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Membrane stabilising effects of natural polyphenols and flavonoids from *Sempervivum tectorum* on hepatic microsomal mixed-function oxidase system in hyperlipidemic rats

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Abstract

The extensive role of the microsomal mixed-function oxidase (MFO) system in the oxidation of endo-and xenobiotics, in the detoxication, in the generation of reactive free radicals and in the decomposition of the end products of lipid peroxides is well documented in the literature. Steatotic liver is a very frequent damage with different etiology. Drug metabolising reactions are suppressed in fatty liver, in which pathologically increased production of reactive oxygen intermediates may lead to the peroxidation of microsomal membrane lipids and to the change of membrane bound enzyme activities because of overwhelmed protective mechanisms. The subnormal activity of the MFO system may diminish the non specific resistance of the organism. Therefore we have studied the effects of natural flavonoids and polyphenolic compounds on the mixed-function oxidases. Antioxidant, $O_2^{-\cdot}$ and $^{\cdot}OH$ scavenger properties of *Sempervivum tectorum* extract (STF1) were proved by EPR spectroscopic and chemiluminometric techniques. Potential bioactive constituents were determined by chromatography (HPLC, TLC) and spectrometric (UV, UV–VIS) methods. In the present study we reflect on the membrane stabilising, antioxidant and lipid metabolism modifying effects of this extract. It was established that activities of NAD(P)H reductase and content of cytochrome P450 were normalised in liver microsomes of hyperlipidemic rats, if the animals were treated with STF1 (2 g/bwkg for 9 days in drinking water parallel with fat-rich diet feeding). Fatty acid composition, examined by HRGLC analysis, was changed beneficially. NADPH induced lipid peroxidation was also decreased in microsomes in in vivo and in vitro experiments. At the same time the STF1 had no significant influence on MFO system in normolipidemic animals and on cytochrome *b*5 concentration of microsome fractions of hyperlipidemic rats.

Keywords: *S. tectorum* L. *Crassulaceae*; Antioxidants; Hyperlipidemy; Microsomal MFO system

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1. Introduction

There has been a growing interest in non-toxic natural antioxidants of vegetable origin. Flavonoids and other polyphenols derived from plant materials have potentially beneficial effects on human health (Bors et al., 1996). However, flavonoids carrying aromatic rings highly substituted with phenolic–OH groups generate toxic oxygen species during autooxidation at biological relevant pH presumable via semiquinones (Hodnick et al., 1986; Laughton et al., 1989).

Therapy with preparations of *Sempervivum* species can be traced back to the origins of antiquity herbal medicine, but extracts of *S. tectorum* leaves have not been introduced into medical practice yet. The earliest publications concerning the therapeutic use of this drug dealt with its actions on pharingitis, tracheitis, thrush, combustion and otitis (Bremness, 1994; Blaschek et al., 1998).

S. tectorum extract showed membrane protecting action in several liver experiments, in vitro and in vivo. The hepatoprotective effect of this extract must be related to its antioxidant, free radical scavenger and lipid level lowering properties. Presumably, *S. tectorum* extract contains compounds with immunomodulatory properties that can restore the lipid metabolism in hyperlipidemy, which exerts a suppressive effect on cellular immune response, modifies the cytokine production, therefore the regulatory role of cytokine on the lipid mechanism is altered (Kawakami et al., 1990; Kéry et al., 1992; Fritsche et al., 1993; Blázovics et al., 1992a,b,c, 1994). At the same time antioxidant characteristic of this extract protects against lipid peroxidation processes.

Many of the known constituents of *S. tectorum* leaves are present in STF1 extract (oligomeric and polymeric polyphenols, phenol carboxylic acids, ascorbic acids, flavone- and flavonol-mono and diglycosides, with kaempferol and quercetin aglycones, tannins, cumarines, oligo- and polysaccharides), but alkaloids are not present in STF1 extract. Polyphenols are present in 4.2 w/w%, flavonoids in 0.7 w/w%, polysaccharides in 11.2 w/w%. The dominant cation concentrations are: Ca (76.2 mg/g), K (40.47 mg/g), Mg (817.85 mg/g). Characteristic monosaccharides are rhamnose, arabinose, xylose, mannose, galactose, uronic acids after strong hydrolysis (Kéry et al., 1992; Szentmihályi et al., 1999; Abram and Donko, 1999).

Data for toxicity after i.p. administration are: LD50 value of 2276 mg/bwkg in male and 2098 mg/bwkg in female rats, maximal tolerance i.p. 500 mg/bwkg and p.o. 5000 mg/bwkg. in both sexes. These data indicate that the administration of STF1 extract represents a very low risk.

Free radicals can be produced in the microsomal MFO system as a result of the physiological and pathological processes (Goeptar et al., 1995). Our previous studies revealed some biochemical and morphological changes caused by free radical reactions in experimental hyperlipidemy in the pathomechanism of fatty liver. Steatotic liver, reaching peak lipid accumulation, shows a declining activity and inductibility of enzymes of microsome MFO system as well. The whole lobuli of the liver shows diffuse degenerative hepatocellular changes. Vacuolisation and necrosis can be observed in almost all liver cells, simultaneously elevated liver enzyme levels can be detected in the sera. The formation of radicals in the tissue results, among others, in lipid peroxidation, which gives rise to severe modifications in the structures of membranes, so that they can no longer maintain their proper biological functions and natural scavenger capacity is decreased significantly (Blázovics et al., 1992). Therefore the aim of this study was to examine whether enzyme inductive and antioxidant and/or antilipaemic properties of this drug extract are the main actions which protect against injury processes of MFO in the steatotic liver cells.

2. Materials and methods

2.1. Drug source and chemicals

S. tectorum (L. *Crassulaceae*) plants were from the Botanical Garden in University of Horticulture, Budapest. The plant leaves were washed,

excess water was removed, and leaves were then used for the preparation of extract following the method in SOTE Patent, No. 207657/1993. The leaves after washing with potassium sorbicum solution (0.2% w/w) and water were sliced directly into water (1:1). After 15 min of constant stirring in a blender at room temperature, the extract was obtained after centrifugation. The supernatant was filtered and lyophilized (3.2 g from 100 g fresh leaves).

Glucose-6-phosphate-dehydrogenase and serum bovine albumin were obtained from Calbiochem AG (Lucerne, Switzerland) NADPH, NADH and polyunsaturated fatty acid standard from SUPELCO FAME from SIGMA (St. Louis, USA) and all other reagents from Reanal (Budapest, Hungary)

2.2. Animals

Young male Wistar albino rats weighing 150–200 g were used. 20–20 animals were administered in each group. Normolipidemic animals (group 1) were fed with standard chow by Charles River Hungary Rt. Hyperlipidemic animals (group 2) were kept on fat rich diet consisting of 2% cholesterol, 0.5% cholic acid and 20% sunflower oil added to the normal chow for 9 days. Both normo- (group 3) and fat rich diet fed animals (group 4) were treated with STF1 extract dissolved in drinking water 2 g/bwkg/day (ad libitum) parallel with feedings. The rats were killed by decapitation at 9th day (Blázovics et al., 1994). Permission number SOTE TUKEB 24/1996.

2.3. Preparation of microsomes

Liver microsomes (in each group 5–5 liver were pooled) were prepared by ultracentrifugation method from exsanguinated liver homogenates made by Potter-Elvehjem homogenizer. The procedure was carried out in ice cold 0.15 M KCl solution. Microsomal fraction was sedimented by centrifuging the post-mitochondrial supernatant at 100 000 *g* for 90 min in VAC 60 Janetzky ultracentrifuge (Jordan and Schenkman, 1982).

2.4. Biochemical assays

Protein concentration of liver microsomes were assayed by the methods of Lowry et al. (1951). Enzymatically induced lipid peroxidation was measured in a medium of total vol. 0.5 mL with a protein content of 1 mg/mL. The medium contained 20 mM sodium phosphate buffer, pH 7.5, 0.5 mM KCl, 50 μM $FeCl_3$, 50 μM sodium pyrophosphate, glucose-6-phosphate-dehydrogenase 0.6 IU, 0.5 mM NADPH, and 10 mM glucose-6-phosphate, plus various concentrations of STF1 extract. NADPH cytochrome *c* reductase, NADH ferricyanide reductase activities (Jordan and Schenkman, 1982), cytochrome-P450 and cytochrome-*b*5 contents were also measured (Omura and Sato, 1964a,b) by the standard methods.

2.5. Preparation of fatty acids

Fatty acid analysis of liver homogenates were carried out by gas chromatographic techniques. 1 cm^3 wet liver tissue (from 5–5 pooled liver homogenates) was used for analysis. Lipid fraction was isolated with chloroform: methanol 2:1 mixture. Chloroform phase was analysed. The lipids were saponified by 5% sodium hydroxide in 50% aqueous methanol and the fatty acids were esterised by 10% boron trifluorid methanol reagent. The fatty acids were extracted by hexane and purified by silica gel column chromatography according to Alexander and Justice (1985).

2.6. HRGLC analysis of fatty acids

Gas chromatograph: Hewlett-Packard 5890 with flame ionisation detector and split capillary injector was used for the analysis. Column: SP2340 silica (60 m × 0.32 mm, 0.25 μM film thickness), injector: split, split ratio:1:1000, temperature: column oven 180°C, injector:220°C, detector 220°C, carrier gas: hydrogen: 1.5 cm^3/min, detector gas: hydrogen: 30 cm^3/min, air: 400 cm^3/min, nitrogen: 30 cm^3/min (Kemény et al., 1990).

The peaks were identified by the comparison with the behaviour of a known standards of SUPELCO FAME mixtures.

2.7. Statistical analysis

Statistical analysis was performed with the Student's t test. Confidence limits were added at $P < 95\%$ (each data represents average of four consecutive animal experiments (20–20 rats in each group) with 3–3 parallel examinations of pooled samples (5–5 animals)).

3. Results

Liver weight/body weight ratios, cytochrome P450, cytochrome $b5$ concentrations and the activities of NADPH cytochrome c reductase and NADH ferricyanide reductase were not significantly different in the liver microsomes between STF1 lyophilised extract treated animals and controls. Significant difference ($P < 0.05$) were obtained regarding the cytochrome $b5$ and cytochrome P450 contents and the activity of NADH ferricyanide (cytochrome $b5$) reductase, and no significant decrease was measured in the activity of NADPH cytochrome c (cytochrome P450) reductase as a consequence of necrotic processes in the fatty liver. STF1 extract treatment caused beneficial effects on the microsomal enzymes, except on cytochrome $b5$ content. The rate of its improving effect, except the cytochrome P450 content, was not significant in experimental hyperlipidemy (Table 1).

Table 1
In vivo effect of *Sempervivum tectorum* extract on the hepatic microsomal enzymes in normo- and hyperlipidemic rats

Samples	Normollpidemy[a]		Hyperlipidemy[a]		Significance $P<0.05$
	Without STF-1 (group 1)	With STF1 (group 3)	Without STF1 (group 2)	With STF1 (group 4)	
NADPH cytocrome c reductase (nmol. red.cyt.c/mg prot. min)	89.92 ± 18.64	84.22 ± 11.24	56.82 ± 17.97	78.86 ± 13.3	1 vs. 3 n.s. 2 vs. 4 n.s. 1 vs. 2 n.s. 1 vs. 4 n.s.
NADH ferricyanide reductase (nmol. ferrocyanide/mg prot. min)	2461.8 ± 386.4	2273.0 ± 279.7	1666.8 ± 397.0	2187.0 ± 379.9	1 vs. 3 n.s. 2 vs. 4 n.s. 1 vs. 2 s. 1 vs. 4 n.s.
Cytocrome P450 content (nmol/mg prot.)	0.530 ± 0.106	0.556 ± 0.132	0.350 ± 0.043	0.480 ± 0.070	1 vs. 3 n.s. 2 vs. 4 s. 1 vs. 2 s. 1 vs. 4 n.s.
Cytocrome $b5$ Reductase (nmol/mg prot.)	0.419 ± 0.025	0.411 ± 0.031	0.286 ± 0.079	0.287 ± 0.081	1 vs. 3 n.s. 2 vs. 4 n.s. 1 vs. 2 s. 1 vs. 4 s.
Liver weight/body weight ratio	0.0413 ± 0.011	0.0419 ± 0.012	0.0506 ± 0.009	0.0467 ± 0.008	1 vs. 3 n.s. 2 vs. 4 n.s. 1 vs. 4 n.s.

[a] ($\bar{x} \pm h1$; $h2$).

Table 2
Most important changes in fatty acid composition of liver in normo- and in hyperlipidemic rats

Fatty acids	Normolipidemy (group 1)[a]	Normolipidemy + STF1 treatment (group 3)[a]	Hyperlipidemy (group2)[a]	Hyperlipidemy + STF1 treatment (group 4)[a]	Significance ($P<0.05$)
C16	18.5 ± 1.3	19.0 ± 1.1	8.5 ± 0.4	9.8 ± 1.2	1 vs 2
C18	18.6 ± 0.6	16.8 ± 0.4	9.3 ± 0.4	11.4 ± 0.9	1 vs 2
C18 1ω9	13.4 ± 0.6	11.8 ± 0.5	18.1 ± 1.6	16.5 ± 0.4	1 vs 2
C18 2ω6	11.9 ± 1.5	15.8 ± 1.6	39.6 ± 2.2	33.8 ± 1.9	1 vs 2
C20 4ω6	18.6 ± 0.2	20.4 ± 0.3	10.2 ± 0.5	14.4 ± 0.9	1 vs 2
C22 6ω3	5.1 ± 0.5	4.9 ± 0.4	2.3 ± 0.2	2.7 ± 0.3	1 vs 2

[a] $\bar{x} \pm s_x$.

The active components of *S. tectorum*, in the applied concentration, did not influence the normal liver fatty acid parameters in this 'short term' experiment, but the fatty acid composition was proved slightly in hyperlipidemy (Table 2).

The NADPH induced and Fe^{3+} stimulated lipid peroxidation in vitro and in vivo was inhibited by this drug extract (Figs. 1–3) (It has to be noted that STF1 in the presence of NADPH can reduce cytochrome *c* in vitro (Blázovics et al., 1994.)) The significant lower activity of NADPH cytochrome P450 reductase in hyperlipidemy resulted in lower induction of lipid peroxidation as compared to that of control microsome. (Figs. 1 and 2)

4. Discussion

Alimentary fatty liver is a well-established model of liver damage which causes a significant loss of the microsomal MFO system (Fehér et al., 1998). Moreover the $n-6$ and $n-3$ fatty acid contents of dietary lipids influence the enzyme activity of mixed function oxidases differently (Saito and Yamaguchi, 1994). Furthermore hyperlipidemy exerts a suppressive effect on cellular immune response, influences the cytokine production, inhibits the mitogen induced lymphoblast transformation, diminishes the tumor necrosis factor alpha production of spleen cell macrophages and enhances the spontaneous interleukin-1 activity. Cytokines play an important role in the regulation of lipid metabolism both in anabolic and in catabolic pathways (Kawakami et al., 1990). *S. tectorum* modifies the cytokine production in alimentary hyperlipidemy. The beneficial effect of *S. tectorum* extract was observed on the concanavaline — A stimulated blast transfor-

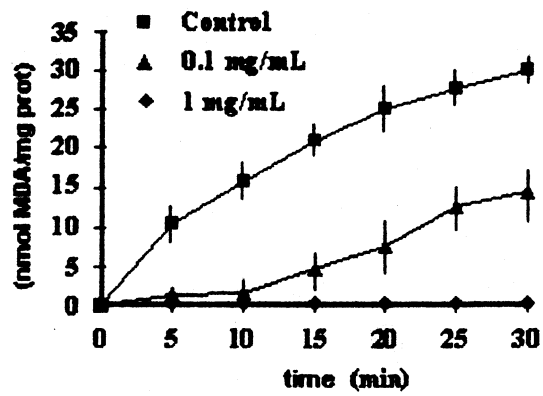

Fig. 1. Effect of STF1 on NADPH induced and iron stimulated lipid peroxidation of rat microsome in vitro (Figure shows the $\bar{x} \pm h1$; $h2$).

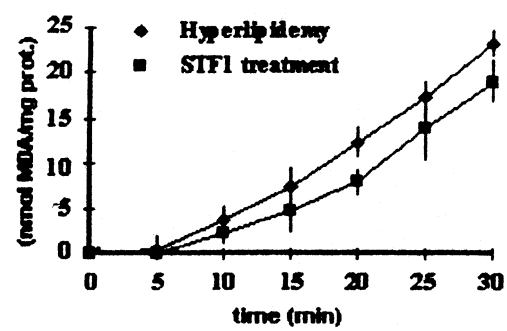

Fig. 2. In vivo effect of STF1 on NADPH induced and iron stimulated lipid peroxidation of liver microsome of normolipidemic rats (Figure shows the $\bar{x} \pm h1$; $h2$).

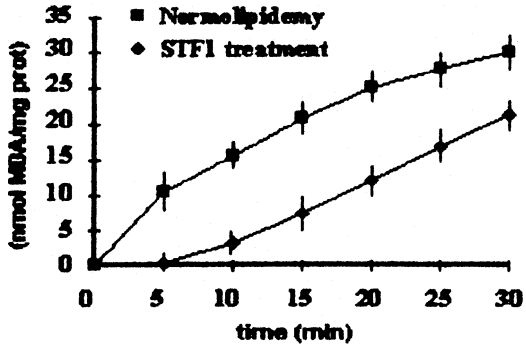

Fig. 3. In vivo effect of STF1 on NADPH induced and iron stimulated lipid peroxidation of liver microsome of hyperlipidemic rats (Figure shows the $\bar{x} \pm h1$; $h2$).

mation of splenocytes, on the lipopolysaccharide triggered tumor necrosis factor-alpha activity and on the spontaneous interleukin-1 activity of spleen macrophages of hyperlipidemic rats. At the same time diffuse hepatocellular degeneration caused by fat rich diet was improved and the natural scavenger capacity of the liver was increased by STF1 extract treatment initiating restoration of liver function (Blázovics et al., 1994).

It is the main question whether the radical scavenging activity of flavonoids, mainly quercetin and kaempferol and other polyphenols can be extended from in vitro to in vivo (Lonchampt et al., 1989.). On the other hand, the bioactive compounds of *S. tectorum* lyophilised extract can influence the MFO system in the liver. Can the antioxidant and antilipaemic treatment modify the activity of membrane bound enzymes of this system through the modification of lipid metabolism (Blázovics et al., 1994)?

On the basis of Figures (Figs. 1–3) it can be hypothesized that the active components of this extract were responsible for the lipid peroxidation decreasing effect both in normo- and hyperlipidemic rat liver microsomes. The difference of quantity of thiobarbituric acid reactive compounds between the control and sick animals depended on preservation of membrane structure and function in the necrotising processes of fatty liver (Blázovics et al., 1992). The gas chromatographic studies revealed the significant changes of fatty acid composition in fatty liver compared to that of controls, which modify the activity of membrane bound enzymes, e.g. NADPH cytochrome P450 reductase (Goeptar et al., 1995; Saito and Yamaguchi, 1994). Figs. 2 and 3 showed that the antioxidant property of this examined extract can be detected both in normo- and in hyperlipidemic groups. Other measured parameters such as activity of NADPH cytochrome *c* reductase, NADH ferricyanide reductase and concentration of cytochrome P450 of microsome were also changed beneficially, but not significantly during 'short term' antioxidant treatments as a consequence of membrane and/or protein (including enzymes) protection against free radical reactions in fatty liver (Fehér et al., 1998). The liver weight/body weight ratio was also changed in the treated hyperlipidemic animals and approached to the control value, indicating the regressive effect of this extract. These data are in accordance with immunostimulatory activity of *S. tectorum* extract, and it can be supposed that this activity modifies the lipid metabolism via cytokine production while its antioxidant effect protects against harmful lipid peroxidation and subsequent necrotic processes. It is well-known that flavonoids, especially quercetin and kaempferol have significant vitamin-protecting effect, and also have important role in the regeneration of vitamin E (Hertog and Hollman, 1998). Vitamin E is the main lipid-phase antioxidant that inhibits the oxidation of fatty acids caused by free radicals in cell-membrane bilayer. It is supposed that the bioactive compounds of the *S. tectorum* extract such as flavonoids can also stabilise the membranes via saving physiological antioxidant defence system.

5. Conclusion

The antioxidant activity of *S. tectorum* is shown both in vivo and in vitro. This extract was able to modify fatty acid ratio of liver in hyperlipidemy and this fact and the lipid lowering activity of STF1 together contribute to membrane restitution (Blázovics et al., 1992).

The component composition of STF1 extract did not induce the examined enzymes of microso-

mal P450 system, however, the enzyme activities were enhanced because of its membrane stabilising property in hyperlipidemy.

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