

## Hepatoprotective effect of aqueous extract of *Phyllanthus niruri* on nimesulide-induced oxidative stress *in vivo*

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Nimesulide (NIM), an atypical non-steroidal anti-inflammatory drug (NSAID) is also used as analgesic. In the present study, we evaluated its effect on the prooxidant-antioxidant system of liver and the hepatoprotective potential of aqueous extract of the herb *Phyllanthus niruri* (PN) on NIM-induced oxidative stress *in vivo* using a murine model, by determining the activities of hepatic anti-oxidant enzymes superoxide dismutase (SOD) and catalase (CAT), levels of reduced glutathione (GSH) and lipid peroxidation (expressed as malonaldehyde, MDA). Aqueous extract of PN at a dose of 50 or 100 mg/kg body wt was administered either intraperitoneally or orally for 7 days, before NIM administration at a dose of 8 mg/kg body wt twice daily for 7 days in mice. Animals were sacrificed 24 h after administration of final dose of NIM. In another set of experiments, both aqueous extract of PN (at a dose of 50 or 100 mg/kg body wt) and NIM (8 mg/kg body wt) were administered simultaneously for 7 days. Animals were sacrificed 24 h after administration of final dose of the extract and NIM, liver tissues were collected, and the activities of SOD and CAT and levels of GSH and lipid peroxidation end-product (as MDA), were determined from the livers of all the experimental animals. Appropriate NIM control was maintained for all sets of experiments. NIM administration (8 mg/kg body wt) for 7 days caused significant depletion of the levels of SOD, CAT and reduced GSH, along with the increased levels of lipid peroxidation. Intraperitoneal administration of the extract at a dose of 50 mg/kg body wt for 7 days, prior to NIM treatment, significantly restored most of the NIM-induced changes and the effect was comparable to that obtained by administering 100 mg/kg body wt of the extract orally. Thus, results suggested that intraperitoneal administration of the extract could protect liver from NIM-induced hepatic damage more effectively than oral administration. Antioxidant property of the aqueous extract of PN was also compared with that of a known potent antioxidant, vitamin E. The PN extract at a dose of 100 mg/kg body wt along with NIM was more effective in suppressing the oxidative damage than the PN extract at a dose of 50 mg/kg body wt. Results suggested that beneficial effect of the aqueous extract of PN, probably through its antioxidant property, might control the NIM-induced oxidative stress in the liver.

**Keywords:** Nimesulide, Oxidative stress, *Phyllanthus niruri*, Antioxidants, Hepato-protective activity.

The *Phyllanthus* spp. have been shown to exhibit beneficial effect against various pathological states<sup>1-5</sup>, including liver diseases<sup>6,7</sup> like hepatitis, fatty liver, cirrhosis, etc. and no side-effects have been reported in clinical studies<sup>8</sup>. Although the herb *Phyllanthus niruri* (PN) has been found to be effective against a number of drug and toxin-induced hepatic disorders<sup>9-11</sup>, but no report is available about the effect of its aqueous extract against nimesulide (NIM)-induced hepatic damage. NIM, a widely used non-steroidal anti-inflammatory drug (NSAID), is only weakly acidic<sup>12,13</sup> and at the therapeutic doses, is a selective cyclooxygenase-2 (COX-2) inhibitor, with only residual activity against COX-1<sup>14-18</sup>. It is almost exclusively metabolized by the liver<sup>19</sup>. So, individuals

with hepatic insufficiency, and with genetic/or acquired abnormalities in drug-metabolizing enzymes or trans-membrane carriers, may be at an increased risk for higher NIM concentrations in the liver.

NIM is reported to cause injury to the isolated rat liver cells, mediated mainly by the impairment of ATP production by mitochondria<sup>20</sup>. It also causes liver damage, ranging from mild abnormal function such as increase in serum amino transferase activity to severe organ injuries such as hepatocellular necrosis or intrahepatic cholestasis<sup>21-23</sup>, mainly due to generation of reactive oxygen species (ROS)<sup>24,25</sup>. The production of ROS has the potential to inflict considerable damage on the tissues<sup>26</sup>. The superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $HO^\cdot$ ) are the main ROS produced in mitochondria<sup>27</sup>. Every cell has its own defense mechanism against ROS and contains antioxidative enzymes such as SOD, CAT, GPx, GST, etc. and several antioxidant molecules like GSH<sup>28,29</sup>.

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In this study, we evaluated the effect of the aqueous extract of PN against NIM-induced oxidative stress, by determining the activities of hepatic anti-oxidant enzymes SOD and CAT, levels of GSH (a ROS scavenger) and lipid peroxidation. In addition, protective effects of the extract were also compared with a known antioxidant vitamin E.

## Materials and Methods

### Materials

Nimesulide, bovine serum albumin (BSA), Bradford reagent, 1-chloro-2,4-dinitrobenzene (CDNB), and protein estimation kit were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. 5,5'-Dithiobis (2-nitrobenzoic acid) [DTNB, (Ellman's reagent)], sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), EDTA, glacial acetic acid, hydrogen peroxide, NADH disodium salt, nitroblue tetrazolium chloride (NBT), phenazine methosulphate (PMT), potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), reduced glutathione (GSH), trichloroacetic acid (TCA), thiobarbituric acid (TBA), and vitamin E were bought from Sisco Research Laboratory, India. All other chemicals and reagents used in this study were of certified analytical grade.

### Preparation of aqueous extract of *Phyllanthus niruri* (PN)

Fresh plants were collected from Bose Institute Experimental Farm and local markets. The fresh leaves and stems of the young plants were homogenized in 50 mM sodium phosphate buffer, pH 7.2, at 4°C and the homogenate was centrifuged at 12,000 g for 30 min to remove unwanted debris. Supernatant was dialyzed against ice-cold water, centrifuged again under the same condition, collected and lyophilized. The freeze-dried material was weighed, dissolved in the same phosphate buffer and used in different experiments.

### Animals and treatment

Swiss male albino mice (weighing  $25 \pm 2$  g) were acclimatized under standard laboratory conditions for a fortnight, before starting experiments. Animals had free access to standard diet and water *ad libitum*. The effect of PN on NIM induced oxidative stress was determined as described below. Animals were divided into five groups, each group having 8 mice. Group I received NIM at a dose of 8 mg/kg body wt/twice daily for 1 wk. Groups II and III were pretreated orally and intraperitoneally respectively with PN at a

dose of 50 mg/kg body wt twice a day for 1 wk and then treated with NIM at a dose of 8 mg/kg body wt/twice daily for 1 wk. Groups IV and V were treated similarly like groups II and III, except that PN was used at a dose of 100 mg/kg body wt. Respective controls were maintained appropriately for all the groups. Animals were sacrificed 24 h after the administration of the final dose of NIM and liver tissues were collected. To validate the study, effect of a known antioxidant, vitamin E was also determined on the NIM-induced oxidative stress.

The combined effects of PN and NIM on the oxidative stress in liver was determined by intraperitoneal administration of aqueous PN either 50 or 100 mg/kg body wt/twice daily along with NIM at a dose of 8 mg/kg body wt/twice daily for 1 wk. Respective controls were maintained for the study. Animals were sacrificed 24 h after the administration of the final dose of the extract and NIM, livers were collected and oxidative stress related parameters were determined.

### Preparation of liver homogenate

About 200 mg of liver tissue was homogenized in 10 vol of 100 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.4 and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected and used for following experiments as described below. Protein concentration of the supernatant was measured according to the method of Bradford using crystalline BSA as standard as described below.

### Assay of antioxidant enzymes SOD and CAT

The activity of SOD was assayed as described previously<sup>30,31</sup>. Liver homogenate containing about 5 µg protein was mixed with 50 mM sodium pyrophosphate buffer, 6 µM PMT and 10 µM NBT. The reaction was started by the addition of NADH and the reaction mixture was incubated at 30°C for 90 s. The reaction was stopped by the addition of 1 ml of glacial acetic acid and the absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity was defined as the enzyme concentration required to inhibit chromogen production by 50% in 1 min under the assay condition.

CAT activity was determined as described earlier<sup>32</sup>. CAT converts  $\text{H}_2\text{O}_2$  formed via the action of SOD on superoxide radical into water. About 5 µg protein contained in liver homogenate was mixed with 2.1 ml

of 7.5 mM H<sub>2</sub>O<sub>2</sub> and time scan was performed for 10 min at 240 nm at 25°C. The disappearance of peroxide depending on the CAT activity was observed. One unit of CAT activity was defined as the amount of enzyme, which reduced 1 μmol of H<sub>2</sub>O<sub>2</sub> per min.

#### Determination of GSH level

Hepatic GSH level was determined by the method of Ellman<sup>33</sup>. Tissue homogenate (720 μl) was double-diluted and 5% TCA was added to it to precipitate the protein content. After centrifugation (at 10,000 g for 5 min) the DTNB solution was added to the supernatant and the absorbance was measured at 417 nm. A standard graph was drawn using different concentrations of a standard GSH solution (1 mg/ml) and GSH content was calculated.

#### Estimation of lipid peroxidation

Lipid peroxidation (LPO) was assayed using a colorimetric method using the thiobarbituric acid (TBA)<sup>34</sup>. Briefly, the experimental samples containing 10% tissue homogenates (approx 1 mg protein) was diluted to 500 μl using extraction buffer. The reaction mixture was incubated for 1 h at 37°C, treated with 1 ml 20% TCA and 2 ml 0.67% TBA and kept in boiling water bath for 30 min. After cooling, samples from all the experimental groups were centrifuged at 3000 rpm for 15 min. Amount of TBARS formed was measured by taking the absorbance of the supernatant at 532 nm. About 99% of TBARS was malondialdehyde (MDA). The TBARS concentration of the samples was calculated using the extinction coefficient of MDA as  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

Protein concentrations in the liver homogenates were determined according to the method of Bradford<sup>35</sup>.

#### Statistical analysis

All the values were represented as mean ± SD (n = 8). Data on biochemical investigation were analyzed using analysis-of-variance (ANOVA) and the group means were compared by Duncan's multiple range test (DMRT). A probability of  $p < 0.05$  was considered as significant.

#### Results

The SOD activity in NIM-treated liver tissue homogenate was significantly reduced ( $394 \pm 17$  unit/mg total protein), compared to that of control group ( $575 \pm 23$  unit/mg total protein). In the PN pretreated group, intraperitoneal administration with a dose of 50 mg/kg body wt for 7 days, before NIM

administration, SOD activity was significantly higher ( $489 \pm 10$  unit/mg total protein) than NIM control; the activity was comparable to that from 100 mg/kg body wt orally administered group ( $499 \pm 15$  unit/mg total protein). The activity was even higher, when the extract was intraperitoneally administered at a dose of 100 mg/kg body wt ( $543 \pm 11$  unit/mg total protein) and was comparable to that of the control group (Fig. 1A).

Like SOD, the CAT activity in the liver homogenate in NIM-treated group showed marked reduction, compared to the normal (control) mice ( $99.5 \pm 2.0$  vs.  $35.0 \pm 1.8$  unit/mg total protein, in control and NIM-treated group respectively). In PN pretreated group, intraperitoneal injection with a dose of 50 mg/kg body wt for 7 days, before NIM administration, CAT activity was significantly higher ( $61.0 \pm 1.5$  unit/mg total protein) than NIM control and was comparable to the 100 mg/kg body wt orally administered group ( $63.0 \pm 1.7$  unit/mg total protein). The activity was even higher ( $85.0 \pm 0.8$  unit/mg total protein), when the extract was administered intraperitoneally at a dose of 100 mg/kg body wt (Fig. 1B).

NIM administration caused significant reduction in liver GSH levels, compared to normal liver ( $31.0 \pm 4.0$  vs.  $84.6 \pm 3.4$  nmole/mg total protein in NIM-treated and normal mice respectively). Intraperitoneal administration of the aqueous extract at a dose of 50 mg/kg body wt for 7 days, prior to NIM treatment, significantly elevated GSH level ( $55.7 \pm 1.5$  nmole/mg total protein); this level was comparable to 100 mg/kg body wt orally administered group ( $57.0 \pm 2.5$  nmole/mg total protein). Like SOD and CAT activities, intraperitoneal administration of the extract at a dose of 100 mg/kg body wt also showed the higher GSH ( $79.0 \pm 1.0$  nmole/mg total protein) level (Fig. 1C).

The level of lipid peroxidation (expressed as MDA) was significantly increased in the liver tissue of NIM-treated group, compared to the control group ( $74.3 \pm 4.5$  vs.  $36.6 \pm 3.0$  nmole/mg protein, in NIM-treated and control mice respectively). Pretreatment with PN intraperitoneally at a dose of 50 mg/kg body wt significantly reduced the NIM-induced increase of TBARS content ( $48.0 \pm 2.0$ ) and was comparable to that obtained by administering 100 mg/kg body wt of the extract orally ( $51.0 \pm 3.0$  nmole/mg protein). Thus, intraperitoneal administration showed better result, compared to the oral administration (Fig. 1D).

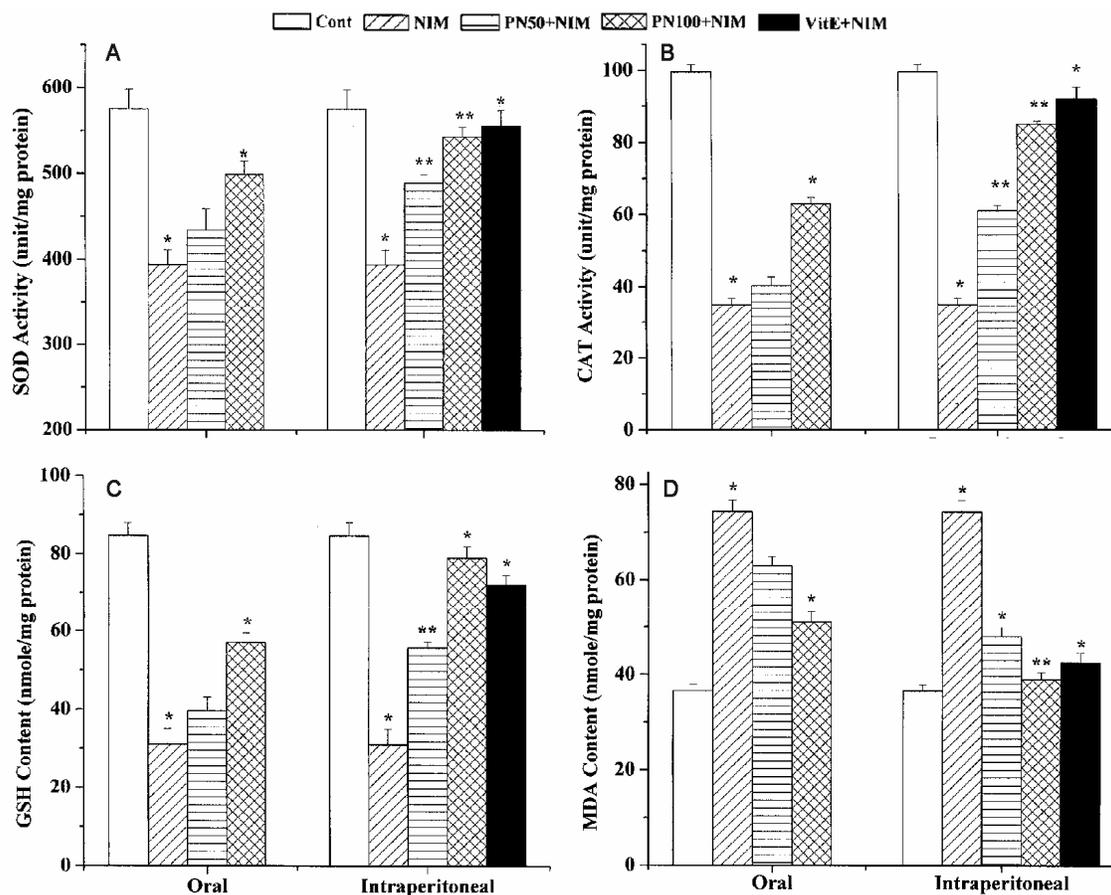


Fig. 1—Effect of PN on the SOD activity (A), CAT activity (B), GSH level (C) and on TBARS formation (expressed as MDA) (D) of liver in NIM-induced oxidative stress [PN was administered at two different doses, 50 and 100 mg/kg body wt, either orally or intraperitoneally 7 days prior to NIM administration. For experimental details, see the ‘Materials and Methods’. Left panel and right panel show the effect of oral and intraperitoneal administration, respectively. Cont, normal mice; NIM, NIM-treated mice; and PN50 + NIM and PN100 + NIM, aqueous extract of PN given at a dose of 50 and 100 mg/kg body wt respectively prior to NIM administration. Each value represents mean  $\pm$  SD,  $P^* < 0.05$ ,  $P^{**} < 0.01$ ]

Table 1—Combined effect of NIM and PN on antioxidant enzyme activities, GSH level and lipid peroxidation (MDA level) [NIM at a dose of 8 mg/kg body wt/twice daily and PN at a dose of either 50 or 100 mg/kg body wt/twice daily were used for 1 wk. Animals of all the groups were sacrificed 24 h after the administration of the final dose of the extract and NIM, livers were collected and the parameters (as mentioned above) were determined. Control; normal mice, NIM; NIM-treated mice, PN-50 + NIM and PN-100 + NIM; PN administered intraperitoneally at a dose of 50 and 100 mg/kg body wt respectively along with NIM. Values represent mean  $\pm$  SD,  $n=8$ ]

Groups	SOD (unit/mg protein)	CAT (unit/mg protein)	GSH (nmole/mg protein)	MDA (nmole/mg protein)
Control	615 $\pm$ 26	87.5 $\pm$ 2.8	86.0 $\pm$ 3.0	32.3 $\pm$ 2.4
NIM	414 $\pm$ 13**	29.0 $\pm$ 2.3*	24.0 $\pm$ 3.5*	76.5 $\pm$ 1.3**
PN-50 + NIM	507 $\pm$ 21*	54.0 $\pm$ 1.7*	59.0 $\pm$ 2.0*	57.0 $\pm$ 1.5*
PN-100 + NIM	593 $\pm$ 17**	81.7 $\pm$ 1.2**	75 $\pm$ 0.05**	40.3 $\pm$ 1.0**

$P^* < 0.01$ ,  $P^{**} < 0.001$

In all the pretreatment studies, vit E effectively resisted the alterations of the oxidative stress related parameters (Fig. 1A-D). Results of the combined effect of NIM and PN are shown in Table 1. The

pretreatment study showed that PN could overcome the NIM-induced oxidative stress in the liver and also intraperitoneal administration of PN was more effective than oral administration in reducing that

stress, thus only intraperitoneal administration procedure was followed for this particular study. Results suggested that administration of NIM, in combination with aqueous extract of PN prevented alterations of the levels of all the parameters. The PN extract at a dose of 100 mg/kg body wt along with NIM was more effective in suppressing the oxidative damage than at a dose of 50 mg/kg body wt.

### Discussion

In this study, we evaluated the protective effect of the aqueous extract of PN against NIM-induced hepatic damage. NIM administration at a dose of 8 mg/kg/twice daily for 1 wk significantly reduced the SOD and CAT levels and the non-protein thiol GSH, accompanied by the significant enhancement of lipid peroxidation. In experimental animals, administration of PN prior to NIM-treatment or alongwith NIM, restored the levels of antioxidant enzymes and GSH near to the control (normal liver). PN also significantly inhibited the NIM-induced lipid peroxidation. Similar results were obtained, on the treatment of animals with vitamin E, prior to NIM administration.

Oxidative stress is a disturbance in the pro-oxidant/antioxidant balance (increased pro-oxidant levels) and is caused due to the increased formation of ROS or by a decrease of the antioxidant defense. About 90% of the cellular oxygen is consumed in mitochondria, of which 1-4% is converted into superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $HO^\cdot$ ) under physiological conditions and by some enzymes such as xanthine oxidase, NADPH oxidases and cytochrome P450(s)<sup>37</sup>.  $H_2O_2$  is produced by a wide variety of enzymes including monooxygenases and oxidases<sup>38</sup>. Although the cells cope up with the ROS and other free radicals generated in the respiratory chain through various enzymatic and non-enzymatic systems, it is generally accepted that with increasing age, in the cell and particularly in mitochondria, a condition of oxidative stress is established when the defense capacity against ROS becomes insufficient. The free radicals react with proteins leading to their denaturation, carbonylation etc<sup>39,40</sup>. ROS can also react with DNA bases and oxidize them, resulting in single and double strand breakage<sup>41</sup>. This process may cause peroxidative tissue damage, resulting in inflammation, cancer, toxicity of xenobiotics and aging.

MDA is one of the end products in the LPO process. It reacts with primary amino groups of

proteins and forms Schiff bases, leading to polymerization and inactivation of enzymes<sup>42-44</sup>. In addition, it reacts with nucleic acid bases of DNA at physiological pH forming adducts, resulting in inhibition of transcription and translation<sup>45,46</sup>. Cells have a number of mechanisms to protect themselves from the toxic effects of ROS. Among these SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues<sup>47</sup>. CAT is also a key component of antioxidative defense system. SOD removes  $O_2^-$  by converting them to  $H_2O_2$  and  $O_2$ . This  $H_2O_2$  is then converted by CAT and GPx to  $H_2O$  and oxygen.

GSH, a small peptide consisting of amino acids cysteine, glutamic acid and glycine, directly involves in the detoxification by the liver. It binds to toxins, such as heavy metals, solvents, and pesticides, and transforms them into a form that can be excreted in urine or bile<sup>48,49</sup>. In eukaryotic cells, two distinct enzymes catalyze the process of dismutation. The Mn-SOD is found in matrix of mitochondria, whereas the Cu- and Zn-SOD occur primarily in the cytosol. In addition, significant amount of GSH, an important antioxidant, is present in hepatocytes and in RBCs for detoxification of xenobiotics or free radicals. In preliminary study, dietary GSH intake from fruit and raw vegetables has been associated with protection against some forms of cancer<sup>46,48</sup>.

Although use of NIM as a drug has already been banned in many countries, it is still widely used in India and some other countries for reducing pain, inflammation and fever associated with respiratory tract infections<sup>19</sup>. However, an association has been found between NIM-treatment and the development of liver injury,<sup>22,50</sup> probably due to the generation of ROS<sup>24,25</sup>. As previously discussed, antioxidants provide protection against oxidative damage by scavenging reactive oxygen molecules or by chemically reducing oxidized compounds<sup>51</sup>. Besides, antioxidant compounds can prevent oxidative damage from spreading by decreasing the concentration of lipid free radicals and terminating the propagation of LPO<sup>52</sup>.

As NIM caused a severe depletion in the hepatic content of SOD, CAT and GSH, accompanied by a high level of LPO, suggesting that it induced oxidative insult and suppressed antioxidant defense. Significant restoration of the altered levels of these anti-oxidant parameters on treatment with the extract of PN, prior to NIM administration or in combination

with NIM suggested that the active principle(s) of this herb might possess antioxidant activity. Significant difference was observed in hepatoprotective activity between oral and intraperitoneal administration at the same dose of PN extract, the exact reason for which is not clearly understood. Possibly, when fed orally, the active ingredients get degraded in the stomach, which remain intact in intraperitoneal administration and thus help them to exhibit their function better. It has been reported<sup>53,54</sup> that some plant proteins possess antioxidative and hepatoprotective activities. Also, in a recent study<sup>55</sup>, we observed that the protein isolate of the PN exhibit antioxidant property against NIM-induced oxidative stress and heat treatment or enzymatic digestion destroyed its biological activity. Thus, a protein(s) might also be involved in the hepatoprotective action of PN.

In conclusion, the protective role of PN against NIM-induced hepatic damage is probably due to its antioxidant property. The improvement in hepatic injury and liver functions by the extract may be possibly due to the presence of some protein molecules and/or other active ingredient(s). However, further investigation is needed to identify and fully characterize the active principle(s) responsible for its hepatoprotective action.

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