Radical Scavenger and Antioxidant Activities of Selected Medicinal Plants

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Abstract

Oxidative damage that results in lipid peroxidation can inactivate cellular components and can have serious effects on the cells, probably leading to ageing as well as several diseases. In recent years much attention has been focused on this subject, especially in the field of clinical medicine. Several natural compounds from plants exhibit antioxidant and/or radical scavenger properties. It also has become clear that the direct free radical scavenging effect and/or membrane protection play an important role in the action mechanism of several old established drugs. The natural antioxidant nutrients also deserve attention as they offer the possibility to replace the optimal overall antioxidant status. The aim of the present work was to prove the phytotherapeutical significance of some officinal and popular medicinal plants on the base of their antioxidant activity due to their influence on pathological free radical reactions.

Experimental methods were planned and developed in order to measure the antioxidant, free radical scavenging, immunmodulant, membrane protecting activities and to monitor the cholesterol-, lipid peroxidation-lowering capacity of plant extracts and enriched/isolated fractions. Complex phytochemical screening and analytical standardization was carried out considering the potential bioactive constituents, which were determined by chromatographic and spectroscopic techniques. The non-specific scavenger activities of the medicinal plant extracts were studied by the chemiluminometric technique. Relationships were proved between the quantity / composition of active ingredients and scavenger activities of the individual extracts. Our results so far suggest that medicinal plants, their phytotherapeutical preparation or isolated constituents as supplements of human diets are promising as preventative agents when free radical reactions are involved in the pathogenesis of diseases. As natural antioxidants seldom work singly by themselves but as part of a synergistic action of antioxidative systems, further studies are needed.

INTRODUCTION

Free radicals are formed constantly by the body's normal use of oxygen, such as for respiration and some cell mediated immune functions. They are also found in or generated from environmental pollutants, cigarette smoke, car exhaust fumes, radiation or ultraviolet light, air pollutants, pesticides, and certain industrial solvents. Some medications and anesthetics form free radicals in vitro; they are also produced to excess in inflammatory responses and hemorrhaging. Free radicals can damage cell membranes and other vital cell components, such as genetic material in the cell nucleus, and can inactivate enzymes. Damage to body cells and molecules by oxygen containing free radicals has been implicated in a wide variety of diseases.

The unsaturated fatty acids of lipids and lipoproteins are especially susceptible to

free-radical mediated oxidation, and oxidative modification of LDL particles in the blood is believed to be an important part of the atherosclerotic process. Recently, lipid peroxidation has been suggested as a factor in degeneration of myelin in certain neurological diseases. Free radical damage to DNA is believed to play a role in initiation of carcinogenesis. Oxyradicals can also attack proteins, thereby changing their structure and ability to function.

A variety of mechanisms provides defenses against free radical damage. Important antioxidants can be synthesized readily within the body. Metal binding proteins catalyse reactions, which eliminate reactive oxyradicals. Some of these antioxidant enzymes incorporate essential trace metals, like selenium, which are often classified as antioxidant nutrients.

Certain dietary micronutrients play important roles in antioxidant defence. Vitamin C is effective in the body's aqueous compartments (plasma, cell cytosol). Vitamin E (tocopherol) and ubiquinol (coenzyme Q) provide antioxidant protection in the body's lipid phase. Carotenoids also are believed to provide antioxidant protection to lipid rich tissues. There are several so called coupled reactions or antioxidant interactions between them, which is also an important pathway for antioxidant protection in humans (Sami, 1995).

Although complex, the protection provided by the natural antioxidant scavenger system of the organism seems to be inappropriate in certain diseases or in their prevention. Therefore the so called natural - and synthetic antioxidants have been more widely employed for additional and adjuvant treatments.

Research on the antioxidative effects of medicinal plants has rapidly become an active field of modern pharmacology. Presently, antioxidants are widely used in food processing. Their effects on organisms are so important that an extensive literature has appeared related to many aspects. These include experimental studies of antioxidant phenoloids distributed in traditionally used medicinal plants and plants with potential and beneficial effects on disorders related to free radical attacks. Taking into account the possible prooxidant character of the various phenoloids, and the pros and cons of antioxidant drugs, it is important to emphasize their potential importance and their place in medicine. We have to move away from considering them as drugs for treatment of diseases, and begin to concentrate on the idea that they may be drugs for the prevention of diseases (Cao et al.,1997).

Our research group became interested about 10 years ago in proving the phytotherapeutical significance of some official or traditionally used medicinal plants on the base of their antioxidant activity due to their influence on pathological free radical reactions.

Because of the Janus-face properties of some poly-OH substituted phenoloids, many-sided in vitro and in vivo experiments had to be performed (Blázovics et al., 1993; Hänsel, 1994; Kéry et al., 1992).

MATERIALS AND METHODS

Plant Materials

Vegetable drugs involved in screening works were purchased at drugstores; specimens are deposited in the Department of Pharmacognosy, Semmelweis University. *Sempervivum tectorum* L. (Crassulaceae), for detailed studies, was collected from the Botanical Garden of the University of Horticulture, Soroksár. An herbarium specimen is deposited in the Department of Pharmacognosy, Semmelweis University, where it was identified. Medicinal plant parts used: *Anthriscus cerefolii* herba, *Anthriscus cerefolii* radix, *Calendulae* flos, *Cichoriae* herba, *Cichoriae* radix, *Epilobii* herba, *Equiseti* herba, *Filipendulae* herba, *Helychrisi* flos, *Hyperici* herba, *Millefolii* herba, *Petroselini* radix, *Petroselini* folium, *Raphani sativae* radix, *Sambuci* flos, *Sempervivi tectori* folium, *Solidaginis* herba, *Taraxaci* folium, *Taraxaci* radix, *Tiliae* flos, *Urticae* folium and *Veronicae* herba. The plant samples were extracted with water, methanol and in some

cases with *n*-hexane, chloroform, ethyl acetate and methanol. Methanol solutions were used in the chemiluminometric experiments and water solutions in the antilipoperoxidant ones. Water extracts were lyophilised before use.

Animals

Young male Wistar rats weighing 150-200 g were used. Animals were killed by decapitation, and the livers were removed. The microsomes were prepared by ultracentrifugation procedures (Blázovics et al., 1989).

Hyperlipidaemic Rats

Young male Wistar albino rats weighing 150-200 g were used. The animals were divided into three groups of 10 animals. The animals of group I were fed a normal LATI chow (Gödöllő, Hungary). Ten animals of group II were fed an atherogenic diet consisting of 2,0 % cholesterol, 20 % sunflower oil and 0,5 % cholic acid added to the control LATI chow. Ten animals of group III were fed the same lipid rich diet and treated with *Sempervivum tectorum* extract, 2 g/kg body weight, for 9 days, dissolved in the drinking water. The rats were killed by decapitation on day 9.

Reagents

Xanthine oxidase (XO) was obtained from Boehringer (Mannheim), hypoxanthine (HT), desferrioxamine (DFO) and PBS from Sigma (St. Louis), 5,5-dimethyl-pyrroline-N-oxide (DMPO) from Shonan Analytic Center (Tokyo) and recombinant human superoxide dismutase (SOD) from Nikon Kayaku (Tokyo).

Cytochrome c and NADPH were obtained from Sigma (St. Louis), glucose-6-phosphate dehydrogenase and serum bovine albumin from Calbiochem AG (Lucerne, Switzerland).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) stable radical, microperoxidase, 5-amino-2,3-dihydro-1,4-phtalazinedion (luminol), cytochrome c and NADPH were obtained from Sigma (St. Louis) and glucose-6-phosphate dehydrogenase, and serum bovine albumin from Calbiochem AG (Lucerne). All other reagents were purchased from Reanal (Budapest).

Biochemical Measurements

Malondialdehyde (MDA) production was detected as an estimate of lipid peroxidation by the thiobarbituric acid test (Ottolenghi, 1959). A molar absorption coefficient E_{532} 1 cm of 156 $mM^{-1}cm^{-1}$ was used.

The non-enzymatically induced lipid peroxidation was studied by incubating the protein suspension (1 mg/mL) in a medium of total volume 0,5 mL and containing 50 mM Tris-maleate buffer pH 6.8, 1 mM KH_2PO_4, $5x10^{-5}$ M $FeCl_3$ and various concentrations of ascorbic acid and plant extract. The incubation temperature was 37°C. Protein content of the preparation was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Natural scavenging capacity of lyophilised samples was detected by a chemiluminometric method with a Lumat LB 9051 luminometer, according to the method of Blázovics and co-workers (1999). The I_{50} was determined and expressed in μg, that is the amount of the lyophilisate which diminished emitted light of $H_2O_2/{\cdot}OH$-luminol-microperoxidase by 50 %.

Phytochemical Analysis

Determination of Polyphenol Content

The polyphenol contents of the drugs, infusions and lyophilisates were measured according to the Hungarian Pharmacopoeia (Pharmacopoea Hungarica Editio VII, 1986) spectrophotometric method at 750 nm, using pyrogallol as the reference standard. This method is based on the formation of blue coloured products by redox reaction with Folin

reagent.

Flavonoid Content Determination

Flavonoid contents were determined spectrophotometrically in the samples according to the German Pharmacopoeia (Deutsches Arzneibuch, 1996) method, measuring the flavonoids, in $AlCl_3$-complex form, of the purified ethyl acetate phase obtained after acid hydrolysis. Glycosides and aglycones were determined together in aglycone form.

High-Performance Liquid Chromatography

Chromatography was performed using a Spectra Physics HPLC system consisting of a P4000 quaternary gradient pump, a FOCUS scanning UV-VIS detector (Spectra-Physics Analytical, Fremont, CA, USA) in combination with a Rheodyne 7125 injector (20 µl loop volume) and an IBM PS/2 computer. Analysis was performed on an Eurospher 100-C8 /5µm/ reversed-phase Vertex column (250x4 mm i.d.), with precolumn (5x4 mm i.d.) (Knauer, Berlin, Germany). The eluant was acetonitrile-tetrahydrofuran-30 mM citric acid in water (pH 3.0)-methanol (29:28:526:417, v/v/v/v). The flow-rate was 1 ml/min. Peaks were identified by co-chromatography with authentic standards and/or by diode-array detection (DAD).

Statistical Analysis

Results were assessed by one-way analysis of variance (ANOVA) and represent the mean ±S.E.M. of three different measurements with two parallels.

RESULTS AND DISCUSSION

Non-specific free radical scavenging activity was measured by the chemoluminescence method. To demonstrate the scavenger activity of the extracts we investigated the reduced intensity of the $H_2O_2/^{\cdot}OH$-isoluminol, microperoxidase system in vitro. The changes in chemiluminescence intensity of the system with increasing concentrations of H_2O_2 and the samples were measured. Optimal parameters were determined in model experiments. This method can be used as the first step in the screening work as routine analyses to select the most promising plants, plant extracts or fractions (Fig. 1). Inhibition of chemiluminescence light by 50% (µL/mL sample) was calculated for some interesting lyophilised aqueous extracts (Fig. 2).

Based on this screening, some plants were selected for more detailed studies.

Sempervivum tectorum L. (Crassulaceae) is a well known plant in traditional medicine for the treatment of ear inflammation. The lyophilised extracts showed a marked dose dependent topical anti-inflammatory activity, inhibiting the oedematous response in rabbit ears to croton oil (28-64 %) within 6-24 h. Phytochemical screening of the plant extract proved the presence of a notable quantity of low molecular mass, potentially antioxidative compounds. Flavonoids, kaempferol as the only aglycone of various mono-di- and triglycosides, phenol-carboxylic acids and polysaccharides were detected and analysed (Fig. 3).

Sempervivum tectorum extract exhibits a strong inhibition of chemiluminescence, indicating a non-specific free radical scavenging activity in the chemoluminescence test. Repeating the experiment on hyperlipidemic rats it was demonstrated that *Sempervivum* extract significantly improves the total scavenger capacity of fatty liver in the $H_2O_2/^{\cdot}OH$ luminol system. The extract studied had a true superoxide scavenger activity in a cell free system. This suggests that it may also act as a direct scavenger of $O_2^{\cdot-}$ (superoxide anion) in biological samples (Fig. 4).

Sempervivum extract inhibits the lipid peroxidation induced by ascorbic acid (10^{-3}M) and iron (Fe^{3+}) (5 x 10^{-5} M) in vitro, both dose and time-dependently. Changes in the free radical level in experimental steatosis could be proved by UV-spectrophotometric detection of conjugated dienes, which appeared in peroxidized unsaturated fatty acid following free radical attack. The *Sempervivum tectorum* extract inhibited the reactions, leading to the formation of conjugated double bounds (Fig. 5). The extract showed

antilipoperoxidant activities on rat liver homogenates induced by either ascorbic acid or NADPH + Fe^{3t} in terms of malondialdehyde production (Fig. 6).

Histological investigations of the liver tissue were necessary to make sure of the lipid lowering and membrane stabilizing effects of *Sempervivum tectorum* extract.

Histopathological investigation of the fatty liver revealed the following pathomorphological changes: fatty degeneration, ballon cell degeneration, necrosis and vacuolisation. The extent of the size of the fat droplets are highly variable. After the treatment with *Sempervivum tectorum* fat tends to accumulate at the periphery of the lobulus. The hepatocytes seem to have the normal appearance without any fat or they contain only a few droplets of fat. Most of the cells showed a normal appearance. On the basis of these experiments it was confirmed that *Sempervivum tectorum* improves the structure of the hepatocytes.

Relationships exist between the quantity / composition of active ingredients and scavenger activities of the individual extracts. The best correlations were established with total phenolics of some medicinal plants (*Veronica officinalis, Epilobium parviflorum, Sempervivum tectorum),* while activities in other plants were principally influenced by caffeoyl derivatives (*Cichorium intybus)* or flavonoids (*Solidago canadensis, Anthriscus cerefolium, Taraxacum officinale).*

The versatile effects of *Sempervivum tectorum* might be due to its antioxidant and lipid lowering activities. It diminished the oxidative injuries and enhanced the defense mechanisms against the free radicals. The lipid lowering effect connected with antioxidant activity diminished the membrane alterations in experimental hyperlipidemia.

The study of antioxidant and radical scavenger properties of medicinal plants represents one of the many attempts to understand traditional phytopharmaceuticals.

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Figures

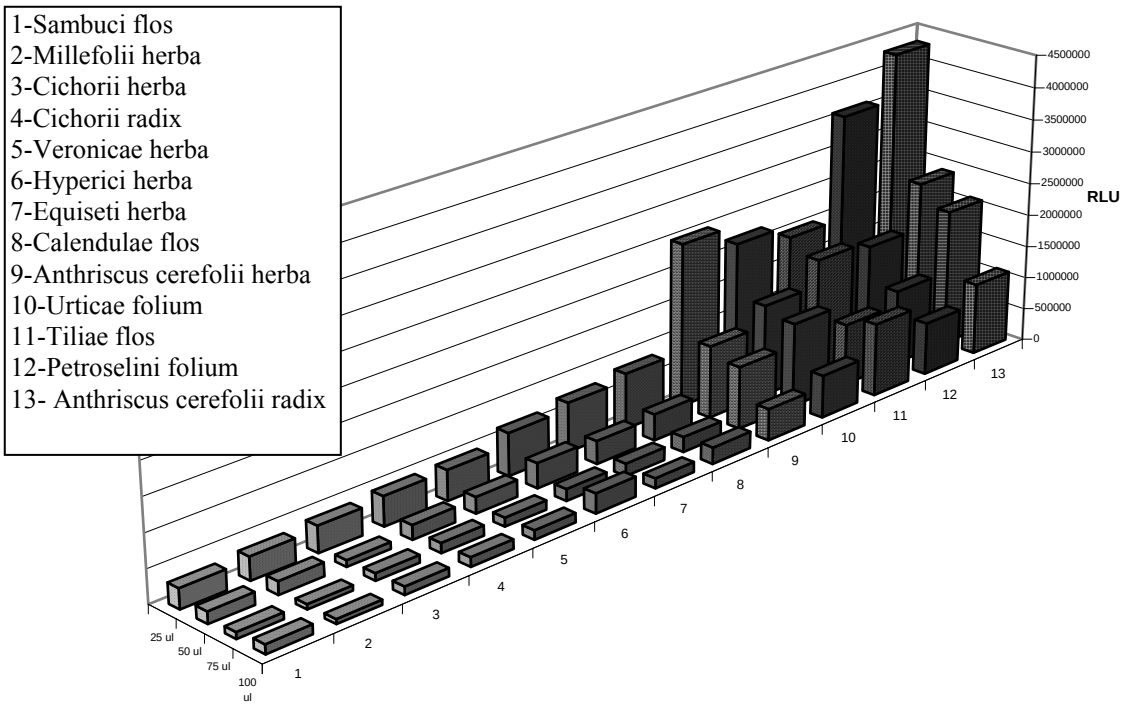

Fig. 1. Effect of different plant extracts on scavenger capacity in $H_2O_2/^{\bullet}OH$-isoluminol system

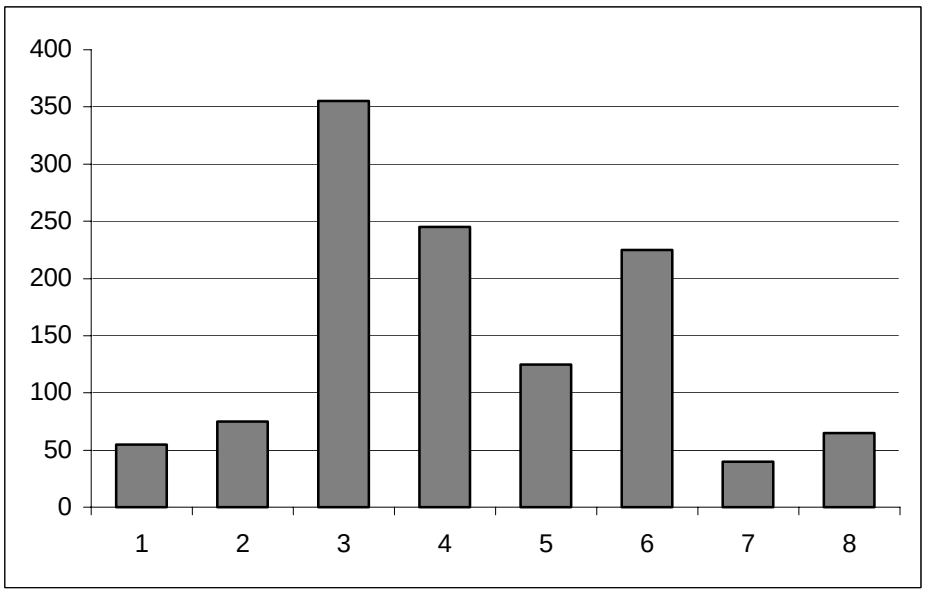

Fig. 2**.** Inhibition of chemiluminescence light by 50% (μg/mL sample) - (*1, Epilobium parvifolium; 2, Sempervivum tectorum; 3, Taraxacum officinale; 4, Taraxacum officinale; 5, Silibinin; 6, Petroselinum crispum; 7, Filipendula ulmaria; 8, Solidago canadensis)*

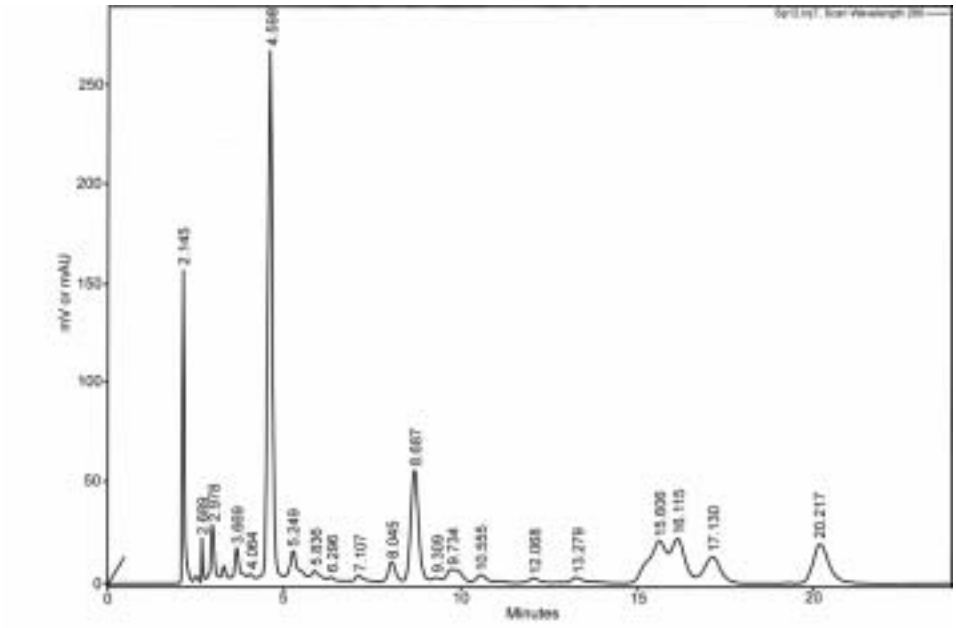

Fig. 3. HPLC fingerprint of *Sempervivum tectorum* extract

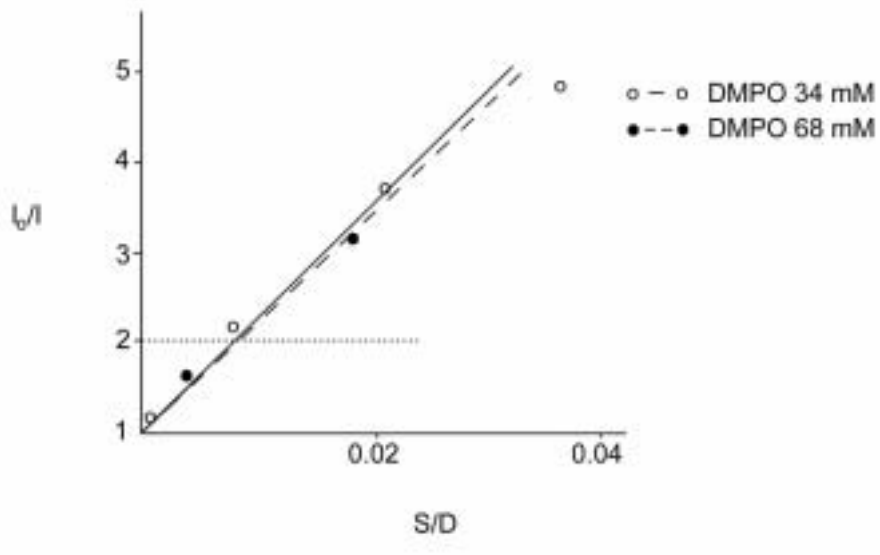

Fig. 4. Superoxide scavenger activity of *Sempervivum tectorum* extract determined by EPR spin trapping method. ESR spectra were recorded on a JES - FE / JOEL Co. Ltd. Tokyo with field set at 355 ± MT, modulation frequency of 9.42 GHz, modulation of 0.125 MT, amplitude of 1000, response of 0.3 sec, sweep time of 2 Min. and microwave power at 8 MV.I_0/I represent the ratio between the intensity of DNPO-O_2^- spin adduct in the absence (I_0) of the scavenger and that in the presence (I) of the agent. S/D is the molar ratio between the scavenger (S) and DMPO (D). Correlation was higher than 0.985.

Samples	Correlation needed to 50% inhibition of DMPO-O-formation I_{50}	Direct effect on the HT/XO system
Sempervivum tectorum extract	DMPO 34.00 mM 68 mM 0.25 mg 0.50 mg	no effect

(HT/XO = hypoxantine - xanthine oxidase)

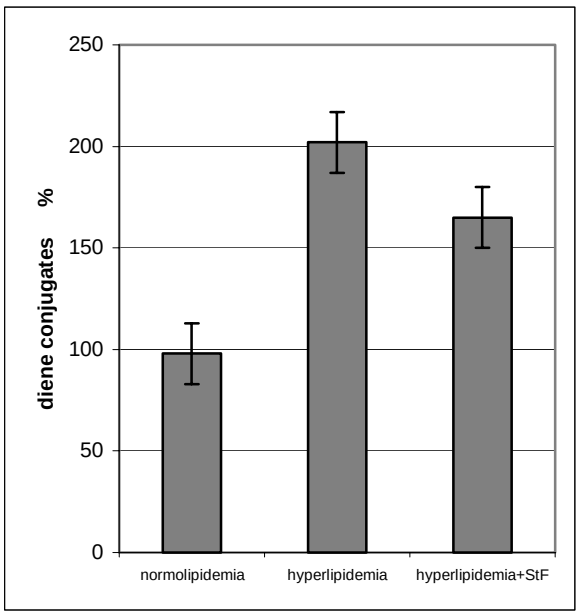

Fig. 5. Effect of *Sempervivum tectorum* on diene conjugate content in rat liver homogenates

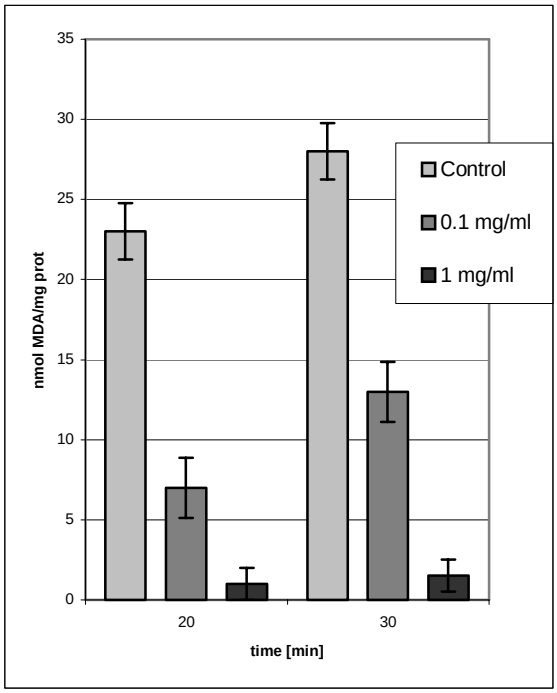

Fig. 6. Effect of *Sempervivum tectorum* extract on the NADPH+Fe^{3+} induced lipid peroxidation of rat liver microsomes