Acta Biologica Hungarica 57 (4), pp. 415–422 (2006)
DOI: 10.1556/ABiol.57.2006.4.3

EFFECTS OF CITRUS FLAVONOIDS ON REDOX HOMEOSTASIS OF TOXIN-INJURED LIVER IN RAT

Erika Rapavi,[1]* Klára Szentmihályi,[2] Erzsébet Fehér,[3] Andrea Lugasi,[4] Edit Székely,[5] Tímea Kurucz,[6] Zs. Pallai[6] and Anna Blázovics[1]

[1] II. Department of Medicine, Semmelweis University, Szentkirályi u. 46, H-1088 Budapest, Hungary
[2] Chemical Research Center, Hungarian Academy of Sciences, P. O. Box 17, H-1525 Budapest, Hungary
[3] II. Department of Anatomy, Semmelweis University, Tűzoltó u. 58, H-1450 Budapest, Hungary
[4] Fodor József National Center of Public Health, National Institute of Food Hygiene and Nutrition, Gyáli út 3/A, H-1097 Budapest, Hungary
[5] Central Hospital of the Hungarian State Railways, Podmaniczky út 109–111, H-1062 Budapest, Hungary
[6] Diachem Kft., Hőgyes Endre út 4, H-1092 Budapest, Hungary

(Received: January 9, 2006; accepted: June 6, 2006)

In order to evaluate the effect of diosmin-hesperidin containing drug on redox balance and Cu, Zn, Fe and Mn concentrations of toxin-injured liver, Wistar albino rats were subjected to thioacetamide administration (500 mg TAA/l in their drinking water) with and without drug (425 mg/kg body weight/day). Animals were treated for 30 days. No significant change in the concentration of Zn, Cu, Mn and Fe in the liver was measured in TAA-treated animals compared to control. Diosmin-hesperidin mixture treatment increased levels of Fe and Zn and decreased concentration of Cu of the liver in TAA-treated animals. These alterations were not significant. Decrease of both the total scavenger capacity (TSC) and the activity of superoxide dismutase (SOD) in liver homogenates were observed in TAA-treated rats. The diosmin-hesperidin-supplemented diet also significantly decreased the TSC and activity of SOD in liver of both the control and toxin-treated animals. On the basis of results it seems that high dosage of the diosmin-hesperidin mixture induces slight changes in the Cu, Zn, Mn and Fe content of the liver, however it may decrease the scavenger capacity and the activity of SOD when applied either alone or together with thioacetamide.

Keywords: Diosmin – hesperidin – thioacetamide-induced hepatotoxicity – redox homeostasis

INTRODUCTION

The liver is an important target of the toxicity of drugs, xenobiotics and oxidative stress. Thioacetamide (TAA) is a thiono-sulfur-containing compound having liver damaging and carcinogenic action. TAA is often used in studies to induce experimental liver necrosis in various species. TAA was originally used to control the decay of oranges and then as a fungicide [5]. Recently it is being employed in leather,

* Corresponding author; e-mail: raperika@freemail.hu

textile and paper industries as an accelerator in the vulcanization of buna rubber and as a stabilizer for motor fuels [19]. Additionally, TAA serves as a replacement for hydrogen sulphide in the qualitative analysis of inorganic compounds [23]. It is very poisonous if swallowed or inhaled and is readily absorbed through the skin and may cause serious liver damage.

It is well known that free radical reactions, lipid peroxidation and disturbances in transition elements metabolism play an important role in the pathomechanism of liver damage [3, 7, 20]. Essential elements, such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) are crucial components in antioxidant defence system of the human body.

Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals [18, 28]. Mechanism of their antioxidant action can include suppressing reactive oxygen species formation, either by inhibition of enzymes or by chelating transition metal elements involved in free-radical production, intercepting reactive species, and upregulating or protecting antioxidant defence [25]. Numerous studies have shown that citrus flavonoids and their metabolites are potent antioxidants, and thus, are able to suppress many of the events of malignization and inflammation [11, 12, 14]. Hesperidin (a flavanone glycoside) and diosmin (a flavone glycoside) found in citrus fruits are active ingredients of Detralex® (a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin) used for the treatment of several illnesses of the circulatory system [8].

Some authors reported that oxidative stress is involved in TAA-induced cell injury [1, 17, 21, 27]. However, little is known about the effects of TAA on the transition elements metabolisms. In addition, the effect of the diosmin-hesperidin mixture on redox state and status of essential elements of TAA-injured liver has not been clarified so far. Therefore, the purpose of the present study was to evaluate the effect of diosmin-hesperidin containing drug on the changes in copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) concentrations and antioxidant capacity of toxin-injured liver in rats.

MATERIALS AND METHODS

Liver damage was produced in female Wistar albino rats (160–180 g) by oral administration of thioacetamide (TAA). Four groups of animals were studied: control group (C-group), control plus Detralex® (450 mg diosmin-50 mg hesperidin containing drug; Servier Laboratories) (CD-group), TAA alone (T-group), TAA plus diosmin-hesperidin containing drug (TD-group). Each treatment group consisted of seven rats. The C-group was given normal water *ad libitum.* T-group received continuous administration of 0.05% TAA solution as drinking water. C- and T-groups were fed a standard solid diet (ssniff R/M–Z+H, Ssniff Spezialdiäten GmbH, Germany). The animals in CD-group were fed with 5 g hesperidin-diosmin mixture added to the 1 kg standard chow (425 mg/kg body weight/day). The TD group was kept on the same

diet, additionally received 500 mg TAA/l in their drinking water. Animals were treated for 30 days.

After the treatment, rats were anaesthetized with Nembutal 35 mg/kg b.w. for deep narcosis. They were exsanguinated via vena cava inferior [3]. The livers were immediately removed, rinsed and homogenized 0.15 M KCl solution. Protein concentration of liver homogenates was determined by Lowry's method [10], and was adjusted to 10 mg/ml, using serum bovine albumin as the standard.

Sections of the left lobes of the rat livers were processed for light microscopy. This processing consisted of fixing the specimens in a 6% neutral buffered formalin, embedding them then in paraffin, cutting sections of 5 μm thickness, and staining with hematoxylin-eosin.

Element concentration in tissue wet weight liver homogenates was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) with Atom Scan 25 (Thermo Jarrel Ash). Sample preparation for element measurement was performed by digestion with a mixture of HNO_3 (5 ml) and H_2O_2 (2 ml) in Teflon vessels. After digestion, the samples were diluted to 25 ml with deionised water. The concentrations of Cu, Fe, Mn, Zn were determined in the samples. Three times three sec integration time, blank subtraction and background correction were applied during the measurements [22].

Total scavenger capacity was determined by a chemiluminometric method in a H_2O_2/˙OH-microperoxidase-luminol system with the use of a Lumat LB 9051 luminometer (Lumat, Berthold, Germany), according to the method of Blázovics et al. [4]. The results are expressed as the percentage of standard light of the H_2O_2/OH˙–luminol-microperoxidase system (RLU% = Relative Light Unit %).

The activity of superoxide dismutase was measured with RANSOD kit (RANSOD SD125). The determination employs xanthine and xanthine oxidase to generate superoxide-radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction.

The distribution of data was tested using the Kolmogorov Smirnov test. The results were assessed by the Student's *t*-test and represent mean ± SD, values of $p < 0.05$ were considered significant. Statistical analyses were performed with STATISTICA software (version 6.0; StatSoft Inc, USA).

This study was approved by the Regional Committee of Science and Research Ethics Semmelweis University, Permission Number TUKEB 24/1996.

RESULTS AND DISCUSSION

Liver injury induced by TAA is a well-known method of drug-induced hepatotoxicity. There is no report available on the effect of diosmin-hesperidin mixture on TAA-injured liver.

Figure 1 shows the light microscopic pictures of liver sections of control and treated animals. No histological change was observed in the control group treated with

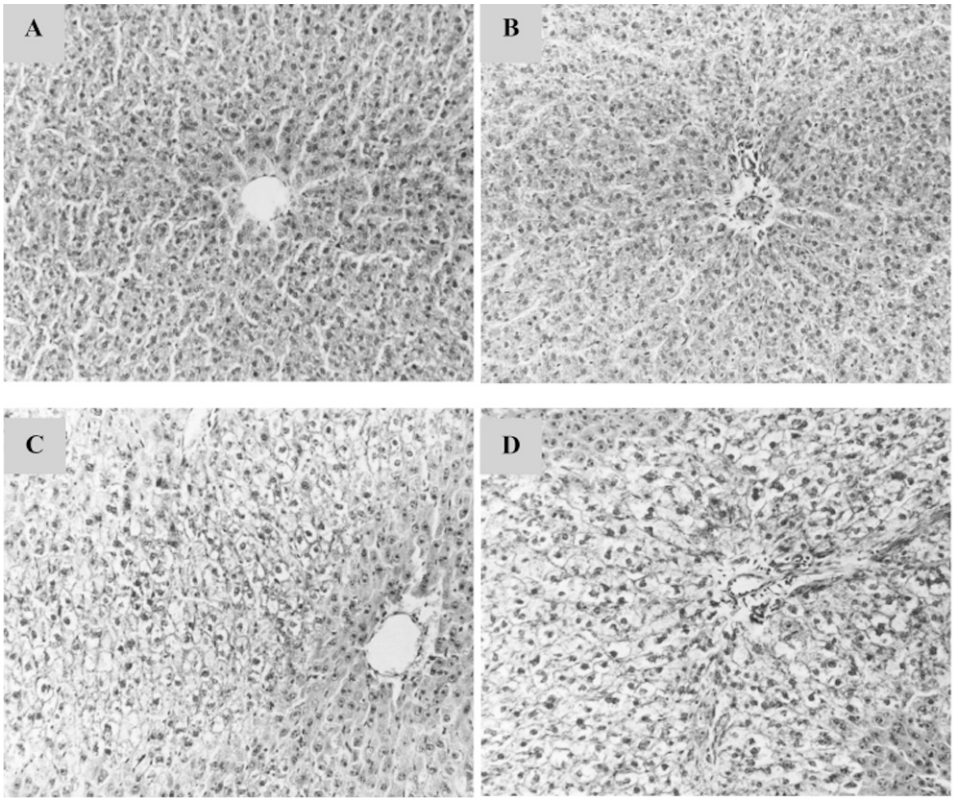

Fig. 1. Effects of thiaceatamide and diosmin-hesperidin-containing drug on liver histology. (A) Liver section from a control rat that received standard diet for 30 days. H&E, original magnification, ×250. (B) Standard diet and treatment with diosmin-hesperidin mixture for 30 days. H&E, original magnification, ×250. (C) Liver section from a TAA-treated rat that received 500 mg TAA/l in their drinking water for 30 days. H&E, original magnification, ×250. (D) TAA plus diosmin-hesperidin mixture supplemented diet for 9 days. H&E, original magnification, ×250

diosmin-hesperidin mixture as compared to the control group (Fig. 1A, B). Histopathological investigation of the liver in TAA-induced hepatotoxicity revealed vacuolisation and necrosis in the perilobular area (Fig. 1C). The livers of rats treated with parallel TAA and flavonoid mixture showed necrotic lesions as well, additionally some fibrocytes and collagen fibers were observed in the centrilobular area of the lobuli (Fig. 1D). The tissue destruction during the feeding period could be deteriorated by high dosage of diosmin-hesperidin mixture.

Pro-oxidant or antioxidant activity of flavonoids is dependent on the redox state of their biological environment (e.g. metal concentration) [18]. Accumulation of the metals in higher concentration may inhibit enzyme activities, influence the gene

expression and the pro-oxidant and antioxidant forms of scavenger molecules [20]. In our present study, the animals received continuous administration of 0.05% TAA solution as drinking water for 30 days. The effect of this treatment on hepatic essential elements content has not been clarified so far. Study by Al-Bader et al. [2] reported that liver manganese levels were significantly reduced in the TAA-induced cirrhotic rats. Metze et al. [13] noted that acute TAA intoxication causes a significant increase in copper and zinc levels in the dystrophic liver of rats. Dashti's study [6] demonstrated that copper content of the liver was increased significantly by a single subcutaneous administration of TAA after 24 and 48 h. Liver zinc content increased at 24 h and returned to normal after 48 h [6]. In the present study, no significant decrease in the concentration of zinc, copper and manganese, and non-significant increase one of iron in the liver was measured in TAA-treated animals compared to control (Table 1). These results might be attributed to differences in severity of liver damage induced by the different route of TAA administration.

Santus et al. [16] reported that diosmin-hesperidin mixture is a strong inhibitor of Cu(II)-induced arachidonic acid peroxidation. Diosmin is a good complexant of Cu(II) ions but not of Fe(II) ions. In the contrary, in the study by van Acker et al. [25] it was observed that diosmin is good Fe(II)-chelator, hesperidin chelates Fe(II) weakly. In this study we measured that diosmin-hesperidin mixture treatment increased levels of Fe and Zn and decreased concentration of Cu of liver in TAA-treated animals (TD-group) compared to the only TAA-treated animals. However in CD-group the citrus flavonoid-treatment had an inverse effect on the levels of examined elements but these alterations were not significant (Table 1). Ortega et al. [15] reported that chronic oral intake of TAA caused not only hepatic alterations but also small intestine alterations. In addition, essential element metabolism could be disturbed as well.

The determination of the tissue total scavenger capacity with the use of chemiluminometry is advantageous because the tissue defence mechanism can be measured with this technique [4]. The alteration of redox parameters was observed in this animal model, i.e. decrease of both the total scavenger capacity and the activity of superoxide-dismutase (C: 3.99 ± 0.13; T: 3.69 ± 0.26; C vs T $P < 0.05$) in liver homo-

Table 1
Concentration of Cu, Fe, Mn, Zn in liver homogenates of control and treated rats. Values are expressed in mean ± standard deviation

	C	CD	T	TD
Cu	2.09 ± 0.10	2.14 ± 0.03	2.06 ± 0.04	1.85 ± 0.02**
Fe	159.0 ± 24.04	136.7 ± 1.53	176.5 ± 17.7	187.0 ± 14.14
Mn	1.26 ± 0.11	1.19 ± 0.02	1.09 ± 0.04	1.10 ± 0.06
Zn	16.5 ± 0.71	15.0 ± 1.00	14.5 ± 0.71	17.5 ± 2.12

** Significance vs T group.

genates in TAA-treated rats (Figs 2 and 3). These data are in concordance with the literature [1, 17, 21, 27].

Hesperidin and diosmin have antioxidant and anti-inflammatory properties [11, 12, 14]. Hesperidin (200 mg/kg) has a protective effect in CCl_4 induced oxidative stress in rat liver and kidney [24]. Villa et al. [26] found that diosmetin (main metabolite of diosmin) protected against both erythromycin estolate and tert-butyl-hydroperoxide toxicity. Lahouel et al. [9] also reported that oral administration of diosmin and quercetin in the form of propolis extract (60 mg/kg/day) for 14 days

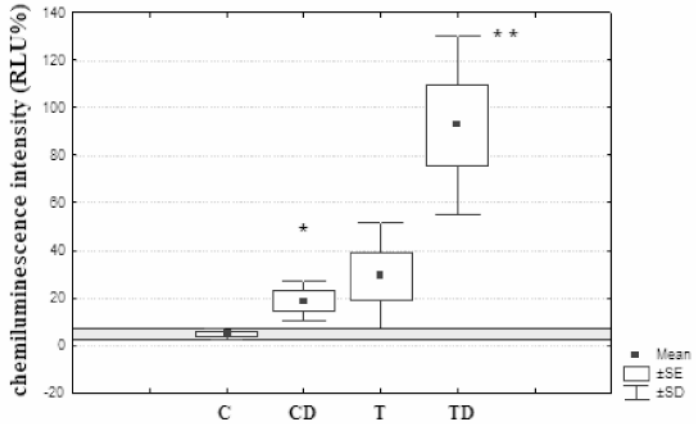

Fig. 2. Total scavenger capacity of liver homogenates in control and treated rats. Values are expressed in mean ± standard deviation. * Significance vs C-group; ** Significance vs T-group; Higher chemiluminometric intensity indicates a lower total scavenger capacity

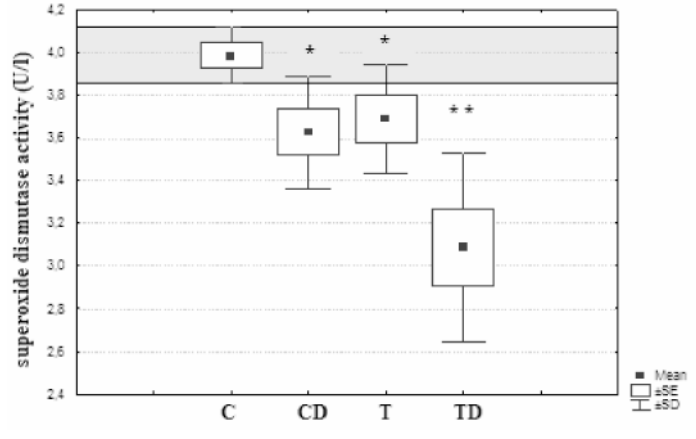

Fig. 3. Activity of superoxide-dismutase in liver homogenates of control and treated rats. Values are expressed in mean ± standard deviation. * Significance vs C-group; ** Significance vs T-group

reduced the effect of cyclophosphamide, vinblastine and paracetamol hepatotoxicity. Our results indicate that treatment with a drug containing diosmin and hesperidin, in high concentration (450 mg/kg body weight/day), significantly decreased the total scavenger capacity in CD- and TD-group compared to the C- and T-group (C: 4.81 ± 2.12; CD: 18.75 ± 8.27; T: 29.36 ± 22.37; TD: 92.60 ± 37.46; C vs CD and T vs TD $P < 0.02$, respectively) (Fig. 2). Moreover there was a trend toward decreased activity of SOD in the samples (C: 3.99 ± 0.13; CD: 3.63 ± 0.26; T: 3.69 ± 0.26; TD: 3.09 ± 0.44; C vs. CD, C vs. T and T vs. TD $P < 0.05$, respectively) (Fig. 3). The data from our study show that the high dosage diosmin-hesperidin mixture supplementation may augment oxidative stress and hepatotoxicity induced by the TAA-treatment in rats.

In conclusion, on the basis of results it seems that high doses of diosmin-hesperidin mixture induce slight changes in the Cu, Zn, Mn and Fe content of the liver, however it may decrease the scavenger capacity and the activity of SOD of liver when applied either alone or together with thioacetamide. Further studies would be necessary to determine the exact mechanism underlying this pro-oxidant effect.

ACKNOWLEDGEMENTS

The authors wish to express gratitude to Mrs. Sarolta Bárkovits, Mrs. Edina Pintér and Mrs. Erzsébet Bíró for their excellent technical assistance. The study was supported by 2/1. Ph.D Program of Semmelweis University, by Széchenyi Projects, nos. 1/016/2001 and 1/047/2001 and by the NKFP 1/047/2004 project.

REFERENCES

1. Abul, H., Mathew, T. C., Dashti, H. M., Al-Bader, A. (2002) Level of superoxide dismutase, glutathione peroxidase and uric acid in thioacetamide-induced cirrhotic rats. *Anat. Histol. Embryol. 31,* 66–71.
2. Al-Bader, A. A., Mosawi, M. H., Hussain, T. A., Dashti, H. M. (2000) Effect of dietary selenium, zinc and allopurinol supplements on plasma and tissue manganese levels in rats with thioacetamide-induced liver cirrhosis. *Mol. Cell. Biochem. 173,* 121–125.
3. Blázovics, A., Fehér, E., Fehér, J. (1992) Role of free radical reactions in experimental hyperlipidemia in the pathomechanism of fatty liver. In: Csomós G, Fehér J (eds) *Free Radicals and Liver.* Spinger, Berlin, pp. 98–126.
4. Blázovics, A., Kovács, Á., Lugasi, A., Hagymási, K., Bíró, L., Fehér, J. (1999) Antioxidant defense in erythrocytes and plasma of patients with active and quiescent Crohn disease and ulcerative colitis: a chemiluminescent study. *Clin. Chem. 45,* 895–896.
5. Childs, J. F. L., Siegler, E. A. (1945) Uses of thioacetamide in agriculture. *Science 102,* 68–72.
6. Dashti, H., Jeppsson, B., Hagerstrand, I., Hultberg, B., Srinivas, U., Abdulla, M., Joelsson, B., Bengmark, S. (1987) Early biochemical and histological changes in rats exposed to a single injection of thioacetamide. *Pharmacol. Toxicol. 60,* 171–174.
7. Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., Lemasters J. J. (2002) Mechanisms of hepatotoxicity. *Toxicol. Sci. 65,* 166–176.
8. Labrid, C. (1994) Pharmacologic properties of Daflon 500 mg. *Angiology 45,* 524–530.

9. Lahouel, M., Boulkour, S., Segueni, N., Fillastre, J. P. (2004) [The flavonoids effect against vinblastine, cyclophosphamide and paracetamol toxicity by inhibition of lipid-peroxydation and increasing liver glutathione concentration]. *Pathol. Biol. (Paris). 52,* 314–322.
10. Lowry, S. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin-phenol reagent. *J. Biol. Chem. 193,* 265–275.
11. Manthey, J. A., Guthrie, N., Grohmann, K. (2001) Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Current Med. Chem. 8,* 135–153.
12. Manuel, Y., Keenoy, B., Vertommen, J., De Leeuw, I. (1999) The effect of flavonoid treatment on the glycation and antioxidant status in type 1 diabetic patients. *Diabetes Nutr. Metab. 12,* 256–263.
13. Metze, K., Brandt, G. (1981) Copper and zinc content of liver, heart, skeletal muscle, and brain, in acute thioacetamide intoxication of rats. *Hepatogastroenterol. 28,* 99–101.
14. Miyake, Y., Yamamoto, K., Tsujihara, N., Osawa, T. (1998) Protective effects of lemon flavonoids on oxidative stress in diabetic rats. *Lipids 33,* 689–695.
15. Ortega, M. A., Torres, M. I., Fernández, M. I., Rios, A., Sánchez-Pozo, A., Gil, A. (1997) Hepatotoxic agent thioacetamide induces biochemical and histological alterations in rat small intestine. *Dig. Dis. Sci. 42,* 1715–1723.
16. Santus, R., Perdrix, L., Haigle, J., Morliere, P., Maziere, J. C., Maziere, C., Labrid, C. (1991) Daflon as a cellular antioxidant and a membrane-stabilizing agent in human fibroblasts irradiated by ultraviolet A radiation. *Photoderm. Photoimmun. Photomed. 8,* 200–205.
17. Sanz, N., Diez-Fernandez, C., Fernandez-Simon, L., Alvarez, A., Cascales, M. (1995) Relationship between antioxidant systems, intracellular thiols and DNA ploidy in liver of rats during experimental cirrhogenesis. *Carcinogenesis 16*, 1585–1593.
18. Shahidi, F., Wanasundara, P. K. J. P. D. (1995): Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr. 61,* 549–554.
19. Sittig, M. (1985) *Handbook of Toxic and Hazardous Chemicals and Carcinogens.* 2nd ed. Noyes Publications, Park Ridge, NJ., pp. 856–879.
20. Stohs, S. J., Bagchi, D. (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Rad. Biol. Med. 18,* 321–336.
21. Sun, F., Hayami, S., Ogiri, Y., Haruna, S., Tanaka, K., Yamada, Y., Tokumaru, S., Kojo, S. (2000) Evaluation of oxidative stress based on lipid hydroperoxide, vitamin C and vitamin E during apoptosis and necrosis caused by thioacetamide in rat liver. *Biochim. Biophys. Acta 1500,* 181–185.
22. Szentmihályi, K., Then, M. (2000) Teas of *Equiseti herba, Myrtilli folium* and *Salviae folium*. *Acta Aliment. Hung., 29,* 43–49.
23. The United States Pharmacopoeia 24, USP Convention Inc., Rockville, 2000.
24. Tirkey, N., Pilkhwal, S., Kuhad, A., Chopra, K. (2005) Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. *BMC Pharmacol. 31,* 5: 2.
25. van Acker, S. A. B. E., de Groot, M. J., van den Berg, D.-J., Tromp, M. N. J. L., den Kelder, G. D.-O., van der Vijgh, W. J. F., Bast, A. (1996) A quantum chemical explanation of the antioxidant activity of flavonoids. *Chem. Res. Toxicol. 9,* 1305–1312.
26. Villa. P., Cova, D., De Francesco, L., Guaitani, A., Palladini, G., Perego, R. (1992) Protective effect of diosmetin on in vitro cell membrane damage and oxidative stress in cultured rat hepatocytes. *Toxicol. 73,* 179–189.
27. Wardi, J., Reifen, R., Aeed, H., Zadel, L., Avni, Y., Bruck, R. (2001) Beta-carotene attenuates experimentally induced liver cirrhosis in rats. *Isr. Med. Assoc. J. 3,* 151–154.
28. Williams, J. R., Spencer, P. E. J., Rice-Evans, C. (2004) Flavonoids: antioxidants or signalling molecules? *Free Rad. Biol. Med. 36,* 838–849.