Original article

Hepatoprotective activity of a phytotherapeutic formula on thioacetamide - Induced liver fibrosis model

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Abstract. Hepatic fibrosis is a widespread alteration in the liver that primarily consists of increased collagen deposition in the tissue. The aim of the present study was to evaluate the protective effects of poly-phytocompound EH-1501 containing small amounts of silymarin but also other potentially effective substances on thioacetamide (TTA)-induced liver fibrosis and to elucidate the mechanisms underlying these protective effects in rats. Forty rats were randomly divided into four groups. Liver fibrosis was induced by intraperitoneal injection of 200 mg/kg body weight. TAA dissolved in saline was administered thrice a week, for 8 weeks. Groups 1 (normal healthy control) and 2 (liver injury model) received water for 8 weeks or silymarin (50 mg/kg p.o. daily) for 8 weeks (group 3) or a poly-phytocompound EH-1501 (containing grape leaf, wild strawberry, dandelion and milk thistle, EuroHealth, Italy) (200 mg/kg, daily respectively) for 8 weeks (group 4). Biochemistry and serum fibrosis markers were AST, ALT, GGT, bilirubin, thiobarbituric acid reactive substances (TBARs), hyaluronic acid and type IV collagen 7s. Liver tissue was used to assay glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), TBARs, hydroxyproline and gene expression of collagen α1 (col α1) and transforming growth factor-β1 (TGF-β1). Silymarine and EH-1501 were equally effective in reducing serum markers of liver damage and fibrosis as well as oxidative stress. However, as compared to silymarine, EH-1501 was significantly more effective in improving tissue level of GPx while decreasing TBARs and hydroproline content ($p<0.05$). When looking at gene expression of col α1 and TGF-β1, EH-1501 showed a significantly higher degree of gene down-regulation as compared to silymarine ($p<0.05$). Taken altogether, these data suggest that a natural antioxidant-containing phytocompound EH-1501 exerts an effective hepatoprotective property in experimental chronic fibrotizing liver injury to a significantly higher degree than silymarin. (www.actabiomedica.it)

Key words: liver fibrosis, antioxidants, silymarin, poly-phytocompound EH-1501, liver fibrosis

Introduction

Chronic liver disease is one of the leading causes of mortality and morbidity in the world and hepatic fibrosis is a characteristic feature of longstanding inflammatory hepatocellular damage due to a number of noxious agents such as viruses, alcohol, drugs, autoimmune phenomena and others. In particular, hepatic dysfunction due to ingestion and inhalation of hepatotoxin is worldwide increasing (1). Moreover, fibrosis can be regarded as one of the processes of tissue repair. However, when an overwhelming and qualitatively uneven deposition of extracellular matrix takes place, it occurs a liver dysfunction with sustained necrosis of

the hepatic parenchymal cells. This is associated with perisinusoidal and pericellular fibrosis and impairment of the blood flow which interferes with the exchanges of oxygen and nutrients. As a result, the remodelling of hepatic parenchyma is mainly directed by reparative mechanisms which prevails the regenerative ones.

Such dynamic tissue reparative response to hepatocellular injury is usually associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status (2, 3). Maintaining the balance between ROS and antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) is therefore crucial and could serve as a major mechanism in preventing molecular damage by oxidative stress. In this regard, reduction of oxidative stress may be a potential and effective therapeutic strategy for prevention and treatment of liver fibrosis. In the clinical practice liver fibrosis is a consequence of chronic hepatitis and involves abnormal accumulation of extracellular matrix proteins, particularly collagen I (4). The amount of collagen is assessed by determining hydroxyproline content and indicates the extent of fibrosis (5). It has been reported that regenerative nodules and liver fibrosis more prominently occur in the model injected with thioacetamide (TAA) than the one injected with CCl4, and also that the histology of the model injected with TAA is quite similar to that of human cirrhosis (6). Moreover, in the liver, TAA is S-oxidized at the thioamide group to TAA sulfoxide [CH_3-C(SO)NH_2] and subsequently to di-S-oxide [CH_3-C(SO_2)NH_2]. Reactive intermediates in this pathway covalently bind to hepatic macromolecules and eventually bring about liver damage (7, 8). Shortly after administration, TAA undergoes an extensive metabolism to acetamide and to the hepatotoxic metabolite TAA-S-oxide by the mixed function oxidase system (7-9). It has been clearly shown that free radical-mediated lipid peroxidation contributes to the development of TAA-induced liver fibrosis (10, 11).

Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices and in traditional systems of medicine. Indeed, plant–derived compounds have been an important source of several antioxidant agents where their antioxidant property is claimed to be the mechanism of hepatoprotective activity in many plants. For instance silymarin, a most published hepatoprotective agent, provides hepatoprotection against poisoning by acetaminophen, ethanol, galactosamine, thioacetamide, halothane and carbon tetrachloride (12) and hence it was used a reference drug in this study. Therefore, the present study was performed to evaluate the protective effects of poly-phytocompound EH-1501 containing small amounts of silymarin but also other potentially effective substances on TTA-induced liver fibrosis and to elucidate the mechanisms underlying these protective effects in rats.

Materials and methods

Animals. Male Wistar rats, 6-7 weeks old (weighing 150-180 g) were housed in conventional cages in an air-conditioned and temperature controlled room (23 ± 2°C) with lighting maintained at a 12-hour light/dark cycle a 12 h. Rodent chow and water were provided ad libitum. All procedures including laboratory animal use were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (13). Forty rats were randomly divided into four groups. Groups 1 (normal healthy control) and 2 (liver injury model) received water for 8 weeks or silymarine (50 mg/kg p.o. daily) for 8 weeks (group 3) or a poly-phytocompound EH-1501 (100 mg composition: 23 mg grape leaf, 23 mg wild strawberry, 2 mg dandelion, 1 mg milk thistle and 49 mg fiber. EuroHealth, Italy) (200 mg/kg, daily respectively) for 8 weeks (group 4).

TAA-induced liver fibrosis

Liver fibrosis was induced by intraperitoneal injection of TTA 200 mg/kg body weight. TAA dissolved in saline was administered thrice a week, for 12 weeks. The control group was given intraperitoneal injection of saline (0.1 ml/10 g body weight) thrice a week, and distilled water (0.1 ml/10 g body weight, p.o., daily) for 12 weeks. The phytochemical drugs were administered while the chronic injury model was induced and lasted for the whole 8 weeks observation.

At the end of the experimental period, the rats were sacrificed under CO_2 anesthesia and blood was withdrawn from the abdominal vein. Liver samples were collected and frozen in liquid nitrogen and stored at -80°C until examined for histological and biochemical studies.

Biochemistry and serum fibrosis markers

Serum level of AST, ALT, GGT and bilirubin was estimated through enzymatic procedures on a Roche/Hitachi Modular System (Roche Diagnostics GmbH, Mannheim, Germany). Lipid peroxidation was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS) in the plasma following the procedures of Niehaus and Samuelson, (14) and Jiang et al. (15), respectively. Type IV collagen 7s in the serum was assayed as ng/ml using a radioimmunoassay (Nippon DPC Corp., Tokyo, Japan) with a normal range of nomore than 6 ng/mL and hyaluronic acid was measured as ng/ml by a sandwich binding-protein assay (Corgenix, Denver, CO, USA).

Hepatic hydroxyproline concentration

Portions of the frozen right lobe of the liver were used. Liver tissue was homogenized with 10 volumes of dilution buffer. A complete hydrolysis was performed by adding 3 ml 10 N HCl to 2 ml liver homogenate in screw capped glass tubes followed by incubation at 110°C for 16 h. After cooling, the hydrolysate was filtered through a 0.45m m filter (Millipore). Each tube and filter was rinsed with 2 ml 6 N HCl, and the rinse solution was added to the first filtrate. The hydrolysates were stored at -20°C until analysis. Hydroxyproline determination was performed as described elsewhere with some modifications (16). Briefly, a 1 ml sample of the hydrolysate was added to a mixture of 1 ml of 6 N NaOH and 1 ml of citrate buffer. One millilitre 0.05 M chloramine-T solution (20% p-toluenesulfon-chloramine) in citrate–acetate buffer (pH 6.0-6.5) in the presence of *n*-propanol (1.32 M) was added to this mixture. The solution was well mixed and incubated for 20 min at room temperature. Incubations were stopped by adding 1 ml perchloric acid/*p*-dimethylaminobenzaldehyde solution in *n*-propanol (70% perchloric acid 32.5 ml, dimethylaminobenzaldehyde 18.75 g, *n*-propanol 75 ml). The color was developed by incubation for 20 min at 60°C. After cooling, the absorbance was determined at 550 nm with a UV–visible spectrophotometer within 2 h. Hydroxyproline content was calculated from standard curves using pure hydroxyproline (Sigma Chemicals Co., USA) as a standard and expressed as $\mu g\ g^{-1}$ wet weight.

Liver antioxidant assay

Glutathione peroxidase (GPx) activity was measured through the method of Lawrence and Burk (17). One milliliter 50 nM PBS, pH 7.4, containing 5 mM EDTA, 2 μM NADPH, 20 μM GSH, 10 μM NaN3, and 23 mU glutathione reductase was incubated at 37°C for 5 min. Then, 20 μL 0.25 mM H2O2 and 10 μL supernatant were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank containing all components except the supernatant was also monitored. One unit of GPx activity is reported as μmol NADPH consumed per min (i.e., μmol/min), using a molar extinction coefficient of 6.22 x 10^3 M/cm. GPx specific activity was calculated as μmol·min-1·mg protein-1. The activity of catalase (CAT) was assayed according to the method of Carrillo et al. (18) with minor modifications. The assay mixture consisted of 1.0 ml phosphate buffer (0.05 M, pH 7.0), 0.975 ml hydrogen peroxide (0.019 M), 0.025 ml of hepatic PMS (10% w/v) in a total volume of 2.0 ml. The activity was calculated at 240 nm by measuring the rate of H_2O_2 utilization with the molar extinction coefficient for H_2O_2 being 43.6 M-1 cm-1. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 M of H_2O_2 in 1 min. The activity of superoxide dismutase (SOD) enzyme in liver homogenate was determined according to the method described by Nandi and Chatterjee (19). This method is based on the ability of SOD to inhibit the auto-oxidation of pyrogallol at alkaline pH. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autooxidation with a known SOD activity. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogalol auto-oxidation.

TBARS determination in liver was assayed through spectrophotometric method and the concentration was expressed in nmol/mL (20).

RT-PCR analysis of liver fibrosis genes

Total RNA was isolated from rats liver and from Kupffer cells cultured using the acid guanidinium thiocyanate-phenol-chloroform extraction method, as described by Chomczynski and Sacchi (21). A 5-μg sample of total RNA from each liver sample underwent reverse transcription (RT) by moloney murine leukemia virus reverse transcriptase in a 50 μL reaction volume. Aliquots of the RT mix were used for amplification through the polymerase chain reaction of fragments of the collagen α1 and transforming growth factor-β1 (TGF-β1) by using the following primers: Collagen α1 74 bp Sense 5'-GGTCCCA AAGGTGCTGATGG-3'; Antisense 5'-GACC AGCCTCACCACGGTCT-3'; 5'-CATTCCTG GCGTTACCTTG-3' and 5'-CAGTGAGCACTG AAGCGAAA-3' for TGF-β1, 5'-ACATCATCCCT GCATCCACT-3' and 5'-TCTGGGATGGAATT GTGAGG-3'for β-actin. Amplified DNA underwent electrophoresis in 2% agarose, stained with SYBR Green 1 (Molecular Probes Ltd., Eugene, Oregon. USA). The expected sizes of amplified DNA were 143 bp, and 457 bp for TGF-βl and β-actin, respectively. The expression levels of all the transcripts were normalized to that of β-actin mRNA in the same tissue sample. PCR product identities were confirmed using sequence analysis., and the bands were quantified with a densitometer. The mean ratio of each group was calculated as the average from 10 animals.

Statistical analysis

Results were expressed as mean ± standard error of mean. Statistical significance was tested by employing analysis of variance (ANOVA) and comparison between means was calculated by either Duncan's multiple range test. Differences with $p < 0.05$ were considered as statistically significant.

Results

Serum markers of liver damage and liver fibrosis

Table 1 shows the activity of serum hepatic marker enzymes in normal and TTA hepatotoxic rats. The activities of AST, ALT, GGT, bilirubin and TBARs increased in TTA model rats. Treatment of rats with phyto-formula and silymarin markedly control the activity of above marker enzymes without significant difference among different treatment schedules. As for serum markers of liver fibrosis, TTA model rats showed a significant increase of hyaluronic acid and, to a lesser extent, of type IV collagen 7s ($p<0.01$, table 2). This increase was partly mitigated in both treated groups when tested at 4 weeks observation ($p<0.05$ vs untreated TTA-model) but at 8 weeks observation EH-1501 phytocompound was significantly better than silymarin ($p<0.05$) enabling a normalization of values.

Hepatic hydroxyproline concentration

Liver concentration of hydroxyproline content showed a significant increase of over 40% in TTA-

Table 1. Serum markers of liver damage and oxidative stress in TTA-model: effect of silymarin and of phytocompound EH-1501 (values referred to 8 weeks observation)

	AST U/L	ALT U/L	GGT U/L	Bilirubin mg/dL	TBARS (μM/mg protein)
Healthy control	44±7	32±9	0.3±0.1	0.6±0.1	0.60±0.02
TTA model	135±14*	87±12*	6.3±1.4*	1.2±0.3*	2.4±0.3*
TTA model + sylimarine	102±12§	57±13§	3.3±1.7§	0.7±0.1§	0.79±0.7§
TTA model + EH-1501	104±11§	49±22§	2.9±2.1§	0.7±0.2§	0.82±0.9§

Legend: Serological markers in control and TTA-model rats: alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), bilirubin and thiobarbituric acid reactive substances (TBARS) for the control group and for the TTA-model rats treated with silymarin and EH-1501 phytocompound. *$p<0.001$ vs control § $p<0.001$ vs untreated TTA-model group

Table 2. Serum markers of fibrosis markers, of liver damage and oxidative stress in TTA-model: effect of silymarin and of phyto-compound EH-1501

Weeks	Control	TTA	TTA + sylimarin	TTA +EH-1501
		Hyaluronic acid (ng/ml)		
0	8.3±4.3	5.6±3.7	6.2±4.0	5.2±3.6
4	6.7±3,6	133.8±55.6*	69.8±24.7*§	1.1±18.9*§#
8	11.3±5.4	224.6±77.5*	19.5±7.2§	12.3±5.6§#
		Type IV collagen 7s (ng/ml)		
0	4.3±0.2	4.4±0.2	4.2±0.3	4.7±0.4
4	4.2±0.2	6.2±0.5	5.1±0.5§	4.9±0.9§
8	4.3±0.6	8.7±0.4	5.3±0.1§	5.6±0.7§

Legend: Serological markers of liver fibrosis in control, TTA-model rats and TTA-model animals treated with silymarin and EH-1501 phytocompound. *p<0.001 vs control, § p<0.001 vs untreated TTA-model group group, #p<0.05 vs TTA+ silymarin

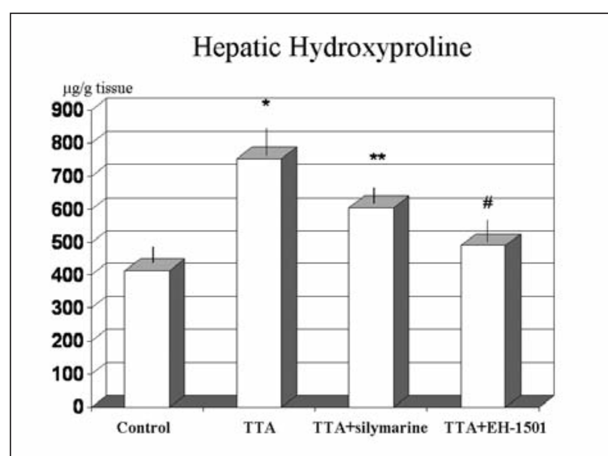

Figure 1. Hepatic hydroxyproline concentration in TTA-model: effect of silymarin and of phytocompound EH-1501(values referred to 8 weeks observation). *p<0.001 vs control, **p<0.001 vs TTA group, #p<0.05 vs TTA+ silymarin

model rats (p<0.05, fig. 1). Silymarin co-administration enabled a significant reduction of this value (p<0.05) while EH-1501 phytocompound showed an even lower concentration (p<0.05 vs silymarin-treated group). No correlation appeared between liver hydroxyproline content and serum markers of liver fibrosis or of liver damage (data not shown).

Lipid peroxidation and Enzymatic antioxidants

Table 3 shows the concentration of TBARS in the liver tissue of normal and TTA hepatotoxic rats. The levels of TBARS were significantly increased in TTA hepatotoxic rats. Oral administration of EH-1501 phyto-formula and silymarin significantly reversed these changes, the former at a more significant extent than silymarin (p<0.05). The activities of SOD, and CAT, and GPx were significantly improved in rats treated with EH 1501 phyto-formula and silymarin (p<0.01). However the latter parameter was fully restored only in EH-1501 phyto-compound (p<0.05 vs TTA-model rats treated with silymarin)

Table 3. Hepatic tissue level of lipid peroxidation and antioxidants in TTA-model: effect of silymarin and of phytocompound EH-1501 (values referred to 8 weeks observation)

	GSH-Px (µmol GSH/min·mg protein)	SOD U/mg protein	CAT (U/mg protein)	TBARs (nmol/100 mg)
Healthy control	7.4±0.5	73.4±11.9	327.8±22.3	1.2±0.3
TTA model	3.2±0.9*	44.7±10.6*	189.9±19.7*	2.6±0.3*
TTA model + sylimarine	5.5±1.1**	67.2±12.1**	278.6±14.6**	1.9±0.5**
TTA model + EH-1501	7.4±0.8#	72.1±11.8**	318.3±23.1**	1.1±0.4#

Legend: Serological markers of oxidative stress in liver tissue in control, TTA-model rats and TTA-model animals treated with silymarin and EH-1501 phytocompound. *p < 0.01 vs control values **p < 0.05 vs baseline values; #p<0.05 vs TTA+ silymarin

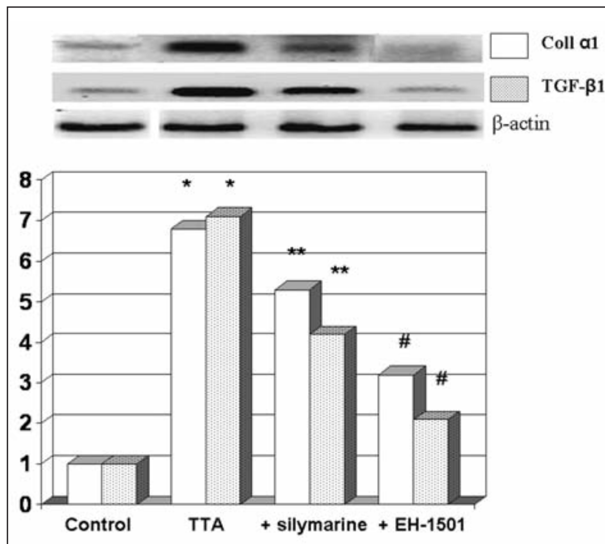

Figure 2. Relative expression activities of genes involved in extracellular matrix modulation in the treated groups and control group. The means identified with different letters (a, b, c) were significantly different ($p < 0.05$). Col-α1 = collagen-α1; TGF-β1 = transforming growth factor-β1; *p < 0.01 *vs* control values **p < 0.05 *vs* baseline values; #p<0.05 vs TTA+ silymarin

Collagen (α1) and TGF-β1 mRNA Expressions

The collagen α1/ β-actin and TGF-β1/β-actin ratios in the TTA group were over 6 times higher than those in the control group (Fig. 2). Treatment with silymarine and EH-1501 led to a significant down-regulation of both genes ($p<0.05$) although the reduction of collagen α1/ β-actin and TGF-β1/β-actin ratios exerted by EH-1501 was significantly more pronounced ($p<0.05$ vs silymarin).

Discussion

Chronic liver injury invariably leads to excessive deposition of collagen resulting in the irreversible endpoint of cirrhosis, which is one of the most common causes of death worldwide. Thus, prevention and/or suppression of fibrotic changes in the liver are of vital importance. In such peculiar set up, fibrosis is not a simple deposition of excess matrix in liver tissue since this phenomenon is associated with a change in the type of matrix molecules (including collagens, glycoproteins and proteoglycans) and histological redistribution of the matrices (22). In the normal liver 5 types of collagen molecules (type I, III, IV, V and VI) have been identified so far. Type I and III collagens account for one third of total collagens in the liver, respectively. When hepatic fibrosis develops, all types of collagens increase their amount, among which type I collagen shows a predominant increase, comprising a half of all hepatic collagens. Potential inhibitors of hepatic fibrosis (23-26) have been reported but none of them has been successfully used for the treatment of patients while showing a number of limitations in the clinical use. Among several drugs and phytocompounds available for liver injury, Silymarin is the most clinically popular for patients and is known to have hepatoprotective and anti-fibrotic properties (27). Therefore in this study it was used a reference standard. From our data it appeared that oral administration of phyto-formula EH-1501 was equally effective as silymarin in significantly reducing serum levels of AST, ALT, GGT, bilirubin and TBARs in TTA-model rats as an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage. TAA is a typical hepatotoxin and causes centrilobular necrosis by generation of ROS which, on their turn, triggers lipid peroxidation chain reaction, MDA being one of its most important products. Moreover, its amount can express the degree of lipid peroxidation. The hepatic concentration of TBARS, an index of lipid peroxidation, significantly increased in TTA-administred rats while an over 40-50% reduction of SOD, GPx and catalase occurred in both treated groups. GSH acts as an intracellular or extracellular antioxidant in association with various enzymatic processes and multiple intracellular functions include detoxification of reactive oxygen intermediates and reduction of low-molecular weight thiols, sulfides and mixed disulfides of proteins (28). It has been suggested that the lipid peroxidases generated after liver intoxication are eliminated by GSH peroxidase in the presence of GSH. This is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for GPx. In the current study, we observed a

significant decrease in hepatic GPx levels in TTA-administrated rats which was reversed by treatment. In this respect, the improvement obtained in EH-1501-treated rats, either for GPx and TBARs, proved to be significantly higher than in those treated with only silymarin ($p<0.05$).

Oxidative stress resulting from the increased production of ROS and lipid peroxides is suggested to be associated with the proliferation, activation, collagen synthesis and migration of hepatic stellate cells (HSC) either directly or through paracrine stimulation of neighboring cells including damaged hepatocytes (29, 30). This has already been confirmed in the past by the observation that HCS activation by type 1 collagen is blocked by antioxidants (31), suggesting that lipid peroxidation may play a role in hepatofibrogenesis. Therefore, a number of studies have focused on the pathogenetic significance of oxidative stress in liver injury, as well as on the therapeutic intervention of this process with antioxidant and metabolic scavengers. Interestingly, when looking at the liver content of hydroxyproline, in our study suggested that the antifibrotic effect of EH-1501 was significantly better than silymarin. In particular this phyto-compound exerted a greater down-regulating effect on col α1 which is known to contribute to the increase of collagen content in fibrotic liver and its expression reflect the degree of hepatic fibrosis (32). Similarly, as compared to silymarin-treated rats, EH-1501 treatment yielded a more significant decreased expression of TGF-β1, cytokine that plays a pivotal role in liver fibrosis by activating type I collagen promoters, upregulating the extracellular matrix such as collagen, laminin and fibronectin from HSC and accelerating the transformation of quiescent HSCs into myofibroblasts (33). Moreover a very small amount of silymarin, ingredients of EH 1501 phyto-compound are grape leaf and wild strawberry in major amount and dandelion to a lesser amount. While further studies aimed to specific mechanisms of action are underway, we may suggest that antioxidant properties of grape leaf (34, 35) together with supporting liver antioxidant enzyme effect shown for dandelion (36, 37) and possibly antioxidant and endothelium-dependent vasodilation exerted by berry anthocyanins (38, 39) might have played a beneficial role.

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Accepted: ....
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