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Therapeutic Effect of Indian Ayurvedic Herbal Formulation Triphala on Acetaminophen-Induced Hepatotoxicity in Mice

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Abstract: Triphala, an Indian ayurvedic formulation used widely in ayurveda, is believed to promote health, immunity and longevity. The present study evaluates its hepatoprotective role. Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.) inhibited acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$, i.p.) -induced hepatotoxicity in mice as indicated by the decrease of serum aminotransferases, alkaline phosphatase, inflammatory mediator TNF-α and hepatic lipid peroxidation. Triphala extract also protected acetaminophen-induced hepatic enzymic anti-oxidants and glutathione depletion. These observations demonstrate that Triphala treatment may attenuate acetaminophen-induced hepatoxicity in mice.

Key words: Triphala, acetaminophen, lipid peroxidation, hepatoprotective, antioxidant, tumour necrosis factor-α

INTRODUCTION

Acetaminophen (AAP) hepatotoxicity is currently the single most important cause for acute liver failure in the US and is associated with a significant number of deaths (Björnsson and Olsson, 2006). An overdose of the analgesic drug, acetaminophen (AAP) can lead to severe liver injury in humans and in experimental animals. Although studied intensely for more than 25 years, the mechanism of this injury is still not entirely clear. Drug induced hepatotoxicity is a potentially serious adverse effect of the currently used non-steroidal anti-inflammatory drugs. A conventional drug used for the treatment of such adverse reactions are often inadequate and presently de-challenge of the offending drug is recommended. Therefore, efforts to explore hepatoprotective effect of any natural products against acetaminophen-induced hepatotoxicity carry a great clinical significance.

Triphala is the most commonly used Indian ayurvedic herbal formulation, comprising the fruits of three trees, Indian goose berry (*Emblica officinalis* Gaertn, family-Euphobiaceae), Belleric myrobalan (*Terminalia belerica* Linn, family-Combretaceae), Chebulic myrobalan (*Terminalia chebula* Retzr, family-Combretaceae). In ayurveda, it is an important medicine of the rasayana group and is believed to promote health, immunity and longevity (Sandhyaa *et al*., 2006). This formulation, rich in antioxidants, plays an essential role in the treatment of a wide variety of conditions like infections, obesity, anaemia, fatigue, constipation and in infectious diseases like tuberculosis, pneumonia and AIDS (El-Mekkawey and Merelhy, 1995). Triphala extract has been reported to be a rich source of Vitamin C, ellagic acid, gallic acid, chebulinic acid, bellericanin, β-sitosterol and flavanoids (Jagetia *et al*., 2002). Its components *Emblica officinalis, Terminalia belerica, Terminalia chebula* are reported to possess anti-inflammatory, antimutagenic, antioxidant, cytoprotective, gastroprotective activity, myocardial necrosis, hepatoprotective, antibacterial and anticancer activity (Mukherjee *et al*., 2006; Jose and Kuttan, 2006). However, the hepatoprotective effect of

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Triphala against acetaminophen-induced hepatotoxicity has not yet been reported to the best of our knowledge. This study was, therefore, undertaken to examine the hepatoprotective effect of Triphala on acetaminophen-induced hepatotoxicity in mice.

MATERIALS AND METHODS

Animals

Swiss albino mice, 25-30 g, of either sex were obtained from the Tamil Nadu Veterinary College, Chennai, India. They were acclimatized for a week in a light and temperature -controlled room with a 12 h dark-light cycle and fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water was made freely available. The animals used in this study were treated and cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Culture, Chennai, India.

Drugs and Chemicals

The commercially available Triphala powder (mixture of dried and powdered fruits of three plants, i.e., *T. chebula*, *E. officinalis* and *T. bellerica* in equal proportions (1:1:1)) was obtained from the Indian Medical Practitioners Co-operative Stores and Society (IMCOPS), Chennai, India. Silymarin, a standard hepatoprotective drug (25 mg kg^{-1} b.wt.$^{-1}$) obtained from the SRS Pharmaceuticals, Mumbai, India was administered intraperitoneally. Acetaminophen, thiobarbutric acid, 5, 5'-Dithiobis-p-nitrobenzoic acid, reduced glutathione and bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were standard laboratory reagents of analytical grade and purchased locally.

Preparation of the Triphala Extract

One hundred grams of the Triphala powder was boiled in 1000 mL of distilled water till the volume was reduced to one fourth of the original (250 mL). The extract was cooled; centrifuged using a cold centrifuge and the supernatant was collected and was concentrated by evaporating its liquid contents (Jagetia *et al*., 2002). An approximate 20% yield of the extract was obtained. Triphala extract suspended in saline solution was used at the dose of 100 mg kg^{-1} b.wt.$^{-1}$ intraperitoneally, based on the acute toxicity studies carried out (Litchfield and Wilcoxon, 1949).

Experimental Protocol

In this experiment, mice were randomly allocated into 5 groups, each consisting of six animals. All animals were made to fast 24 h before the experiment. The first group, the control group, received saline. The second group, acetaminophen group, was treated with a single dose of acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$, i.p.) (Sener *et al*., 2006). Acetaminophen was first dissolved in water at 70°C and then cooled to 37°C before administration. The 3rd, group, (Triphala + acetaminophen) were given Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.) 30 m after the single injection of acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$, i.p.). The 4th group (Silymarin + acetaminophen) was given Silymarin (25 mg kg^{-1} b.wt.$^{-1}$, i.p.) 30 mts after the single injection of acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$ i.p.). The fifth group, the Triphala extract group, received Triphala (100 mg kg^{-1} b.wt.$^{-1}$ i.p.) suspended in saline solution. The mice were decapitated at 4 h after acetaminophen injection; the trunk blood was collected, the serum was separated and stored at -70°C. Tissue samples from the liver were obtained for biochemical and histological analysis.

Biochemical Parameters

The activities of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase in serum were estimated by using commercial kits (Span Diagnostics, India).

In the hepatic tissue samples, lipid peroxidation was determined by the procedure of Ohkawa *et al*. (1997). Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of oxidative stress. Superoxide dismutase (was assayed according to the method of Marklund and Marklund (1974). The unit of enzyme activity is defined as the enzyme required to give 50% inhibition of pyrogallol auto-oxidation. Catalase (CAT) was assayed by the method of Sinha (1972). In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. Chromic acetate thus produced was measured colorimetrically at 610 nm. Glutathione peroxidase (GPx) was assayed by the method of Rotruk *et al*. (1973) based on the reaction between glutathione remaining after the action of GPx and 5,5'-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. Glutathione Reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Bellomo *et al*. (1987). Glutathione-S-transferase (GST) was assayed by the method of Habig *et al*. (1974). Total reduced glutathione (GSH) was determined by the method of Moron *et al*. (1979). The protein content was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Effect of Triphala Extract and Silymarin on TNF-α Production

TNF-α level in plasma of control and experimental mice were determined by enzyme-linked immunosorbent assay (ELISA, Cayman Chemicals, USA), according to the manufacturer's instructions.

Histopathological Studies

Immediately after sacrifice, a portion of the liver was fixed in 10% formalin. The washed tissue was dehydrated in descending grades of isopropanol and finally cleared in xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at 5 μm thickness and stained with haematoxylin

Statistical Analysis

Results were expressed as mean±SD and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's Newman-Keul's test. $p<0.05$ implied significance.

RESULTS AND DISCUSSION

The activities of alanine transaminase, aspartate transaminase and alkaline phosphatase in serum were significantly increased in acetaminophen control group compared to normal control group. The levels of the above enzymes were significantly reversed on treatment with Triphala extract (Table 1).

In acetaminophen treated mice, MDA level was increased significantly; whereas superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and glutathione were found to be decreased when compared to the control group. The administration of Triphala extract to acetaminophen induced mice altered the above changes by regulating the MDA level and anti-oxidant enzymes to nearly that of normal levels (Table 2).

Figure 1 shows the levels of pro-inflammatory cytokine tumour necrosis factor-α in the serum of control and experimental animals. The level of tumour necrosis factor-α in the acetaminophen treated mice were systemically overproduced in the serum, while the elevated level of tumour necrosis factor-α was found to be decreased in Triphala extract administered mice treated with acetaminophen.

Table 1: Effect of Triphala extract on liver functional markers in acetaminophen-intoxicated mice in serum

Parameters	Control	1	2	3	4
Alanine transaminase (U dL^{-1})	80±5.71	175±10.9a*	106±80.15b*	98±60.12c*	85±6.53
Aspartate transaminase (U dL^{-1})	89±5.56	256±18.2a*	128±10.6b*	120±70.05c*	95±5.93
Alkaline phosphatase (K.A.units/l)	114±8.76	476±29.75a*	225±14.06b*	180±13.8c*	125±8.92

1: Acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$), 2: Acetaminophen + Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.), 3: Acetaminophen + Silymarin (25 mg kg^{-1} b.wt.$^{-1}$, i.p.), 4: Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.). Each value represents the mean±SD of 6 mice. Comparisons were made as follows: a-control vs. AAP; b- AAP vs. Triphala extract + AAP; c- Silymarin vs. AAP, The symbols represent statistical significance at: *: $p<0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test

Table 2: Effect of Triphala extract on liver antioxidant enzymes-specific activities and lipid peroxidation levels in acetaminophen-intoxicated mice

Parameters	Control	1	2	3	4
Lipid peroxidation (LPO)	0.81±0.08	1.30±0.11a*	0.95±0.07b*	1.05±0.07c*	0.83±0.06
Superoxide dismutase (SOD)	3.25±0.27	1.54±0.12a*	2.75±0.21b*	2.90±0.17c*	3.21±0.17
Catalase (CAT)	24.30±1.51	17.20±1.22a*	22.00±1.37b*	23.40±1.95c*	25.30±1.80
Glutathione peroxidase (GPx)	8.50±6.57	5.40±0.31a*	7.50±0.625b*	8.01±0.50c*	8.70±0.54
Glutathione reductase (GR)	165.30±10.33	110.60±8.50a*	150.40±11.56b*	146.00±8.1c*	162.00±9.52
Glutathione-S-transferase (GST)	95.40±7.33	80.30±6.69a*	89.60±6.89b*	92.30±5.42c*	96.00±7.38
Total reduced glutathione	33.50±2.39	14.30±1.02a*	20.50±1.20b*	34.20±2.63c*	34.20±2.10

1: Acetaminophen (900 mg kg^{-1} b.wt.$^{-1}$), 2: Acetaminophen + Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.), 3: Acetaminophen+ Silymarin (25 mg kg^{-1} b.wt.$^{-1}$, i.p.), 4: Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.). Units: LPO: nmol of MDA formed/mg protein; CAT: µmol of H_2O_2 consumed/min/mg protein; SOD: units/mg protein (1 U = amount of enzyme that inhibits the autooxidation of pyrogallol by 50%); GPx: µg of GSH utilized/min/mg protein; GR: nmol of NADPH oxidized/min/mg protein; GST: nmol of 1-chloro-2,4-dinitrobenzene-GSH conjugate formed/ min/mg protein; Total reduced glutathione-nmol/mg/protein. Each value represents the mean±SD of 6 mice. Comparisons were made as follows: a-control vs. AAP; b- AAP vs. Triphala extract+ AAP; c- Silymarin vs. AAP, The symbols represent statistical significance at: *: $p<0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test

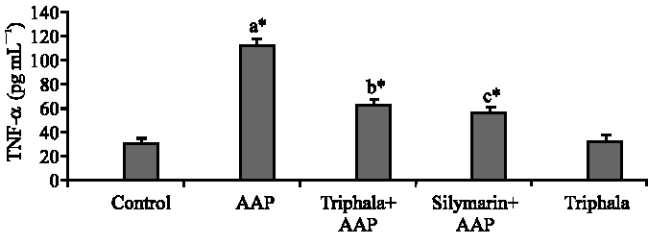

Fig. 1: TNF-α levels in serum samples of the control, acetaminophen (AAP), Triphala extract + AAP, Silymarin+AAP and Triphala extract groups. Each value represents the mean±SD of 6 mice. Comparisons were made as follows: a-control vs. acetaminophen (AAP); b-AAP vs. Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.) + AAP; c-Silymarin (25 mg kg^{-1} b.wt.$^{-1}$, i.p.) vs. AAP, The symbols represent statistical significance at: *: $p<0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test

Liver sections from the control group (Fig. 2a) and Triphala treated mice (Fig 2e) showed normal lobular architecture and normal hepatic cells. The liver section from animals given acetaminophen showed focal hepatocyte damage in the form of necrosis and condensed hepatic nuclei (Fig. 2b). The histological pattern of the liver of the mice treated with Triphala extract (Fig. 2c) and Silymarin (Fig. 2d) showed no necrosis and swollen hepatocytes (Fig. 2c).

Acetaminophen (paracetamol), a frequently used analgesic and antipyretic drug, is known to be hepatotoxic in high doses, which is primarily metabolized by sulfation and glucuronidation to unreactive metabolites and then activated by the cytochrome P-450 system to cause liver injury.

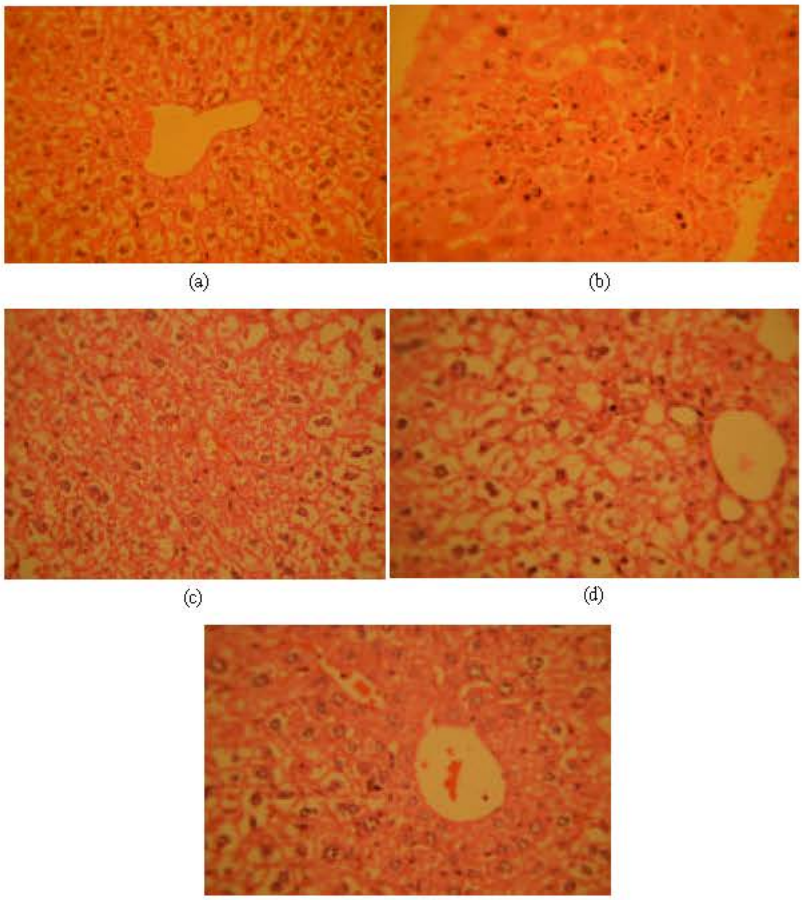

Fig. 2: Photomicrographs of liver sections taken from different groups. (a) Control groups; showing normal lobular architecture and normal hepatic cells (b) AAP group; showing focal hepatocyte damage in the form of necrosis and condensed hepatic nuclei (c) AAP group +Triphala extract (100 mg kg^{-1} b.wt.$^{-1}$, i.p.), showing marked improvement over AAP treated group (d) AAP group + Silymarin (25 mg kg^{-1} b.wt.$^{-1}$, i.p.); showing almost normal architecture of liver and (e) Triphala extract control (100 mg kg^{-1} b.wt.$^{-1}$, i.p.); showing normal lobular architecture and normal hepatic cells. (H and E staining, original magnification 400x)

In the present study, the rise in the serum levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in acetaminophen treated mice has been attributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm and are released into the circulation after cellular damage (Sallie *et al*., 1991). Triphala extract seems to preserve the structural integrity of the hepatocellular membrane as evident from the significant reduction in acetaminophen-induced rise in serum enzymes in mice. The decreased serum enzymes level in acetaminophen-induced liver damage by Triphala extract may be due to the prevention of leakage of the intracellular enzymes by its membrane stabilizing activity, which was supported by the limited extent of histological changes. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew *et al*., 1987).

It is established that acetaminophen is bioactivated to a toxic electrophile, N-acetyl-p-benzoquinone imine (NAPQI), which binds covalently to tissue macromolecules and probably also

oxidizes lipids, or the critical sulphydryl groups (protein thiols) and alters the homeostasis of calcium (Lin *et al*., 1997). In addition, NAPQI can increase the formation of reactive oxygen species and reactive nitrogen species such as superoxide anion, hydroxyl radical and hydrogen peroxide, nitro oxide and peroxynitrite, respectively. Excess levels of reactive oxygen species and reactive nitrogen species can attack biological molecules such as DNA, protein and phospholipids, which leads to lipid peroxidation, nitration of tyrosine and depletion of the antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) that further results in oxidative stress (Hinson *et al*., 2002). In accordance with previous findings, in our study we observed a high lipid peroxidation with a concomitant decrease in the enzymic antioxidant status including glutathione in the hepatic tissue during acetaminophen toxicity. Triphala extract was observed to exhibit hepatoprotective effect as demonstrated by enhanced activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase), glutathione and diminished amount of lipid peroxide against the acetaminophen-induced hepatotoxicity animals. *Emblica officinalis*, *Terminalia belerica* and *Terminalia chebula*, constituents of Triphala extract has been reported to be a rich source of Vitamin C, ellagic acid, gallic acid, chebulinic acid, bellericanin, β-sitosterol and flavanoids. Most of these compounds have been reported to be a potent inhibitor of lipid peroxide formation, a scavenger of hydroxyl and superoxide radicals and to increase the antioxidant enzymes (Jagetia *et al*., 2002; Jose and Kuttan, 1995). Thus the modifying role of Triphala extract observed in our study may be due to the antiperoxidative action of its components that was reported earlier (Lee *et al*., 2005).

TNF-α is a multifunctional cytokine produced by a number of different cells in the liver, including kupffer cells. Blazka *et al*. (1995) hypothesized that excess production of acetaminophen metabolite causes the initial hepatic damage and subsequent activation of inflammatory mediator TNF-α. It is capable of stimulating the production and secretion of other cytokines and infiltrating neutrophils to release reactive oxygen intermediates, which in turn contribute to tissue necrosis. In accordance to this report, our results demonstrate that acetaminophen increases serum TNF- α, indicating the role of this cytokine in acetaminophen induced hepatotoxicity. Furthermore, Triphala extract treatment significantly reduced the elevated TNF- α level in acetaminophen treated mice.

CONCLUSION

Present results provide strong evidence that Triphala extract significantly inhibits the acute liver toxicity induced by high doses of acetaminophen in mice which extract might be due to its antioxidant and TNF-α lowering properties of the extract. However, further pharmacological evidences at molecular level are required to establish the mechanism of the action of the drug which is underway.

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