxidant activity has been recently evaluated by Velioglu et al. [12] and Zheng and Wang [10]. Their results indicate that a certain correlation between phenolic content and antioxidant activity exists, but, in many cases, there was also antioxidant activity in the herbs that may be attributable to other unidentified substances or to synergistic interactions.

Our hypothesis concerning polymer polyphenols and glycosides is in accordance with Lodovici et al. [13] who showed that the antioxidant activity of red wine is not restricted to monomeric polyphenols. The fraction containing monomers displayed about the same activity as the polymeric fraction. On the other hand, constituents other than phenolics may be responsible for the antioxidant activity. Further work is in progress in our laboratory to identify compounds responsible for the antioxidant activity.

Conclusion

Both fractions from the leaves of *S. sonchifolius*, SOX and OF9, showed a potent antioxidant activity in DPPH and xanthine/XOD superoxide radical scavenging tests, inhibited the lipoperoxidation of rat liver subcellular membranes and protected rat hepatocytes against oxidative injury. These results may predetermine *S. sonchifolius* leaves as an active component in dietary supplements for the prevention of chronic diseases.

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References

1. Valentova K, Frcek J, Ulrichova J (2001) Yacon (Smallanthus sonchifolius) and Maca (Lepidium meyenii), traditional Andean crops as new functional foods on European market. Chem Listy 95:594–601
2. Singleton VL, Rossi JA Jr. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16:416–417
3. Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, Leitão SG (2001) Screening of Brazilian plant extract for antioxidant activity by the use of DPPH free radical method. Phytother Res 15:127–130
4. Yinrong L, Yeap Foo L (2001) Antioxidant activities of polyphenols from sage (Salvia officinalis). Food Chem 75:197–202
5. Buedge JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymol 52:302–310
6. Mathiensen L, Malterud K E, Sund RB (1995) Antioxidant activity of fruit exudate and C-methylated dihydrochalcones from Myrica gale. Planta Med 61:515–518
7. Berry MN, Edwards AM, Garrit GJ (1991) Isolated Hepatocytes, Preparation, Properties and Application. Elsevier, New York, pp 1–441
8. Sieuwerts AM, Klijn JGM, Peters HA, Foekens JA (1995) The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures in vitro for the assessment of growth characteristics, IC_{50}-values and cell survival. Eur J Clin Chem Clin Biochem 33:813–823
9. Bergmeyer HU, Bernt E (1974) Lactate dehydrogenase: UV-Assay with Pyruvate and NADH. In: Bergmeyer HU (ed) Methods in Enzymatic Analysis. Academic Press, New York, pp 574–576
10. Zheng W, Wang SY (2001) Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem 49:5165–5170
11. Chen JH, Ho C-T (1997) Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. J Agric Food Chem 45:2374–2378
12. Veioglu ZS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 46:4113–4117
13. Lodovici M, Guglielmi F, Casalini C, Meoni M, Cheynier V, Dolara P (2001) Antioxidant and radical scavenging properties in vitro of polyphenolic extracts from red wine. Eur J Nutr 40:74–77