Herbal (Phyllanthus niruri) protein isolate protects liver from nimesulide induced oxidative stress

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Abstract
Present study was conducted to evaluate the role of a protein fraction (PI, protein isolate) of the herb, Phyllanthus niruri (P. niruri) against nimesulide-induced oxidative stress in vivo using a murine model. Mice were intraperitoneally treated with that at a dose of 5 mg/kg body weight for 7 days before and separately 1–5 days after nimesulide (at a dose of 10 mg/kg body weight for 7 days) administration to evaluate its preventive and curative role. Levels of reduced glutathione (GSH), antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), as well as thiobarbituric acid reactive substances (TBARS) were measured in the liver homogenates of all study groups. Pretreatment with isolated P. niruri protein fraction significantly enhanced nimesulide-induced reduced levels of antioxidant enzymes and GSH as well as reduced the enhanced level of lipid peroxidation. Post-treatment studies showed that the recovery after nimesulide induced oxidative stress was more rapid if PI was administered compared to the spontaneous recovery of liver. Histological studies also suggest that this protein fraction could prevent as well as cure liver from nimesulide induced oxidative stress. DPPH radical scavenging assay showed that it could scavenge free radicals. Its antioxidant property was compared with that of a known potent antioxidant, Vitamin E. Besides, the effect of a non-relevant protein, BSA, was also included in the study. Heat treatment and trypsin digestion destroyed the biological activity of this protein fraction. In conclusion, data obtained suggest that the P. niruri protein fraction may protect liver from nimesulide-induced oxidative stress probably via promotion of antioxidant defense.

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1. Introduction
Liver is the primary target for environmental and occupational toxic exposures as it is the major site of detoxification of our body. This toxic exposure brings about various hepatic disorders mainly by producing reactive oxygen species, ROS [1,2]. At present hardly any drug with minimal or no side effects can protect the liver against damages or stimulate its function. Numerous herbal plants and their formulations are, therefore, used widely for the recovery of liver from various diseases. One well-known herb of this kind is Phyllanthus niruri, which has been shown to possess beneficiary role against various pathological states [3–5]. This herb has also been reported to have protective action against various toxin and drug-induced hepatic disorders [6–8]. Moreover, clinical studies with this herb on humans have also been carried out without any side effect [9]. However, the mechanism for the beneficial role of this herb is not clearly known.

Several compounds including clinically useful drugs can cause cellular damage through the metabolic activation of the parent compound to highly reactive substances and also provoking the generation of oxygen derived free radicals. Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) is such a non-carboxylic acid, nonsteroidal anti-inflammatory drug that has been widely used for the treatment of a variety of inflammatory and pain conditions. If the drug is consumed in overdoses or for longer period of time, people
having weak liver function suffer severely from this drug for unpredictable hepatic problems. It has been reported that the drug can cause several types of liver damage, ranging from mild abnormal function such as increase in serum aminotransferase activity to severe organ injuries such as hepatocellular necrosis or intrahepatic cholestasis [10–13]. Results from our laboratory have shown that aqueous extract of *P. niruri* protects liver from nimesulide-induced oxidative stress and the antioxidative effect of the extract was found to be more pronounced when its administration was done intraperitoneally. This observation suggests that protein(s) present in *P. niruri* may exert its hepatoprotective effect via an antioxidative defense mechanism. We, therefore, initiated the present study to evaluate the role of the protein isolate of *P. niruri* against nimesulide-induced oxidative stress in murine liver.

In this study, hepatic pro-oxidant/antioxidant status was evaluated by measuring the hepatic contents of the non-protein thiol, GSH, antioxidative enzymes SOD and CAT and also by determining the hepatic content of the end products of lipid peroxidation, thiobarbituric acid reactive substances (TBARS). The radical scavenging activity of the protein fraction isolated from *P. niruri* was determined from its DPPH free radical quenching ability. Effects of heat treatment and proteolytic digestion on the biological activity of the protein fraction were determined by incubating it at 95 °C and digesting it with trypsin, respectively. Effect of a non-relevant protein, BSA, and a known antioxidant, Vitamin E, were also studied on the nimesulide-induced oxidative stress. In addition, histological studies were carried out on the livers of normal mice, mice treated with nimesulide and mice treated with PI prior and post to nimesulide administration for the determination of the ultrastructural changes of the liver due to pre- and post-treatment of PI on nimesulide induced oxidative stress.

## 2. Materials and methods

### 2.1. Animals and chemicals

Swiss albino mice (male, body weight 25 ± 2 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals. The animals were acclimatized under standard laboratory conditions for a period of 2 weeks before starting any experiment. Animals had free access to standard diet and water ad libitum.

Nimesulide, DPPH free radical and protein estimation kits were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) USA. 1-Chloro 2, 4-dinitrobenzene (CDNB), ethylenediamine tetraacetic acid (EDTA), thiobarbituric acid (TBA), nitroblue tetrazolium chloride, phenazine methosulphate and nicotinamide adenine dinucleotide reduced (NADH) were procured from Sisco research laboratory, India.

### 2.2. Preparation of protein fraction from *P. niruri*

For the preparation of protein fraction, all the steps were carried out at 4 °C unless otherwise mentioned. The fresh young leaves and stems of *P. niruri* were homogenized in 50 mM phosphate buffer, pH 7.2, and the homogenate was centrifuged at 12,000 × g to get rid of debris. The supernatant was brought up to 60% saturation with (NH₄)₂SO₄. The precipitate was then dialyzed against phosphate buffer to remove (NH₄)₂SO₄. The protein was further concentrated by adding two volumes of acetone (initially chilled at -20 °C) to one volume of the protein solution. Protein was precipitated; it was then brought into solution in phosphate buffer, dialyzed against the same buffer to remove acetone. All the experiments were carried out using this protein fraction, called protein isolate (PI).

To check the effect of heat treatment on the biological activity of this *P. niruri* protein fraction, it was preheated at 95 °C for 10 min, cooled and then applied intraperitoneally at a dose of 5 mg/kg body weight for 7 days after nimesulide administration (10 mg/kg body weight). To check the effect of protease on its biological activity, it was incubated with trypsin at 37 °C for 1 h and then administered intraperitoneally after nimesulide administration as described earlier. The effect was compared with that of biologically active protein fraction.

Protein concentration was determined by using a protein estimation kit obtained from Sigma Chemical Company. The estimation was performed according to the method of Bradford [14] using crystalline bovine serum albumin as standard.

### 2.3. Determination of biological activities

#### 2.3.1. Preventive role

Preventive role of the protein isolate against nimesulide-induced hepatic damage was determined as follows. The animals were divided into six groups, each group having eight mice. Group I was normal control, group II received only 50 mM phosphate buffer, pH 7.2, group III received nimesulide at a dosage level 10 mg/kg body weight for 1 week; group IV was pretreated with the isolated protein fraction at an intraperitoneal dose of 5 mg/kg body weight for 7 days and then treated with nimesulide at a dose of 10 mg/kg body weight for 1 week. Animals were sacrificed under mild ether anesthesia 24 h after the last dose of nimesulide administration. To validate the study, effects of a non-relevant protein, BSA, and a known antioxidant, Vitamin E, were also determined on the nimesulide-induced oxidative stress.

#### 2.3.2. Curative role

Liver has a unique regeneration property after any drug and toxin injury [15,16]. To ascertain whether the protein can cause the healing of the liver faster than its natural regeneration, a time course study was conducted where eight mice in each group were injected with the protein isolate for 1, 2, 3, 4 and 5 days after the administration of nimesulide at
the same dose and time as mentioned earlier. Mice were sac-
rificed after 2nd, 3rd, 4th 5th and 6th day, respectively. In
another set, eight mice in each group were treated with only
nimesulide and sacrificed after 2nd, 3rd, 4th 5th and 6th day.
In addition, eight mice were kept as normal controls.

2.4. Determination of GSH and the antioxidant enzyme
levels in liver homogenate

Hepatic GSH level was determined by the method of Ell-
man [18].

The activity of SOD was assayed following the method
originally developed by Nishikimi et al. [15] and then modi-
fied by Kakkar et al. [16]. CAT activity was measured by the
method of Bonaventura [17].

2.5. Estimation of lipid peroxidation end-products

Extent of lipid peroxidation in the experimental liver sam-
ples was assessed using a colorimetric method in which
mainly malondialdehyde is produced by the thiobarbituric
acid (TBA) reaction as described by Esterbauer and Cheese-
man [19].

2.6. Liver histopathology

Liver specimens from all the experimental groups were
fixed in 10% buffered formalin and were processed for paraf-
fin sectioning. Sections of about 5 μm thickness were stained
with haematoxylin and eosin to study the ultrastructural
changes of the liver under different experimental conditions.

2.7. Statistical analysis

All the values are represented as means ± S.D. (n = 8).
Student’s t-test was applied to calculate the significance of
difference between groups. P values of 0.05 or less were
considered significant.

3. Results

The protein fraction isolated from of P. niruri partially
prevented nimesulide induced hepatic disorder (Figs. 1–6).
The heat treatment and trypsin digestion destroyed the
protective activity of this isolated P. niruri protein fraction
(Fig. 1). When DPPH was incubated with the protein fraction
at various concentrations, the absorbance at 517 nm dimin-
ished. This suggests that it possessed free radical scavenging
activity (data not shown).

The effect of nimesulide alone and effect of pretreat-
ment of the isolated P. niruri protein fraction followed by
nimesulide treatment on SOD activity in the liver
homogenates are shown in Fig. 2A. The hepatic SOD activ-
ity was reduced in nimesulide treated mice (394 ± 7.3 ver-
sus 575 ± 12.5 units/mg protein in control mice liver). The
decrease was significantly prevented by the protein pre-
treatment (533 ± 9.6 units/mg protein). The hepatic CAT
activities are shown in Fig. 3A. They were much lower in
nimesulide treated mice than in the controls (35.9 ± 1.8 and
99.5 ± 2.0 units/mg protein, respectively). In the P. niruri
protein pretreated group, the CAT activity was significantly
higher (85 ± 0.8 unit/mg protein) than the nimesulide treated
group and was almost close to the normal group.

The hepatic GSH levels have been shown in Fig. 4 A.
Nimesulide treatment of mice reduced liver GSH
level (39.9 ± 0.62 in nimesulide treated mice versus
35.9 ± 1.1 nmole/mg protein in normal mice). GSH was
significantly higher in the P. niruri protein fraction pretreated
mice (79.2 ± 1.6 nmole/mg of total protein).

Fig. 1 shows the protective effect of P. niruri protein
fraction on nimesulide induced lipid peroxidation, measured
by the hepatic MDA content. Suggesting oxidative dam-
age, the hepatic MDA levels were much increased in mice
treated with nimesulide compared to controls (84.3 ± 1.1
versus 36.6 ± 1.6 nmole/g liver tissue in controls). Pre-
treatment with the P. niruri protein fraction significantly
reduced the nimesulide induced increase of TBARS con-
tent (42.0 ± 1.5 nmole/g liver tissue). The non-relevant pro-
tein, BSA, could not prevent nimesulide induced oxidative
stress whereas the positive control, the antioxidant, Vitamin E administration did.

As liver regenerates after damage, we conducted a time dependent study to evaluate the curative effect of \textit{P. niruri} protein fraction on nimesulide induced oxidative stress in mice. Fig. 2B shows the changes in SOD activity after nimesulide induced oxidative stress followed by the treatment with this protein fraction. Nimesulide treatment caused reduction of the SOD activity (341 $\pm$ 11.9 compared to 546 $\pm$ 12.0 units/mg protein in controls). SOD activity started to increase when the animals were post-treated with the \textit{P. niruri} protein fraction for 3 days (455 $\pm$ 12.9 for 3 days and 525 $\pm$ 17.3 units/mg protein for 4 days) compared to their nimesulide only treated controls (380 $\pm$ 14.2 and 422 $\pm$ 12.6 units/mg total protein for 3 days and for 4 days, respectively). After 5 days of \textit{P. niruri} protein fraction treatment, the SOD activity came back to almost normal (555.3 $\pm$ 15.4 units/mg total protein) whereas it remained low (440 $\pm$ 13.2 units/mg total protein) in its nimesulide treated controls. Fig. 3B shows the CAT activity after nimesulide induced oxidative stress followed by the post-treatment with Fig. 2. (A) Effect of pretreatment of PI on SOD level in nimesulide induced oxidative stress. For experimental detail, see the materials and methods. Cont: SOD level in control mice, Nim: SOD level in nimesulide treated mice, PI + Nim: SOD activity in PI pretreated mice followed by nimesulide treatment; Vitamin E: effect of Vitamin E pretreatment on nimesulide induced SOD level and BSA: effect of BSA pretreatment on nimesulide induced SOD activity. Each column represents mean $\pm$ S.D., $n=8$; (*$P<0.05$). (B) SOD level in the murine liver after nimesulide administration followed by PI post-treatment. Control: SOD level in normal mice, Nim: SOD levels in nimesulide treated mice at a dose of 10 mg/kg body weight for 1 week and then sacrificed after 24, 48, 72, 96 and 120h, respectively, Nim + PI: SOD levels in the livers of PI treated mice once daily i.p. at a dose of 5 mg/kg body weight 24, 48, 72, 96 and 120h, respectively after nimesulide administration. Each value represents mean $\pm$ S.D. ($n=8$) (*$P<0.05$).

Fig. 3. (A) Effect of pretreatment of PI on CAT activity in nimesulide induced oxidative stress. Legend is exactly same as described in that of Fig. 2A. ‘CAT’ should be read in place of ‘SOD’. (B) CAT activity in the murine liver following protein post-treatment after nimesulide administration. Legend is exactly same as described in that of Fig. 2B. ‘CAT’ should be read in place of ‘SOD’.
**P. niruri** protein fraction. The CAT activity was significantly elevated when the animals were post-treated with the protein fraction for 3 days (77.0 ± 4.2 and 98.6 ± 3.9 units/mg total protein for 4 days, respectively) compared to their respective nimesulide controls (62.6 ± 4.3 and 73.3 ± 3.8 units/mg total protein). After 5 days post-treatment, the CAT activity was almost normal (102.7 ± 4.8 units/mg total protein) whereas for the nimesulide controls, the activity was still lower (76.9 ± 2.9 units/mg total protein). Effect of **P. niruri** protein fraction on GSH level post to nimesulide administration has been shown in Fig. 4B. Nimesulide caused significant depletion of GSH level (40.8 ± 1.1 unit/mg total protein for nimesulide treatment vs. 87.7 ± 3.2 unit/mg total protein for normal control). Like SOD and CAT, post-treatment with PI could not enhance the reduced level of GSH for treatment up to 2 days. After that, the enhancement was significant (59.6 ± 2.1, 73.3 ± 2.3 and 80.7 ± 1.4 mmole/mg tissue for 3, 4 and 5 days, respectively). The normal recovery was, however, slower in the nimesulide controls (47.0 ± 2.2, 58.3 ± 0.9 and 62.5 ± 1.5 mmole/mg tissue for 3, 4 and 5 days, respectively).

The post-treatment effect of **P. niruri** protein fraction on hepatic lipid peroxidation has been shown in Fig. 5B. On day 3, the MDA level in protein treated mice (48.8 ± 0.59 mmole/g
Histopathological assessment have been represented in Fig. 6. Nimesulide treatment caused prominent changes including centrilobular necrosis, bile duct proliferation, disorganization of normal radiating pattern of cell plates around central vein, etc. (Fig. 6B). Qualitatively, the inflammations in \textit{P. niruri} protein fraction treated mice before and after nimesulide administration were less severe (Fig. 6C and D).

4. Discussion

Present study showed that nimesulide caused a reduction of the levels of GSH and various antioxidant enzymes were significantly elevated when the animals were intraperitoneally pre-treated with a partially purified protein fraction isolated from fresh young leaves and stems of \textit{P. niruri}. In addition, nimesulide induced enhancement of lipid peroxidation was also significantly reduced by this pre-treatment. Also post-treatment of animals with this protein fraction restored the altered levels of the antioxidant enzymes, GSH and lipid peroxidation caused by nimesulide. The \textit{P. niruri} protein fraction possessed radical scavenging activity. Histopathological assessment showed that nimesulide induced prominent changes typical of liver necrosis, including cell disruption, bile duct proliferation etc. were less severe in the pre- and post-treated mice. However, this \textit{P. niruri} protein fraction lost its biological activity either when digested with trypsin at 37 \celsius or preheated at 95 \celsius for 10 min. Intraperitoneal administration of BSA at the same dose had no effect on nimesulide induced oxidative stress, although hepatoprotective results were obtained when the animals were treated with the antioxidant, Vitamin E, prior to nimesulide administration.

It is known that liver is the central organ to metabolize all foreign compounds. It is injured by the exposures to various pollutants, toxicants, hazardous chemicals and also by a number of drugs when taken frequently or beyond their therapeutic doses. These toxicants mainly damage liver by producing reactive oxygen species, ROS [1,2]. To encounter the oxidative stress, antioxidant defence mechanism operates in our body to detoxify or scavenge ROS. The antioxidant system comprises different types of functional components including different antioxidant enzymes, together with the substances that are capable of reducing ROS or preventing their formation. Among them, SOD mainly act by quenching of superoxide (\(O_2^-\)), an active oxygen radical [20,21], produced in different aerobic metabolism. CAT acts by catalyzing the decomposition of \(H_2O_2\) to water and oxygen.

Fig. 6. Histopathological pictures of normal mouse liver (A), nimesulide treated mouse liver (B) and hepatic histology mice treated \textit{P. niruri} protein fraction before and after nimesulide administration (C and D, respectively). Liver sections were stained with haematoxylin and eosin (H and E, magnification 40×).
We found that nimesulide treatment caused a severe depletion of the hepatic GSH and various antioxidant enzymes in association with the enhancement of lipid peroxidation. Treatment with *P. niruri* protein fraction prior to nimesulide administration restored the altered levels of GSH and those enzymes to almost normal. In addition, extent of lipid peroxidation was also reduced by pretreatment. Results of the post-treatment studies showed that it took 3 days protein treatments until the promoted healing was visible. Post-treatment for 3rd, 4th and 5th days after nimesulide administration significantly reduced the increased MDA level and elevated the decreased levels of the antioxidant enzymes and GSH. Thus the *P. niruri* protein fraction speeded the recovery.

Liver histology of nimesulide treated mice showed considerable necrosis along the central vein. The sizes of the hepatocytes were also larger and balloon like compared to those in the normal mice liver. The necrosis was mostly centrilobular and extending through the whole liver lobule. On the other hand, the livers were less necrotic in mice pre- and post-treated with the *P. niruri* protein fraction.

The data obtained suggest that the *P. niruri* protein fraction possesses both preventive and curative activities against nimesulide induced oxidative stress in mice. To clarify the mechanism(s) further purification and characterization as well as investigation on its biological activities are needed and currently in progress.

In conclusion, the pre- and post-hepatoprotective action of the *P. niruri* protein fraction against the nimesulide-induced oxidative stress in murine liver seems to be due to its antioxidant properties.

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**References**


