Safety and Bioavailability in Beagles of Zinc and Vitamin E Combined with Silybin and Phosphatidylcholine

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ABSTRACT

Redox stress resulting from an imbalance in the production and removal of oxygen- and nitrogen-centered free radicals can play a major role in various forms of liver dysfunction. Agents that boost the hepatocyte antioxidant network, including vitamin E, zinc, and silymarin, can support liver function. The flavanolignan silybin present in silymarin extracted from milk thistle has been used for liver dysfunction in humans and continues to be studied, especially for non-alcoholic fatty liver disease. A supplement containing vitamin E, zinc, and a phytosomal complex of silybin:phosphatidylcholine (Marin®, Nutramax Laboratories, Inc., Edgewood, Md) was evaluated in beagles for its safety and capacity to increase serum levels of the individual components. A group of 8 beagles (4 males, 4 females) was administered vitamin E, zinc, and silybin at average levels of 27.4 IU/kg, 5.2 mg/kg, and 7.9 mg/kg body weight, respectively. Vitamin E increased from a basal value of 1,314 ± 74 µg/dL to 2,995 ± 222 µg/dL at 7 days, then stabilized at 2,248-2,414 µg/dL at 14-28 days. Zinc increased from a baseline of 0.568 ± 0.029 µg/mL to 0.632 ± 0.126 µg/mL at 7 days, then stabilized at 0.85 µg/mL at 14 and 28 days. Silybin increased to a maximum of 1.31 ± 0.31 µg/mL at 3 hrs, then returned to background levels at 24 hrs. Complete blood count analyses showed no clinically significant changes from baseline in any of the parameters tested. Nine parameters on serum chemistry were detected as statistically different from baseline, but all values were within published reference ranges. This study shows that a supplement with a combination of silybin:phosphatidylcholine complex, vitamin E, and zinc can be given safely to dogs and that it produces changes in blood levels of each of the components. The study supports the use of the silybin:phosphatidylcholine complex, vitamin E, and zinc supplement for liver dysfunction.

INTRODUCTION

The development and progression of various forms of liver disease involves damage caused by reactive oxygen and reactive nitrogen species (ROS/RNS) resulting from an imbalance in the production and removal of free radicals in specific liver cell types.[1-3] Management of the inflammatory state associated with hepatic dysfunction and cirrhosis has been attempted with various supplements, including silymarin, vitamin E, zinc, S-adenosylmethionine, injectable N-acetylcysteine, and glycyrrhizin,[2,4-6] in companion animals and humans. These agents

can augment the antioxidant network in hepatocytes and increase protection against damaging free radicals. A supplement containing a combination of vitamin E, zinc, and silybin, the major active component of silymarin, is now available for companion animals. Because each of these components has different mechanisms of action and entails different issues of bioavailability and safety, a study was done in beagles to address these questions.

The rationale for combining these supplements derives from the range of causes that can produce ROS/RNS changes in the liver. Oxidative or nitrosative stress can develop in hepatocytes and sinusoidal endothelial cells as a consequence of various forms of toxic insults, both chemical and biological, resulting in activation of Kupffer cells, stellate cells, or invasion of neutrophils. Elevated levels of inflammatory cytokines, particularly TNF-α, coming from these cells can amplify free radical production, causing peroxidative damage to cell membranes, hepatocyte dysfunction, and ultimately cell death.[1,2] Inappropriate proliferation, collagen production, and fibrosis[7] may result from stellate cell activation. Increased effectiveness in attenuating oxidative/nitrosative stress may occur when two or more agents with antioxidant activity are co-administered[8] and has been observed in hepatic dysfunction.[9]

Silymarin, a mixture of polyphenols in the flavanolignan category derived from milk thistle, has a long history of use and an expanding body of basic and clinical research assessing antioxidant, anti-inflammatory, and antifibrotic effects in the liver.[10,11] Silymarin contains a mixture of flavanolignans, with silybin isomers A and B the major, active components.[12-14] Studies on silymarin or silybin alone have shown hepatoprotection against iron overload,[15] mushroom toxicity,[16] and chemical toxicity[17] as well as inhibition of stellate cell activation,[18,19] inhibition of Kupffer cell function,[20] and enhancement of liver regeneration and hepatocyte metabolism,[21,22] including bile salt production.[23]

Vitamin E has been recognized for many years for its ability as a lipophilic molecule to defend against peroxidative membrane damage by terminating free radical-induced chain reactions.[24] It also has apparent non-antioxidant functions that play a role in suppression of activated inflammatory cells, including neutrophils and macrophages.[24-27] Hepatic levels of vitamin E are decreased, even with normal plasma values, in various forms of liver disease in humans[28] and metal toxicity in dogs.[29,30] Supplementation with vitamin E has led to improvement in alanine aminotransferase and redox balance (reduced glutathione/oxidized glutathione) in dogs with chronic liver dysfunction.[29] Vitamin E protection has been observed against extrahepatic biliary obstruction,[31] Kupffer cell activation,[32] and stellate cell activation.[33,34] Some of the hepatoprotective effects of flavonoids are associated with reduction of oxidation and sparing of vitamin E.[35] In addition, vitamin E can work synergistically with other supplements in protecting against liver injury.[9]

Zinc has been used to manage conditions of copper or iron overload,[36] functioning in part by competition with metal binding sites on various proteins. Zinc is known to be essential for normal function in a wide range of metabolic, enzymatic, and transcription factor activities, with overall antioxidant activity,[37-39] especially in the liver. Unlike copper and iron, zinc is not redox active. It works as a cofactor in many enzymes and regulatory proteins, binding with very high affinity to sulfhydryl groups in regulating protein structure, enzyme activity, and ultimately redox balance.[38,39] The level of free zinc within the cell determines its net effects. Zinc is normally maintained within the cell at picomolar to low nanomolar concentrations, but is under tight, redox-dependent regulation[40] that depends upon and influences glutathione metabolism. When cytoplasmic zinc falls below these levels, it fails to regulate enzymes and gene expression that cumulatively produce its pro-antioxidant acitivity,[41,42] resulting in oxidative stress. At abnormally

high intracellular concentrations, zinc inhibits many enzymes involved in energy metabolism, resulting in excessive free radical production and oxidative stress.[41]

Additional dietary zinc may be necessary to achieve normal levels, especially in conditions of deficiency associated with inflammation and redox imbalance, in order for zinc to function normally in redox homeostasis. Metallothionein, with its multiple zinc binding sites, plays a pivotal role in zinc metabolism and is regulated by glutathione[38,43] and the redox state of sulhydryl groups at these binding sites.[39] Zinc deficiency results in oxidative damage to lipids and proteins[44-46] and is associated with overt hepatic encephalopathy,[47] a condition that appears to be alleviated with oral zinc supplementation.[48-50] Zinc has been used to treat experimentally induced liver cirrhosis in rats,[51] the copper toxicity associated with Wilson's disease in humans,[52] and a rodent model of this disease,[53] as well as copper toxicosis in dogs.[54,55]

The interplay of endogenous antioxidants in the antioxidant network allows agents acting by multiple, overlapping mechanisms to cumulatively provide more effective prevention or management of liver disease. When used together, vitamin E and zinc have been shown to act synergistically to protect against iron-mediated lipid peroxidation.[36] Silybin complexed with phosphatidylcholine in a phytosomal form has been shown to be markedly more bioavailable in both rodents[56] and humans.[57,58] A combination of this complex and vitamin E was shown to be both hepatoprotective and antifibrotic in a rodent model of chemically induced liver toxicity.[18] This more bioavailable phytosomal form is now available in a stable combination with both vitamin E and zinc. This single supplement has the potential to improve liver function more so than one agent alone. In this study, the safety of this combination and its capacity to increase serum levels of the components were assessed in beagles.

MATERIALS AND METHODS

The study protocol was reviewed and approved before study initiation by an Institutional Animal Care and Use Committee (IACUC) and complied with the Animal Welfare Act. Eight healthy, adult, purpose-bred beagles were used (4 males and 4 females), weighing 10.14-12.49 kg at the start of the study.

Dogs were fed a standard colony diet (Joy Special Meal, Joy Pet Foods, St. Mary's, OH) that contained not less than 26% protein and 12% fat and not more than 4% crude fiber and 10% water. Baseline blood samples were collected for silybin, vitamin E, zinc, and complete blood count (CBC) and serum chemistries prior to dosing. On Day 1 of dosing, dogs received 178 mg silybin (A+B) equivalents complexed with phosphatidylcholine (SPC), 616 IU d-α-tocopheryl acetate, and 118 mg elemental zinc as zinc gluconate (Marin®, Nutramax Laboratories, Inc.), along with 10 mL of water to assure timely entry into the stomach. Blood samples were drawn 24 hours and immediately prior to dosing, then at 0.5, 1, 2, 4, 6, 8, and 24 hours for silybin pharmacokinetics, which are reported elsewhere (Griffin et al, submitted for publication). Dosing was continued for 27 additional days with 89 mg sylibin, 308 IU d-α-tocopheryl acetate, and 59 mg elemental zinc, with averages over this period of 7.9 mg/kg, 27.4 IU/kg, and 5.2 mg/kg body weight of silybin, vitamin E, and zinc, respectively. Blood was drawn again prior to dosing on Days 7, 14, and 28 for vitamin E, zinc, and CBC and serum chemistries. Blood samples drawn for silybin analysis were collected in lithium-heparin tubes, centrifuged, and plasma samples stored at -70°C or -80°C until analyzed. Plasma or serum samples were analyzed for silybin (A+B) (B.T. Biotecnica, Varese, Italy), vitamin E (Colorado Veterinary Diagnostic Laboratories, College of Veterinary Medicine and Biomedical Sciences), zinc (Colorado Veterinary Diagnostic Laboratories, College of Veterinary Medicine and Biomedical Sciences), and

CBC and serum chemistries (Antech Diagnostics Laboratory).

A repeated measures analysis of variance (ANOVA) was used to assess for any overall differences in vitamin E, zinc, or CBC and serum chemistries over time at an alpha level of 0.05. The Tukey-Kramer Multiple-Comparison test was used for post-hoc analysis of differences between the time points.

RESULTS

Palatability and Dosing Regimen

Initial dosing at 118 mg of elemental zinc after a 23-hour fast resulted in vomiting. All dogs consumed this same level when administered on a full stomach with no vomiting. Difficulties of acceptance of zinc have been encountered and vary with individual dogs.[2] It is recommended to administer the supplement on a full stomach.

Complete Blood Count and Serum Chemistry

Over the 28-day period of dosing, only one change in the 13 CBC parameters was observed. There was a statistically significant increase in the hematocrit between baseline and Days 14 and 28, but the mean values were well within reference ranges (Table 1). All serum chemistry values fell within the normal adult reference range during the supplementation period, except for calcium, which was increased on Day 0. Five parameters (urea nitrogen, aspartate aminotransferase, sodium, chloride, and creatine phosphokinase) exhibited a statistically significant change from baseline at either 14 or 28 days; 4 of the parameters (blood urea nitrogen/creatinine ratio, total protein, calcium, and globulin) exhibited a statistically significant change from baseline at both 14 and 28 days (Table 1).

Serum Level of Vitamin E and Zinc

Over the dosing period, vitamin E increased from a basal value of 1,314 ± 74 µg/dL to 2,995 ± 222 µg/dL at 7 days, then stabilized at 2,248-2,414 µg/dL at 14-28 days (Figure 1A). The mean basal level of vitamin E in this group of beagles fell below the mean for all dogs analyzed by the Colorado State Clinical Pathology Laboratory, but increased to a level significantly above this average. Adjustment of vitamin E for plasma total lipid, which may provide a more meaningful and reliable parameter for changes,[59,60] yielded vitamin E/total lipid ratios of 7.65 ± 0.44 µg/mg at baseline, 12.71 ± 0.64 µg/mg at 14 days, and 14.08 ± 1.31 µg/mg at 28 days (Figure 1B).

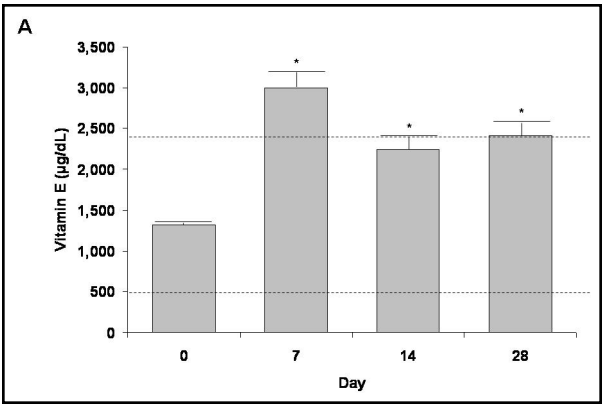
Zinc increased from a baseline of 0.568 ± 0.029 ppm (equivalent to µg/mL) to 0.632 ± 0.126 ppm at 7 days, then stabilized at 0.85 ppm at 14 and 28 days (Figure 2). These values compare with those observed with supplementation of other zinc salts in puppies.[61,62]

Table 1: Mean Parameters (± SEM) That Were Statistically Significant Difference From Day 0

Parameter	Day 0	Day 14	Day 28	Adult Reference Range
Hematocrit (%)	46.59 ± 1.11	50.7 ± 1.486*	49.36 ± 0.819*	36-60
Urea nitrogen (mg/dL)	11.88 ± 0.71	14.38 ± 0.86*	14.13 ± 0.69	6-25
BUN/creatinine ratio	15 ± 0.63	18 ± 0.94*	17.75 ± 0.88*	4-27
Total protein (g/dL)	5.88 ± 0.09	6.25 ± 0.13*	6.21 ± 0.11*	5.0-7.4
AST (SGOT) (U/L)	24.5 ± 2.24	30 ± 2.31*	29.25 ± 1.03	15-66
Calcium (mg/dL)	9.68 ± 0.06	9.0 ± 0.2*	10.21 ± 0.08*	8.9-9.4
Sodium (meq/L)	148.6 ± 0.42	146.1 ± 0.64*	148.1 ± 0.29	139-154
Chloride (meq/L)	113.7 ± 0.77	109.9 ± 0.55*	111.9 ± 0.51	102-120
Globulin (g/dL)	2.39 ± 0.10	2.76 ± 0.15*	2.69 ± 0.09*	1.6-3.6
CPK (U/L)	118.9 ± 9.73	138.9 ± 10.94	146.9 ± 9.97*	59-895

BUN = blood urea nitrogen; AST (SGOT) = aspartate aminotransferase (serum glutamic oxaloacetic transaminase); CPK = creatine phosphokinase.
*P < 0.05 versus Day 0.

Figure 1. Effect of supplementation over 28 days on the serum levels of vitamin E. **A:** Concentration of vitamin E (µg/dL). Dashed lines indicate testing laboratory normal ranges; **B:** Concentration of vitamin E relative to total lipids (µg/mg).*$P < 0.05$ vs Day 0.



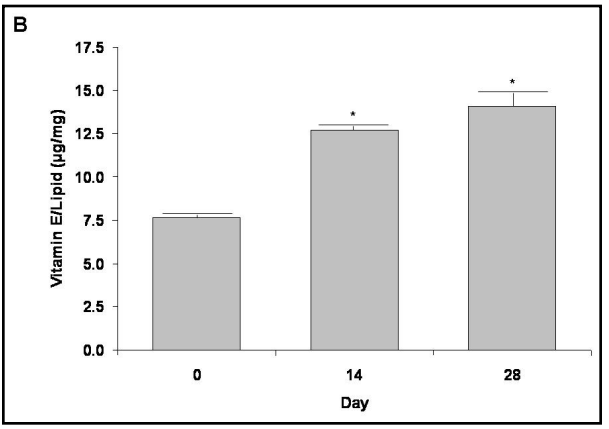
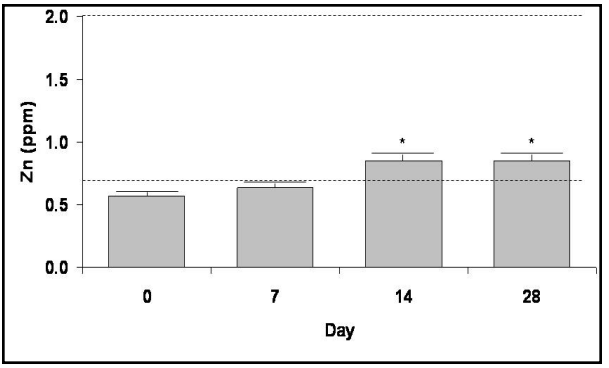


Figure 2. Effect of supplementation over 28 days on serum levels of zinc. Dashed lines indicate testing laboratory normal ranges. *$P < 0.05$ vs Day 0.



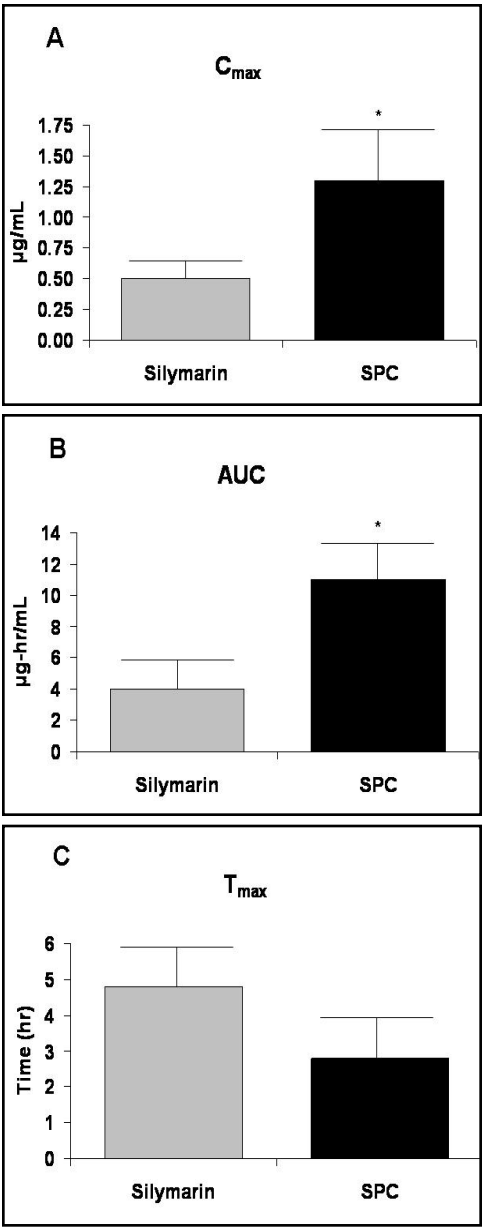
Plasma Level of Silybin

A pharmacokinetic study of plasma silybin levels in these same dogs (Griffin et al, submitted for publication) showed an increase to a maximum of 1.31 ± 0.31 µg/mL at 3 hrs, followed by a return to background levels at 24 hrs, resulting in a mean area-under-the-curve value 300% to 400% that of standardized silymarin extract (Figure 3 adapted from Griffin et al). Continued daily dosing over 28 days resulted in a trend towards an elevated level, but failed to reach statistical significance. Thus, achieving substantial, albeit transient, levels in plasma requires daily dosing.

DISCUSSION

Basic research and clinical studies have shown that a loss of redox homeostasis is both a cause and a consequence of many forms of liver dysfunction. Various precipitating conditions, including excessive intake of alcohol, metals, xenobiotics, or plant toxins or deficient intake of metals, particularly zinc, all result in cell damage and death as a consequence of elevated levels of free radicals, both ROS and RNS. While individual antioxidants, particularly silymarin, vitamin E, and zinc, have been shown to improve liver function in one or more of these conditions,[2,4-6] even greater improvement may be possible with combinations of these agents. A combination of silybin:phosphatidylcholine complex, vitamin E, and zinc (Marin®, Nutramax Laboratories, Inc., Edgewood, Md) was used in this study in beagles to assess safety and bioavailability of each of the components. The recommended administration for the size dogs used in this study is 105 IU vitamin E, 24 mg silybin, and 17 mg of zinc. Therefore, on Day 1 of the study, the dogs received 6 to 7 times the recommended maintenance administration

Figure 3. Comparison of key pharmacokinetic parameters in plasma of silymarin extract and silybin:phosphatidylcholine (SPC) in beagles, administered equivalent amounts of silybin. **A:** maximum plasma concentration (C_{max}); **B:** 24-hour area under the curve (AUC); **C:** time to maximum concentration (T_{max}). *$P < 0.05$ vs Silymarin.



levels, and on Days 2 to 28 of the study, the dogs received 3 to 3.5 times the recommended maintenance administration levels. Dosing following food intake was necessary at these higher levels for full acceptance of the supplement and avoidance of inappetence or emesis that may occur with zinc supplementation in dogs.[2] No clinically significant changes were observed in blood cell counts or in serum chemistries after 28 days of supplementation.

Serum levels of vitamin E (Figure 1) increased substantially over baseline, resulting in the possibility for higher levels in the liver itself. Although a recent meta-analysis of many clinical studies of vitamin E in humans raised concern about potential harmful effects of higher doses in humans,[63] a careful analysis of a robust database by a group of experts concluded that it is safe across a broad range of intakes.[64] Feeding beagles a diet containing a high level of 447 mg α-tocopheryl acetate/kg food for 17 weeks had no adverse effects.[65] Over the 28-day supplementation period of the present study, a daily administration of 27.4 IU/kg body weight had no adverse effects. The Nutrition Research Council (NRC) recommends a maintenance level of 24 IU/kg diet supplying 4,000 kcal of energy/day, which may change depending on the level and nature of fat (saturated versus unsaturated) in the diet.[66] Thus, the levels used here are in the safe range and can induce an increase in dogs that may have a deficiency associated with liver dysfunction. The tocopheryl esters are hydrolyzed to free tocopherol during the absorptive process, which may be affected by factors such as pancreatic lipase and bile secretion.[67,68] A water-soluble form of vitamin E may be more appropriate for conditions of pancreatic or severe hepatic dysfunction.

Supplementation with zinc can produce major benefits in liver diseases. Zinc is an essential trace element that impacts all aspects of metabolism,[37] serves as a cofactor in many enzymes and transcription factors that regulate and/or respond to changes in redox homeostasis,[38] and has pro-antioxidant activity. A zinc deficiency has been observed in humans with liver dysfunction,[69,70] appears in northern-breed dogs,[71] and occurs in dogs on a cereal-based diet.[72]

High liver levels of copper and iron have also been observed in association with histologic lesions in dogs.[73] Bioavailability of zinc depends on the dietary levels of other minerals, especially calcium,[74] and chelators such as phytic acid present in cereal-based diets.[75] Excessive supplemental zinc has a risk of toxicity in humans and may induce a copper deficiency.[76] However, intake in dogs of up to 80 mg/kg body weight/day[77] or 200 mg/kg diet[78] and in cats of 230 mg/kg body weight/day[78] showed no adverse effects. A safe upper limit for dogs or cats has not been established by the NRC.[66] The recommendation for adequate intake is 1.0 mg/kg body weight/day for dogs and 2.0 mg/kg body weight/day for cats.[66] Recommendations for zinc supplementation for liver disease in dogs are 1-5 mg/kg day divided into 2 doses,[2] or a 15-mg/kg induction level followed by 2-3 mg/kg/day on an empty stomach if necessary to avoid vomiting.[79] Administration of zinc at the higher levels in this study did not increase serum zinc levels above the reference ranges, or adversely effect hematocrit or red blood cells. Use at the recommended administration levels may prevent zinc deficiency, which can be associated with liver dysfunction.

Silybin present as a phytosomal complex reached levels substantially higher in the current study than were achieved in dogs with either a different type of silybin:phosphatidylcholine mixture[80] or with a silymarin extract containing an equivalent amount of silybin (Griffin et al, submitted for publication). In both humans[58,81] and rodents,[56] this complex was much more bioavailable than a standardized powdered extract. The maximal concentration shown in dogs (this study and Griffin et al, submitted for publication) and bioavailability assessed as area-under-the-curve over 24 hours were comparable to those observed in humans[58,81] and twice that shown in beagle dogs after adjusting for dose.[82] No data is available to establish an upper safe limit for silybin or silymarin in dogs, but dosing with up to 800 mg/day in humans produced no harmful effects.[10] Treatment of amanitin toxicity in beagles showed that 50-150 mg/kg silymarin extract provided protection if given 5 and 24 hours after intoxication; given 10 minutes after intoxication, a 15-mg/kg dose provided partial protection, while a 100-mg/kg dose gave total protection.[16] Given the much lower bioavailability of these extracts, it is likely that a phytosomal preparation should be more hepatoprotective against less extreme forms of toxicity that may cause liver dysfunction.

In summary, a combination supplement containing silybin, zinc, and vitamin E was administered to beagles and shown to produce significant increases in plasma levels of each of these constituents. The combination of silybin, vitamin E, and zinc in a single product neither prevented the absorption of the individual components nor present any apparent safety concerns. This combination has the potential to improve conditions of liver dysfunction with an inflammatory component and a redox imbalance.

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