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Short Communication

Prophylactic effects of humic acid-glucan combination against experimental liver injury

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ABSTRACT

Aim: Despite intensive research, liver diseases represent a significant health problem and current medicine does not offer a substance able to significantly inhibit the hepatotoxicity leading to various stages of liver disease. Based on our previously published studies showing the protective effects of a glucan-humic acid (HA) combination, we focused on the hypothesis that the combination of these two natural molecules can offer prophylactic protection against experimentally induced hepatotoxicity. **Materials and Methods:** Lipopolysaccharide, carbon tetrachloride, and ethanol were used to experimentally damage the liver. Levels of aspartate aminotransferase, alanine transaminase, alkaline phosphatase, glutathione, superoxide dismutase, and malondialdehyde, known to correspond to the liver damage, were assayed. **Results:** Using three different hepatotoxins, we found that in all cases, some samples of HA and most of all the glucan-HA combination, offer strong protection against liver damage. **Conclusion:** Glucan-HA combination is a promising agent for use in liver protection.

KEY WORDS: Enzymes, humic acid, glucan, liver, protection

INTRODUCTION

Liver disease can be inherited or caused by a variety of factors that damage the liver, such as viruses and alcohol use. In addition, various environmental factors can cause liver damage. With over 36,000 deaths from chronic liver diseases, it is clear that the search for a cure or preventive treatment currently represents one of the main focuses of medicine.

As the current medicine does not offer a substance able to significantly ameliorate the hepatotoxicity leading to liver disease, it is not surprising that the attention is more and more focused on various natural molecules, including immunomodulators. In most of these studies, the described effects of various natural molecules on liver damage were positive [1-5].

The long-term focus of our laboratories is on the synergistic effects of two natural molecules, humic acids (HA), and β-glucan. HA are ubiquitous molecules, which can be found wherever organic matter is being decomposed. Despite long knowledge of HA, with significant studies going back approximately 100 years, as during World War I, peat extracts were used to prevent infections [6]. Some of their health related effects are still unclear. Some studies showed stimulation of lymphocytes [7], some antiviral properties [8].

Biological properties of polysaccharides have been researched since the 1940s [9] and are currently subject of over 10,000 scientific studies. Glucans are treated as pathogens by pattern recognition receptors on macrophages, neutrophils, monocytes, and natural killer cells [10]. Glucans from different sources possess differential receptor affinities, mostly for Complement Receptor 3 (CD11b/CD18) on macrophages and neutrophils, and Dectin-1 receptor on macrophages [11,12]. The significant effects of glucans have been established in anti-infection and anti-cancer immunity [13], lowering cholesterol [14], suppression of stress [15], and stimulating immunity of chronically ill children [16].

Our previous studies revealed that glucan's effects can be further improved by adding HA [17]. Further studies showed that the administration of glucan had beneficial effects on hepatocytes [18] and hepatoprotective effects on experimentally-induced liver damage [19]. In addition, our own data showed strong synergistic effects of HA-glucan combination in hepatoprotection against lipopolysaccharide (LPS) injury [20].

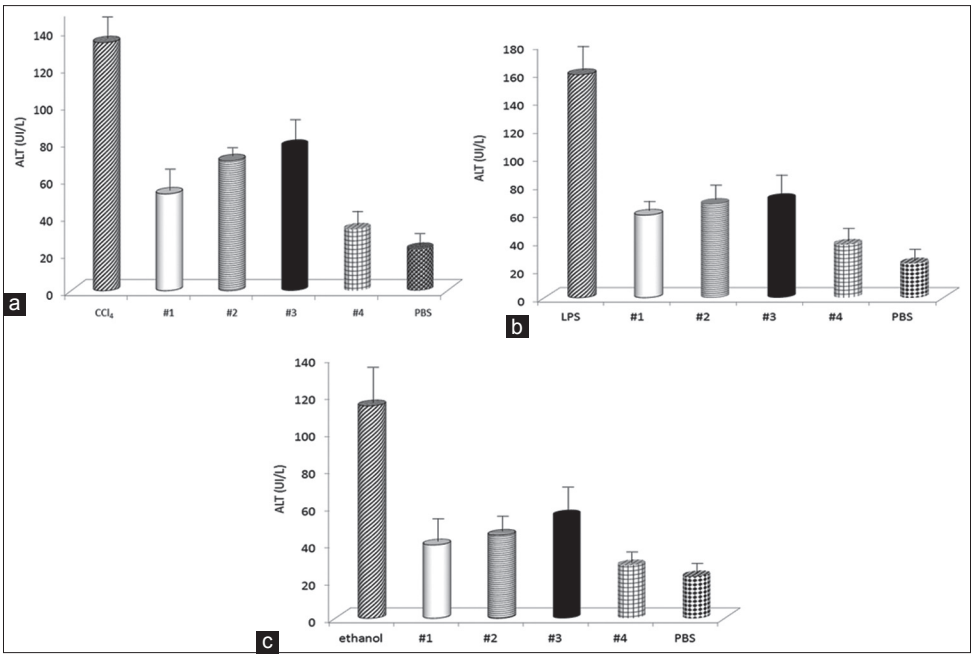

Figure 1: (a, b and c) The alanine aminotransferase activity in the serum after carbon tetrachloride, Lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

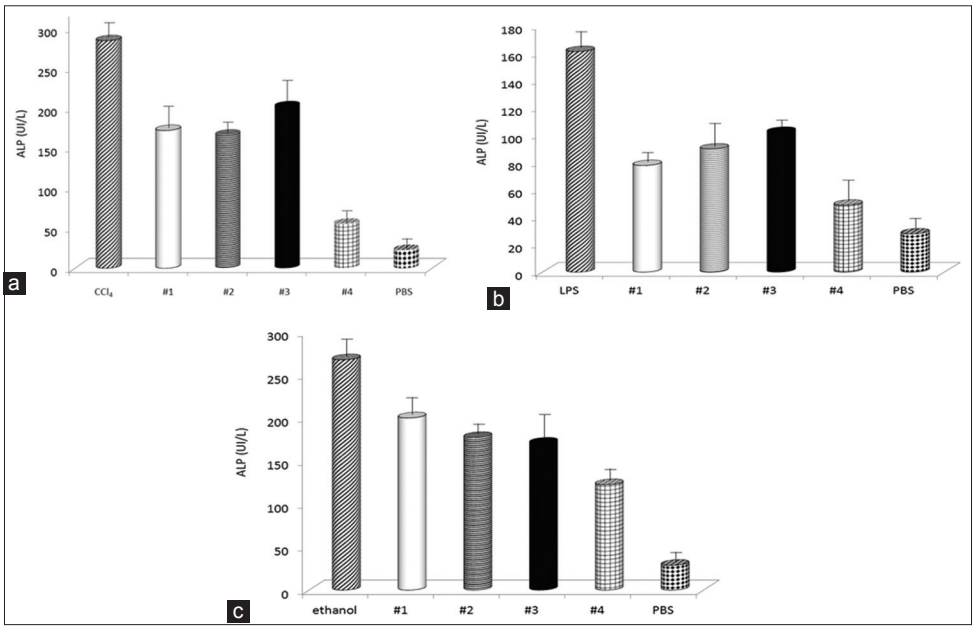

Figure 2: (a, b, and c) The alkaline phosphatase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

However, these data confirmed that the HA-glucan combination can offer healing properties, but offer no information about the effects when used before the liver damage. This shortcoming led us to the current study evaluating the possible prophylactic effects of a HA-glucan combination of experimentally-induced hepatotoxicity. In order to be sure our findings have general reach, we used three different, but well-established, models of hepatotoxicity: LPS induced [21], ethanol-induced [22], and carbon tetrachloride (CCl_4)-induced [23]. The main reason for the fact we decided to use these three models is their widespread in research, combined they represent more than 90% of experimentally-induced hepatotoxic studies.

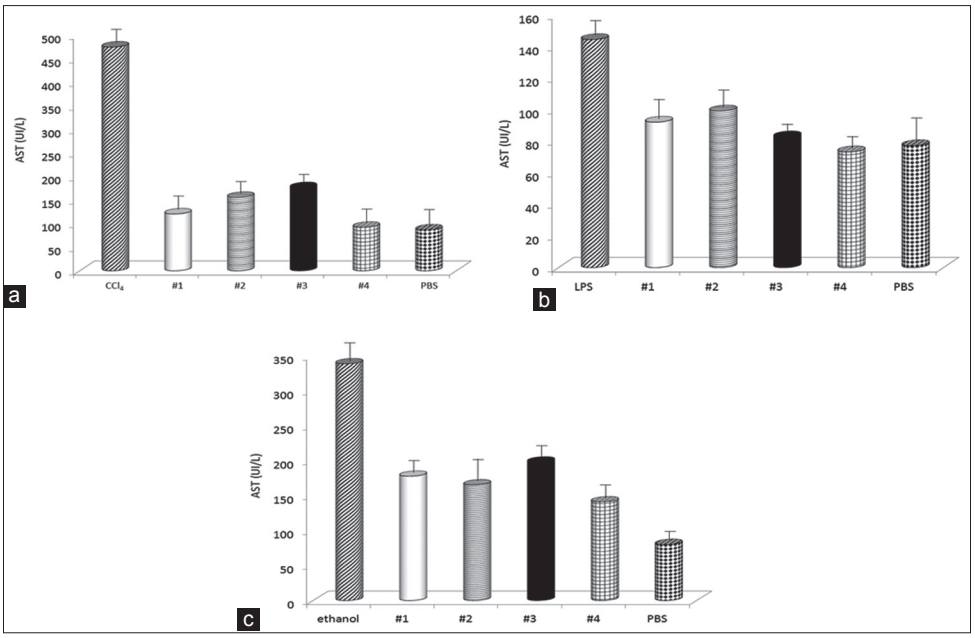

Figure 3: (a, b, and c) The aspartate aminotransferase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

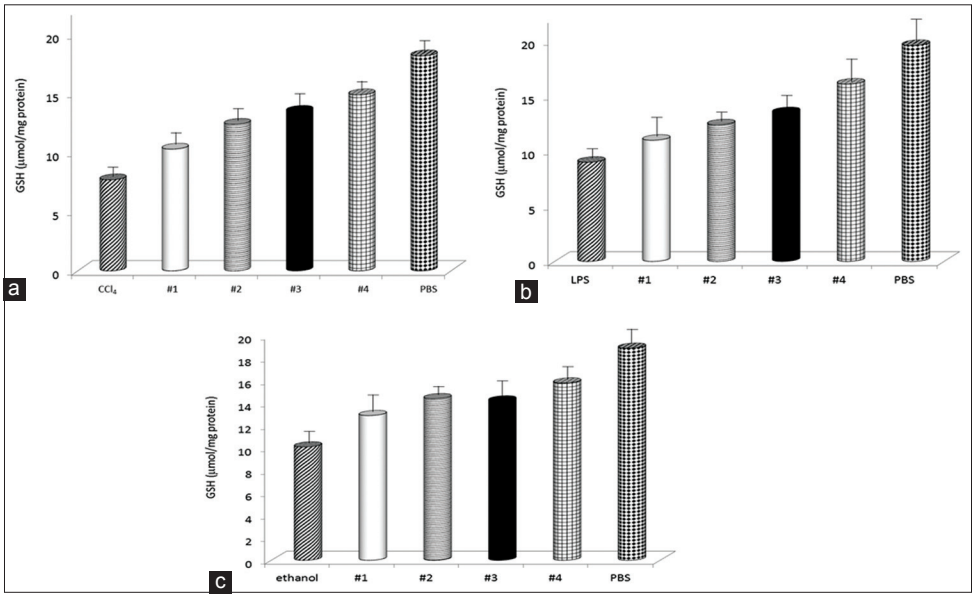

Figure 4: (a, b, and c) The glutathione activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

MATERIALS AND METHODS

Animals

Female, 6-10 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO2 asphyxiation.

Materials

Ethanol, LPS (from *Escherichia coli*), and carbon tetrachloride were purchased from Sigma (St. Louis, MO, USA).

β - 1, 3 Glucan

We used a combination of mannooligosaccharides and β-glucan extracted from saccharomyces cerevisiae by autolysis at high

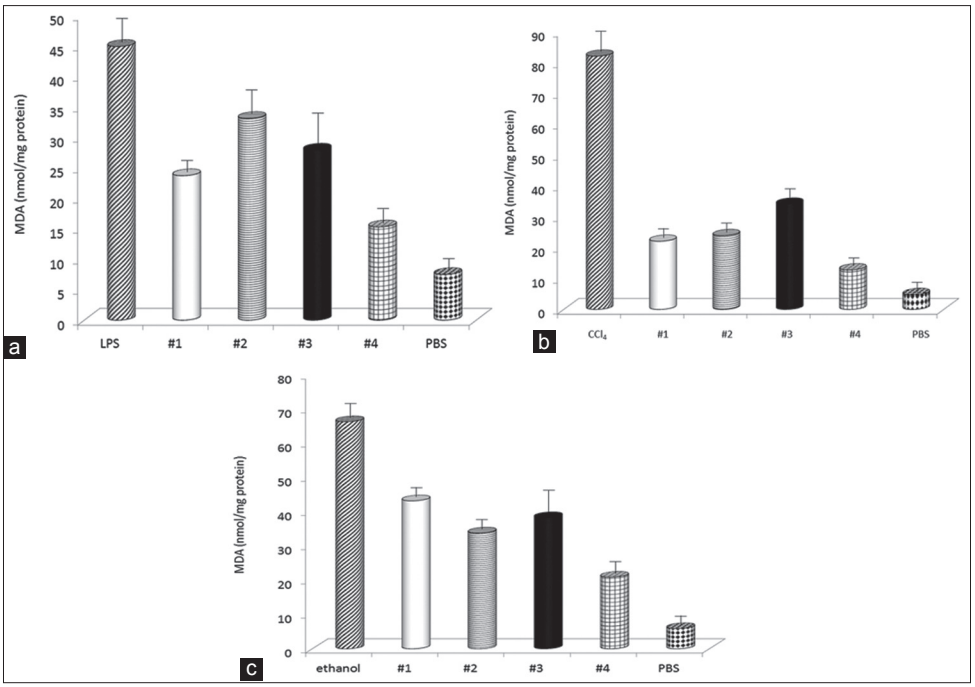

Figure 5: (a, b and c) The lipid peroxidation (malondialdehyde) activity in the serum after carbon tetrachloride, Lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-humic acid combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

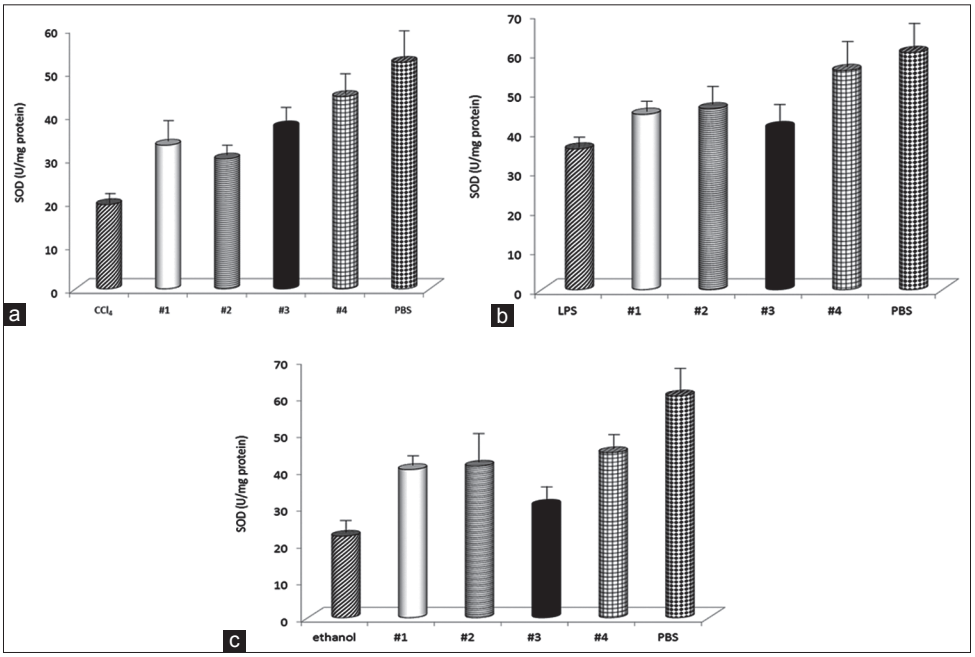

Figure 6: (a, b, and c) The superoxide dismutase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

temperature at controlled pH. When completed, the cell walls and extracts are separated by centrifugation and cell wall is spray dried. The glycosidic composition is 21% mannan, 24% β-glucan (Lallermand Animal Nutrition, Montreal, Canada).

Humic Acid

Two lignin-derived organic systems were obtained from diverse organic materials using the methodology described by the

International Humic Substances Society (IHSS) to extract humic substances and HA, as described in [24]. A first HA was extracted from black peat (Galicia, Spain) (HA8) and the other one from red Quebracho (*Schinopsis* spp.) barks (HA10) [20].

Hepatoprotective Activity

Hepatotoxicity was induced by oral feeding of ethanol (1 g/kg of body weight) for 10 days as described by Park *et al.* [22], by CCl_4 (0.5 ml/kg body weight in olive oil, injected ip.) according to Prasanna and Purnima [25] or by an ip. injection of 100 ng/kg body weight of (LPS) as described by Olleros *et al.* [21]. Alcohol was diluted in water, LPS in phosphate-buffered saline (PBS). Mice were randomly divided into several groups and administered orally by gavage during 14 days as follows: Group 1 - treated with glucan; Group 2 - treated with AH8; Group 3 - treated with AH10; Group 4 - treated with a combination of glucan, AH8, and AH10; and Group 5 - control group treated with PBS. At the end of the study, blood was collected and serum prepared. After that, mice were sacrificed and livers were immediately excised and used for homogenates.

Biochemical Markers

The enzymatic activities of aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were assayed spectrophotometrically by (Antech Diagnostics, Louisville, KY, USA). Liver homogenate was prepared by the following technique: Livers were excised and rinsed in saline. A small section from each liver was placed in 10% PBS-formalin solution to be used in histological slides. The rest was frozen in liquid nitrogen. Frozen liver was ground to a fine powder and 20-25 mg of powder was solubilized. The glutathione (GSH) levels were measured by the GSH test kit (Dojindo Labs, Kumamoto, Japan), superoxide dismutase (SOD) as described by Prasanna and Purnima [25] and malondialdehyde (MDA) [26].

Statistical Analysis

Data were expressed as means ± standard deviation. Statistical analysis was performed by a Pair *t*-test using a GraphPad Prism 502 software (GraphPad Software, USA). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

All three materials used in our study represent widely used experimental models of liver damage. All animals were randomly selected into individual groups. In one group, the liver damage was induced by applying one of the three treatments (LPS, CCl_4 or ethanol). Four other groups were treated with either glucan, two different types of HA, or a combination of glucan and both HA. These four groups were pretreated with these materials for 14 days before the liver-damaging treatment.

Use of CCl_4, LPS or ethanol caused significant stimulation of serum levels of AST, ALT, and ALP [Figures 1-3]. Pretreatment of tested material showed that all four tested groups significantly decreased the levels of these enzymes, and the glucan-HA combination was always the most active. Similar data were found when we focused on hepatic enzymes. Our experimental treatment caused a strong decrease in the levels of GSH [Figure 4], stimulated the level of MDA [Figure 5] and decreased the levels of SOD [Figure 6]. Again, all our prophylactic treatments are active and significantly improved the liver damage tested by enzymatic levels. As in the first part of the study, the glucan-HA combination showed the strongest effects.

DISCUSSION

Liver damage caused by chemotherapeutic agents is of intense interest to both researchers and clinicians. Ethanol, LPS, and CCl_4 represent well-established models of hemotoxic damage of the liver tissue. Ethanol works via live metabolic processes changing over 80% of ethanol to the highly toxic acetaldehyde which is further oxidized into various oxygen species [27]. CCl_4 is an extremely potent liver toxin, as a single exposure to CCl_4 causes a rapid increase in the levels of numerous enzymes, necrosis, and steatosis [28]. In the case of CCl_4, this type of liver injury is the most intensively studied model for xenobiotic-induced oxidative hepatotoxicity [29], making this type of liver damage to be the model of choice for screening efficacy of various possible hepatoprotective drugs. Most of the toxic effects are caused by trichloromethyl free radicals [4].

More and more natural molecules are gaining attraction in the fight against liver damage. Among those, glucans are considered to be the most promising. Mushroom glucan was found to have both hepato- and nephroprotective effects in rats [30], similar effects were described for glycoprotein Antrodan [31]. Oat-derived glucan was found to inhibit LPS-induced liver damage [32] and a glucan-melatonin combination had protective effects against liver injury [33]. Our own data showed the palliative effect of a glucan-HA combination of liver damage caused by LPS, ethanol of CCl_4 [20,34].

All of these studies evaluated the effects of glucans or other natural molecules after the liver injury, which means they were used either simultaneously or after the liver toxic treatment. This study focused on the question of whether the glucan-HA combination, which so strongly reversed the experimentally induced liver damage, also offers a protection. Therefore, we treated the animals with our samples before we used the liver damaging agent.

Many authors have repeated that lipid peroxidation is closely associated with liver pathogenesis. MDA is a byproduct of oxidant-induced liver protein and lipid oxidation, GSH is a component of the antioxidant system. SOD represents endogenous antioxidant and acts via dysmutation of superoxide anions.

The mechanisms of the liver protections are still unknown despite intensive research. Glucans are well established free

radical scavengers wit antioxidant effects [35]. Therefore, we hypothesized that it might have positive effects on liver damage. Similarly, antioxidant effects were described in HA [36], because of the sharp increase in the level of free radicals was described in liver damage [37].

The efficacy of any hepatoprotective molecule strongly depends on its ability to suppress damaging effects. The results of our study showed that daily oral supplementation helped to reduce the levels of ALT and AST in the serum of tested animals. HA alone and the combination in particular were very active, offering a significant decrease in liver damage. The prophylactic effects might be caused by strong potentiation of the antioxidant protective system, supported by protection of the GSH levels depressed by the liver damage by hepatotoxins. CCl_4 is metabolized by the cytochrome 450 to the trichloromethyl free radical [38], which subsequently forms trichloromethyl peroxyl radical attacking lipids on the endoplasmic reticulum leading to cellular necrosis [39]. It is possible that the glucan-HA combination acts by mopping up these free radicals and therefore limiting their damaging effects.

In summary, our findings showed that the inflammatory response to liver toxic agents was significantly decreased by pretreating the animals with orally-supplemented humic-acid combination. These effects were in agreement with inhibiting of the changes in AST, ALT, ALP, GSH, SOD, and MDA, caused by experimentally-induced liver damage. Therefore, we propose that the HA-glucan combination might offer a good possibility of natural molecules helping to reduce damage to the liver.

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