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Research Article

ANTIHEPATOPROTECTIVE POTENTIAL OF LIVINA, A POLYHERBAL PREPARATION ON PARACETAMOL INDUCED HEPATOTOXICITY: A COMPARISON WITH SILYMARIN

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ABSTRACT

Comparative protective activity of polyherbal formulation Livina against a well-established drug Silymarin was assessed against Paracetamol induced hepatic injury in swiss albino mice. To clarify its effect on liver functions, eight-week-old male mice were injected with Paracetamol at the single dose of 200 mg kg^{-1} body weight intraperitonially once daily 28 days. The hepatoprotective effect of polyherbal formulation (Livina) was comparatively evaluated with Silmarin (25 mg kg^{-1}) by measuring levels of serum markers such as alanine aminotransferase (ALT), aspertate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyle transpeptidase (GGT), lipid peroxidase (MDA), and total and direct bilirubin levels in mice serum along with the histopathological studies to support the above mentioned parameters. The study found that the supplementation of Livina significantly ($P<0.05$, $P<0.001$) reduced the damaging effects on liver by Paracetamol. Histopathological changes (congestion of central vein, centrilobular necrosis and sinusoidal congestion) induced by Paracetamol were also reduced to a moderate extent in Livina treated mice. In both the cases the effectiveness of Livina were found to be almost parallel with Silymarin, indicating the herbal formulation to be almost as effective as the standard drug.

Key words: Paracetamol, hepatotoxicity, liver enzymes, Livina.

INTRODUCTION

Liver diseases are considered to be serious health problems, as the liver has one of the highest value of importance for the systemic detoxification and deposition of endogenous and exogenous substances. Steroids, vaccines and antiviral drugs, which are commonly employed as therapies for hepatic diseases, have potential adverse effects, especially when administered for long terms [1]. Therefore, it became necessary to screen a battery of new drugs, which can primarily act as an anti-hepatotoxic agent, and side-by-side ameliorates drug induced critical hepatic damage as a secondary rescuer. Thus to introduce a group of alternative drugs for the treatment of liver diseases and to replace the currently used drugs of doubtful efficacy and safety. In recent years, there has been a substantial increase in the use of so-called complementary and alternative therapies that utilize herbal medicines by patients with liver disease [2-4].

Paracetamol, one of the most widely used hepatotoxic drugs, is safe at therapeutic doses, but causes liver failure in overdoses [5,6]. A number of reports indicate that overdose of paracetamol can produce centrizonal hemorrhagic hepatic necrosis in humans and experimental animals [7,8]. When a normal dose is used, paracetamol is extensively metabolized by conjugation with sulphate and glucuronic acid. A small fraction of the drug is subjected to oxidation reactions catalyzed by CYP450 enzymes in the liver, resulting in generation of N-acetyl-p-benzo-quinoneimine (NAPQI), a highly electrophilic metabolite that triggers ensuing liver damage. Exposure to high doses of paracetamol increases the NAPQI level. Normally, toxic metabolites generated in the liver due to oxidation, are converted into non-toxic metabolites excreted in urine via conjugation with glutathione (GSH). However, high doses of paracetamol tend to limit the ability of GSH to detoxify NAPQI, and result in the consumption of liver GSH stores [9,10]. It was reported that oxidative stress constitutes a major mechanism underlying the pathogenesis of paracetamol-induced liver damage [11,12].

Modern medical science does not have, at present, a therapeutic agent that could cure the different liver disorders .In fact, the available remedies are from the traditional system of medicine. Herbs have recently attracted attention as health beneficial food and as essential materials for some critical disease remedy. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases including liver disease [13,14],

ischemia, reperfusion injury, atherosclerosis, acute hypertension, haemorrhagic shock, diabetes mellitus and cancer [15,16] with relatively little knowledge regarding their molecular mode of action [17]. Livina, a polyherbal formulation is very useful as a natural hepatoprotective medicine [18]. Our recent work also established that Livina protects against gastric mucosal damage and maintain mucosal lipid profile [19]. It also revels that Livina has an antioxidant property that protect liver and stomach against oxidative damage [20]. Recent work established that Livina prevent high fat diet induced obesity experimental mice [21]. Moreover Livina composed of several Indian medicinal plant extract those have hepatoprotective activity.

Silymarin is a composite combination of four flavonolignan isomers, which are silybin, isosilybin, silydianin and silychristin with an empirical formula $C_{25}H_{22}O_{10}$. The structural resemblance of silymarin to steroid hormones is assumed to be accountable for its protein synthesis facilitator measures. Among the isomers silybin is the main and most active component and represents about 60-70 per cent, followed by silychristin (20%), silydianin (10%), and isosilybin (5%) (Saller et al). Silipide (IdB 1016) is the silybin - phosphatidylcholine complex that ensures a huge increase in the bioavailability of silybin [22]. Various researchers against partial hepatectomy models and toxic models in experimental animals have established Hepatoprotective activity of silymarin by using acetaminophen, carbon tetrachloride, ethanol, D-galactosamine, and *Amanita phalloides* toxin [23,24].

Keeping the above information in view, the present study was designed to demonstrate paracetamol induced hepatotoxicity and the possible protective role of Livina, a polyherbal formulation in compared with Silymarin a known hepatoprotective drug in experimental mice.

MATERIALS AND METHODS

Chemicals and Drugs

Paracetamol was procured from Nulife Pharmaceuticals, Pune. Silymarin was purchased as Silybion-140 tablets from Micro labs (Hosur, Tamilnadu, India), bovine serum albumin (Sigma chemical St. Louis, MO, USA), thiobarbituric acid and TCA (Loba Chemie, Mumbai, India). Livina syrup was obtained from Dey's Medical Stores (Mfg.) Ltd., 62, Bondel Road, Kolkta-700019, India. AST, ALT, GGT and ALP kits were obtained from Merck, Germany. All other chemicals and solvents were of analytical grade commercially available.

Animals

Male Swiss albino mice weighing 32 ± 5g were used in the experiment. They were obtained from our CPCSEA approved animal house (Registration No. 50/CPCSEA/1999). The animals were

grouped and housed in cages and maintained under standard laboratory conditions (temperature 25 ± 2°) with dark and light cycle (12h/12h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata) and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. Dey's/IAEC/001/07, dated 14.08.2007).

Experimental Design

The mice were divided into the following groups each containing 10 mice (n=10)

Group-I: Control mice, which fed normal diet and water.

Group-II: Mice treated with paracetamol (200 mg kg^{-1}, p.o.) [25] for 28 days.

Group-II: Mice treated with paracetamol (200 mg kg^{-1}, p.o.)) along with 0.25 ml/day Livina orally once daily.

Group-IV: Mice treated with paracetamol (200 mg kg^{-1}, p.o.)) along with 0.5 ml/day Livina orally once daily.

Group-V: Mice treated with paracetamol (200 mg kg^{-1}, p.o.)) along with 1.0 ml/day Livina orally once daily.

Group-VI: Mice treated with paracetamol (200 mg kg^{-1}, p.o.)) along with Silymarin (25 mg kg^{-1}, p.o.).

Each 2ml of Livina syrup contains extracts of:

1.	*Solanum nigrum*	20 mg
2.	*Holarrhena antidysenterica*	10 mg
3.	*Tephrosia purpurea*	40 mg
4.	*Andrographis paniculata*	10 mg
5.	*Phyllanthus niruri*	20 mg
6.	*Tinospora cordifolia*	10 mg
7.	*Terminalia chebula*	10 mg
8.	*Asteracantha longifolia*	20 mg
9.	*Alstonia scholaris*	20 mg
10.	*Berberis aristata*	40 mg
11.	*Cichorium intybus*	10 mg
12.	*Picrorhiza kurroa*	20 mg

Measurement of body weight

The body weight intake of each mouse was recorded every day using a sensitive balance.

Blood collection

The total duration of experiment was 28 days, at the end of which the animals were fasted overnight, anaesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and killed by cervical dislocation. Blood was collected from the carotid artery and allowed to coagulate at ambient temperature for 30 min.

Preparation of tissue homogenate

Immediately after death, blood samples were collected in heparinized test tubes and plain tubes and centrifuged for the separation of serum. Liver tissue was washed with ice-cold saline. The tissues were then cut into fragments and homogenized with 3 volumes (w/v) of the appropriate buffer using a Potter-Elvehjam homogenizer with a Teflon pestle and centrifuged at 12000g for 20 min at 4°C [26], the supernatant was used for the estimation of lipid peroxidation .

Biochemical estimation

Serum ALT [27], AST [27], GGT [27], ALP [28], and bilirubin [29] (total and direct) were determined by kinetic method using the kit obtained from Merck, Germany in a double beam spectrophotometer (Elico SL-164, Elico Limited, Hyderabad, India). The transaminases activities were determined as change in absorbance/min at 340 nm. Serum ALP activity was determined from the rate of release of paranitrophenol at 405 nm. Total protein and bilirubin (total and direct) were measured at 540 nm. Serum total protein was measured according to the method of Lowry et al 1951[30].

Estimation of Lipid peroxidation

Lipid peroxidation was estimated by the method of Ohkawa et al. (1979) in tissue homogenates. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), ter-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37°C for 90 min, the reaction was stopped by adding 0.2 ml of 8% sodium dodecyl sulfate (SDS) followed by 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% TBA and further heating the mixture at 95°C for 45 min. After cooling, sample were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using 1.53 X 10^5 M^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmoles of TBARS per 90 min/mg protein [31].

Histopathological examination

Small portions of the liver tissue from all the groups were immediately collected after sacrificed. Tissues were fixed in 10% formalin in phosphate buffer (pH 7.0) for 24 hr. at room temperature for histopathology [32]. Tissues were embedded in paraffin wax and sections were cut at 3-5 μm slices and were stained with haematoxylin and eosin (H&E) and observed under light microscope.

Statistical analysis

Data were analysed by one way analysis of variance followed by Duncan's multiple range test using a commercially available statistics software package (SPSS for Windows, ver. 11.0;SPSS Inc., Chicago, IL, USA). Results were presented as mean ± S.D. Values of $P<0.05$ were regarded as statistically significant.

RESULTS

Effects of Livina on body weight and liver weight

The mean body weight was significantly reduced in paracetamol-intoxicated mice compared with control animals. Supplementation of Livina 0.5ml/day and 1ml/day significantly increased mean body weight compared with paracetamol treated mice. Mean liver weight significantly elevated in paracetamol treated mice as compared with the control and that was restored as normal in 0.5ml/day and 1ml/day Livina pretreatment.

Effects of Livina on biochemical investigation

The results of AST, ALT, GGT, ALP, total protein and bilirubin (total and direct) in control mice were 66.39 ± 5.41, 41.57 ± 2.15, 3.24 ± 0.36, 76.91 ± 11.54, 7.16±0.54, 0.86 ± 0.05 and 0.08 ± 0.01 respectively, whereas in paracetamol treated mice these levels were elevated to 112.84 ± 8.06, 88.59 ± 4.82, 7.97 ± 0.85, 198.54 ± 18.47, 3.69±0.29, 4.08 ± 0.06 and 0.65 ± 0.04 respectively. Livina pretreatment at the dose of 0.5 ml/kg significantly ($P<0.001$) prevented the paracetamol induced rise in the AST, ALT, GGT, ALP, total protein and bilirubin (total and direct) to 78.36 ± 4.84, 61.08 ± 3.25, 5.22 ± 0.57, 124.51 ± 13.22, 5.12±0.36, 2.98 ± 0.06 and 0.12 ± 0.02 respectively being compared to paracetamol treated group. With higher dose of Livina (1.0 ml/kg) further reduction of AST, ALT, GGT, ALP, total protein and bilirubin (total and direct) to 71.49 ± 4.33, 48.27 ± 3.44, 4.13 ± 0.46, 102.97 ± 12.44, 6.78±0.43, 1.12 ± 0.05 and 0.09 ± 0.01 respectively were noted. Silymarin (25 mg/kg, p.o) pretreatment also prevented the paracetamol induced rise in AST, ALT, GGT, ALP, total protein and bilirubin (total and direct) to 74.82 ± 5.27, 55.69 ± 2.64, 4.56 ± 0.44, 109.44 ± 12.58, 6.95±0.49, 1.36 ± 0.03 and 0.11 ± 0.02 respectively.

Table -1: Effects of Livina and paracetamol on body weight of Swiss albino mice after 4 weeks treatment

Groups	Initial BW (g)	Final BW (g)	% Weight gain	% Weight loss
I	35.46 ± 1.54	48.65 ± 2.49	37.16	-
II	35.12 ± 1.48	29.58 ± 1.98##	-	10.34
III	34.95 ± 1.32	41.22 ± 2.11*	17.93	-
IV	35.78 ± 1.87	43.03 ± 2.06*	20.26	-
V	34.17 ± 0.98	46.71 ± 1.57**	36.69	-
VI	34.88 ± 1.55	47.68 ± 1.69**	37.69	-

All values are mean ± S.D., n=10 mice in each group. ##*P<0.05* as compared with the control animal.
*P<0.01, **P<0.05, as compared with the paracetamol treated animal.

Table -2: Effects of Livina and paracetamol on serum AST, ALT and GGT of Swiss albino mice after 4 weeks treatment

Groups	Treatment	AST (IU/L)	ALT (IU/L)	GGT (IU/L)
I	Control	66.39 ± 5.41	41.57 ± 2.15	3.24 ± 0.36
II	Paracetamol (200 mg kg^{-1})	112.84 ± 8.06#	88.59 ± 4.82#	7.97 ± 0.85#
III	Livina (0.25 ml)	94.11 ± 6.57*	71.45 ± 4.15*	6.58 ± 0.52*
IV	Livina (0.5 ml)	78.36 ± 4.84**	61.08 ± 3.25**	5.22 ± 0.57**
V	Livina (1.0 ml)	71.49 ± 4.33***	48.27 ± 3.44***	4.13 ± 0.46***
VI	Silymarin(25 mg kg^{-1})	74.82 ± 5.27***	55.69 ± 2.64***	4.56 ± 0.44**

All values are mean ± S.D., n=10 mice in each group. #*P<0.001* as compared with the control animal *P<0.01, **P<0.05, ***P<0.001 as compared with the paracetamol treated animal.

Table -3: Effects of Livina and paracetamol on serum ALP, total bilirubin and direct bilirubin of Swiss albino mice after 4 weeks treatment

Groups	Treatment	ALP (IU/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
I	Control	76.91 ± 11.54	0.86 ± 0.05	0.08 ± 0.01
II	Paracetamol (200 mg kg^{-1})	198.54 ± 18.47#	4.08 ± 0.06#	0.65 ± 0.04#
III	Livina (0.25 ml)	165.48 ± 14.53*	3.33 ± 0.04*	0.25 ± 0.03*
IV	Livina (0.5 ml)	124.51 ± 13.22**	2.98 ± 0.06**	0.12 ± 0.02**
V	Livina (1.0 ml)	102.97 ± 12.44***	1.12 ± 0.05***	0.09 ± 0.01***
VI	Silymarin(25 mg kg^{-1})	109.44 ± 12.58***	1.36 ± 0.03***	0.11 ± 0.02***

All values are mean ± S.D., n=10 mice in each group. #*P<0.001* as compared with the control animal *P<0.01, **P<0.05, ***P<0.001 as compared with the paracetamol treated animal.

Table -4: Histopathological changes in paracetamol induced liver injury in mice

Microscopic observation	Control	Paracetamol treated	Livina (0.25 ml)	Livina (0.5 ml)	Livina (1.0 ml)	Silymarin
Nuclear disintegration	-	+++	-	-	-	-
Chromatolysis	-	++	-	-	-	-
Cytoplasmic vacuolation	-	++	+	+	-	-
Necrobiosis	-	+	-	-	-	-
Necrosis	-	+++	-	-	-	-
Kuppfercell hyparplasia	-	+++	++	+	+	-
Portal inflammation	-	-	-	-	-	-
Sinusoidal dialation	-	++	-	-	-	-
Centralvenous dialation	-	+	-	-	-	-
Increased cytoplasmic eosinophilia	-	+++	+	-	-	+

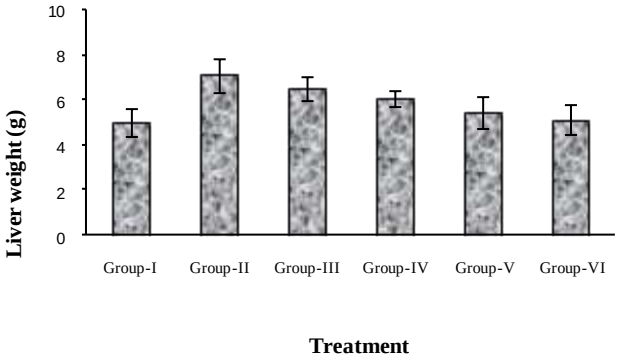

Figure -1: Effect of livina on liver weight in paracetamol induced hepatotxicity in mice. ***P<0.001 significantly different from control group. *P<0.01, **P<0.05 significantly different from paracetamol treated animals. NS not significant.

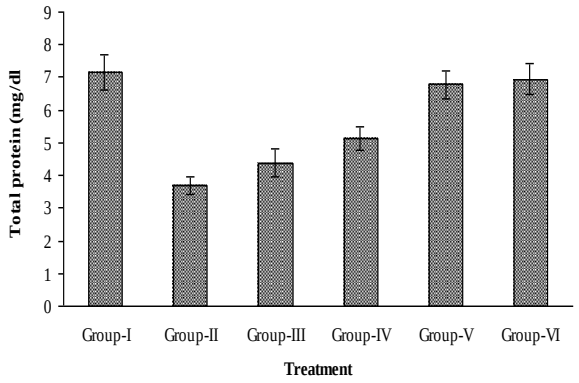

Figure -2: Effect of livina on total protein in paracetamol induced hepatotxicity in mice. ***P<0.001 significantly different from control group. *P<0.01, **P<0.05 significantly different from paracetamol treated animals. NS not significant.

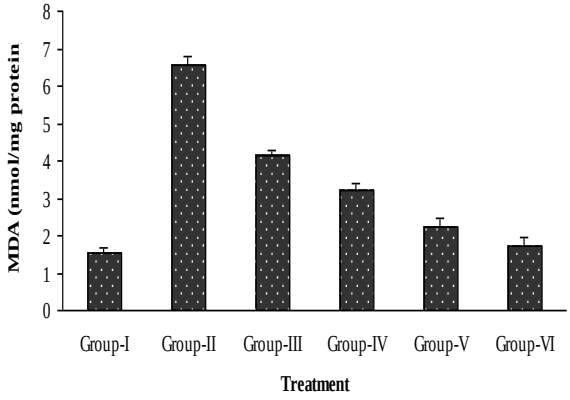

Figure -3: Effect of livina on lipid peroxidation (MDA Content) in paracetamol induced hepatotoxicity in mice. ***P<0.001 significantly different from control group. *P<0.01, **P<0.05 significantly different from paracetamol treated animals. NS not significant.

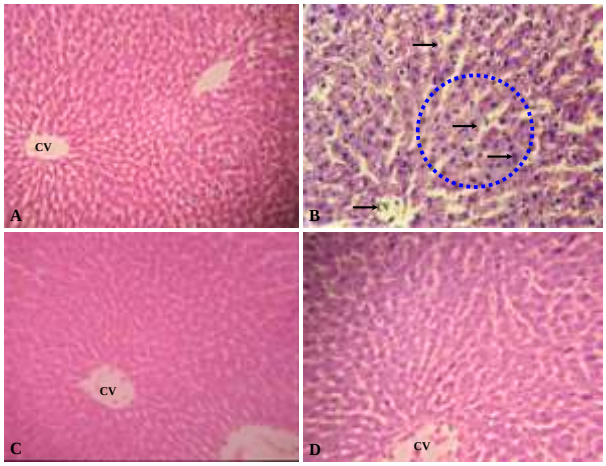

Figure -4: Photomicrographs of mice liver obtained from different treatment groups. A: Control; B: Paracetamol treated; C: Livina treatment (0.5 ml/day); C: Livina treatment (1 ml/day). CV= Central vein; S= Sinusoid

Effects of Livina on histopathological changes

Histological observation of liver tissue of the normal animal showed hepatic cells with well-preserved cytoplasm, nucleus, nucleolus and central vein. In paracetamol treated group, histological observation showed fatty degeneration, damage of parenchymal cells, steatosis and hydropic degeneration of liver tissue. Prominent damage of central lobular region appeared in the liver. Livina restored the histopathological abnormality induced by paracetamol.

DISCUSSION

Paracetamol, a well-known compound for producing chemical hepatic injury in mice has been used as an experimental model to test the potential hepatoprotective activity by several investigator [33-35]. It is mainly metabolized in the liver to glucuronide and sulphate conjugates that are subsequently extracted. The hepatotoxicity of paracetamol has been attributed to the formation a highly reactive metabolite N-acetyl-P-benzoquinoneimine (NAPQI) by the hepatic cytochrome P-450 [36].

The elevated levels of serum enzymes are indicative of cellular leakages and loss of functional integrity of cell membrane in liver. It is established that serum enzymes such as ALT and AST levels were elevated in paracetamol-induced hepatotoxicity [37]. Serum ALP and bilirubin levels on the other hand are related to the function of hepatic cells [38]. Increase in serum level of these hepatic markers signifies structural and functional catastrophe of the hepatic systems, in our current study we are focusing on the magnitude of recovery of the paracetamol injured hepatic unit by Livina.

Serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury [39]. Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells [40]. Hepatocytic necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases. ALT is a sensitive indicator of acute liver damage and elevation of this enzyme in non-hepatic diseases is unusual. ALT is more selectively a liver paranchymal enzyme than AST [41]. Therefore, the marked release of AST, ALT and GGT into the circulation indicates severe damage to hepatic tissue membranes during paracetamol intoxication. In the present study administration of paracetamol caused a dramatic elevation in serum AST, ALT and GGT activities, indicating subchronic hepatotoxicity induced by administration of paracetamol. Pretreatment with Livina, a polyherbal formulation efficiently prevented the paracetamol -induced elevation of serum AST, ALT and GGT activities in a dose-dependent manner, indicating the hepatoprotective activity of Livina against the intoxication of paracetamol. The results were compared with the standard drug Silymarin where Livina showed almost similar results.

Bilirubins, which are enzymes originally present at higher concentration in cytoplasm. When there is heaptopathy, bilirubin comes into the blood stream in conformity with the extent of liver damage [42]. Moreover, it is well known that narcotizing agents produce sufficient injury to hepatic parenchyma to cause elevation in bilirubin content in plasma [43]. However, bilirubin is one of the most useful clinical clues to the severity of necrosis and its accumulation is a measure of binding, conjugation and excretory

capacity of hypatocyte. These effects induced by paracetamol, were confirmed by our results. Livina at the dose of 0.5ml/day and 1ml/day orally for twenty-eight days significantly restored the altered ALP and total bilirubin levels, which is quantitively comparable with the efficacy shown by Silimarin and also directly indicated the gross effectiveness of the herbal formulation on functional status of the liver. Lipid peroxidation has been postulated to be the destructive process of liver damage due to paracetamol intoxication [6]. Lipid peroxide levels were significantly increased in paracetamol-intoxicated mice. The increase in MDA (in terms of TBARS) suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals [44-45]. Paracetamol intoxicated animals treated with 0.5ml/day and 1ml/day Livina orally for 28 days, had significantly reduced lipid peroxide levels in compared with mice treated with paracetamol only. Paracetamol intoxification significantly lowered total protein levels and 0.5ml/day and 1ml/day Livina supplementation significantly increased protein levels more or less up to the normal benchmark. Histopathology of the liver samples revealed that the necrosis was reduced to few inflammatory cells in the mice treated with Livina. Cytoplasmic vacuolations and hydropic changes were less prominent. Inflammation of portal veins was also reduced. Thus the histopathological study shows reduction of degree of necrosis in the mice treated with Livina as compared with paracetamol treatment.

In summary, the results of this study demonstrate that Livina, a polyherbal formulation has a potent hepatoprotective action on paracetamol induced hepatic damage in mice.

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