Hepatoprotective activity of six polyherbal formulations in CCl₄-induced liver toxicity in mice

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To evaluate pretreatment of six polyherbal liquid formulations (PLFs) commercially available in India, on CCl₄-induced liver injury, Swiss albino mice were treated for 7 days with distilled water or PLFs (2.6 and 5.2 ml/kg body weight/day, po) followed by single sc injection of 50% (v/v) CCl₄ in arachis oil at a dose of 1ml/kg. The serum biochemical parameters such as alanine transaminases, aspartate transaminases and alkaline phosphatase were estimated. Phenobarbitone-induced sleeping time and liver histopathology were also carried out. CCl₄-treated animals showed significant increase in the levels of liver enzymes, phenobarbitone-induced sleeping time and revealed fatty changes and centrizonal necrosis on histological examination of liver indicating hepatic damage. When pretreated with PLFs at a dose of 5.2 ml/kg body weight/day, the CCl₄-induced changes were significantly reversed. The pretreatment with PLFs can prevent acute liver damage induced by CCl₄ only at a higher dose. Therefore, it is suggested that a dose adjustment of these PLFs may be necessary for their optimal effects in human liver diseases.

Keywords: CCl₄, Centrizonal necrosis, Hepatoprotective, Hepatotoxicity, Liver enzymes, Polyherbal formulations

Liver plays a pivotal role in metabolism, secretion and storage. Any injury to liver can result in many disorders ranging from transient elevation in liver enzymes to life threatening liver cirrhosis and hepatic failure. The common causative agents of liver injuries are toxic chemicals (e.g., CCl₄, aflatoxin etc.), therapeutic drugs (e.g., antibiotics, anti-tubercular drugs etc.), alcohol, and microbial agents (e.g., hepatitis virus, leptospirosis, malarial parasites)

In recent years, the usage of herbal drugs for the treatment of liver diseases has increased all over the world. The herbal drugs are believed to be harmless and free from serious adverse reactions, as they are obtained from nature, and are easily available. Also, the limited therapeutic options and disappointing therapeutic success of modern medicine has increased the usage of alternative medicine including herbal preparations.

There are about 600 commercial herbal formulations, which are claimed to have hepatoprotective activity and many of them are being sold in market all over the world. In India, about 40 patented polyherbal formulations representing a variety of combinations of 93 herbs from 44 families are available.

Efficacy of the traditional and new herbal products should be tested by standard experimental methods. Also, there should be adequate data from in vivo and in vitro studies to validate the therapeutic potential claimed. Although information about individual polyherbal formulations is available, their comparative evaluation is scarce in the literature.

In the present study, six polyherbal hepatoprotective formulations, namely Liv 52, Livergen, Livokin, Octogen, Stimuliv, and Tefroliv which have been traditionally used for liver diseases were selected. They are claimed to be Ayurvedic medicines and are being sold as liver tonics. The objective of the present study was to evaluate the effects of the pretreatment with above mentioned six polyherbal liquid formulations (PLFs) in CCl₄-induced hepatotoxicity in mice.

Materials and Methods

Drugs and chemicals — Six polyherbal hepatoprotective formulations, namely Liv 52, Livergen, Livokin, Octogen, Stimuliv, and Tefroliv...
were randomly selected from 40 such formulations (Table 1). The criteria for selection were based on: (1) claim as Ayurvedic medicine, (2) commercial availability, (3) availability of liquid formulations for easy administration, (4) hepatoprotective activity of the formulation as claimed by the company, and (5) claim to have sufficient shelf life. CCl₄ was purchased from Ranbaxy, Mumbai, India and phenobarbitone from SPM Drugs, Bhavani, India.

**Animals** — Swiss albino mice weighing 25-30 g of either sex, bred in Central Animal House of JIPMER, Pondicherry were used. The animals were allowed standard food pellets (Pranav Agro Industries Ltd, Sangli) and water *ad libitum*. They were maintained in standard laboratory conditions (12: 12 hr L: D cycle and 25 ± 2° C). The study protocol was approved by “The Institute Animal Ethics Committee,” JIPMER, Pondicherry.

**Dose calculation of polyherbal formulations** — The doses of PLFs were calculated to precisely match with the human doses employed according to the manufacturer’s instructions. The average recommended human dose was extrapolated to that of mice by a standard conversion table. The earlier work on this suggested a partial protection by using 15 ml three times a day as human dose. Similarly, we have used a common minimum of 20 ml/day as the human dose. The formulations were 10 times diluted and were given twice daily for 7 days by oral route as pretreatment [2.6 ml/kg body weight (BW)/d]. All these oral liquid formulations were used within the expiry dates. A double dose pretreatment (po 5.2 ml/kg BW/d) was also studied with the formulations after five times diluting with distilled water. This was done in order to observe if there was hepatoprotection at two-dose levels by reversing the CCl₄-induced hepatotoxicity. Our 40 ml/day human dose fairly matches with the earlier study of 45 ml/day as human dose.

**Study design** — Animals were divided into 28 groups (n=6/group; equal number of males and females) with 14 groups for biochemical and pharmacological studies.

| Table 1—List of six commercially available polyherbal liquid formulations investigated for hepatoprotective activity (compiled from the manufacturer’s instructions) |
|---|---|---|---|
| Sl. no | Name of the formulation | Plants used in the formulation | Indication | Dose |
| 1 | Liv 52 (Himalaya Drug Co, Bangalore) | *Achillea millefolium*, *Capparis spinosa*, *Cassia occidentalis*, *Cichorium intybus*, *Solanum nigrum*, *Tamarix gallica*, *Terminalia arjuna* | Ayurvedic medicine, Protects liver against various hepatotoxins, promotes appetite and growth | 2-3 teaspoon 2 to 3 times daily |
| 2 | Livergen (Standard Pharmaceuticals, Serampore, West Bengal) | *Andrographis paniculata*, *Apium graveolens*, *Asteracantha longifolia*, *Cassia angustifolia*, *Trachyspermum ammi*, *Trigonella foenum-graecum* | Ayurvedic medicine, Gastrointestinal and hepatic regulator | 2-4 teaspoon twice daily |
| 3 | Livokin (Herbo-med, Kolkata) | *Andrographis paniculata*, *Apium graveolens*, *Berberis lycium*, *Carum copicicum*, *Cichorium intybus*, *Cyperus rotundus*, *Eclipta alba*, *Pomoea turpethum*, *Oldenlandia corymbosa*, *Picrorrhiza kurroa*, *Hydrophila spinosa*, *Plumbago zeylanica*, *Solanum nigrum*, *Tephrosia purpurea*, *Terminalia arjuna*, *Terminalia chebula*, *Trigonella foenum-graecum* | Ayurvedic medicine, for hepatic dysfunction | 1-2 teaspoon 2 to 3 times daily |
| 4 | Octogen (Plethico Pharmaceuticals Ltd., Indore) | *Arogayavardhini rasa*, *Phyllanthus niruri* | Ayurvedic medicine, highly potent hepatoprotective | As directed by physician |
| 5 | Stimuliv (Franco-Indian Pharmaceuticals Pvt Ltd, Mumbai) | *Andrographis paniculata*, *Eclipta alba*, *Phyllanthus niruri*, *Justicia procumbens* | Ayurvedic medicine, liver stimulant and tonic | 1-2 teaspoon 2 to 3 times daily |
| 6 | Tefroliv (TTK Pharma Pvt Ltd, Chennai) | *Andrographis paniculata*, *Eclipta alba*, *Ocimum sanctum*, *Phyllanthus niruri*, *Picrorrhiza kurroa*, *Piper longum*, *Solanum nigrum*, *Tephrosia purpurea*, *Terminalia chebula* | Ayurvedic medicine, standardized liver formulation for effective hepatic regeneration | 1 teaspoon thrice daily or as directed by physician |
histopathological parameters and another 14 groups for measuring phenobarbitone-induced sleeping time.

Group 1 - Normal control; the animals received distilled water alone for 7 days.

Group 2 - Pathological control; The animals received distilled water for 7 days and CCl4, 1 ml/kg BW, diluted with arachis oil at 1: 1 ratio, subcutaneously (sc), once on day 8.

Groups 3 to 8 - Pretreatment with polyherbal formulations at 2.6 ml/kg BW/d for 7 days (po) followed by a single dose of CCl4 (sc) on day 8

Groups 9 to 14 - Pretreatment with polyherbal formulations at 5.2 ml/kg BW/d for 7 days (po) followed by a single dose of CCl4 (sc) on day 8

Group 15 - Normal phenobarbitone-induced sleeping time; Animals received distilled water for 8 days followed by a single dose of phenobarbitone, 40 mg/kg, ip

Group 16 - Phenobarbitone-induced sleeping time in pathological control; The animals received distilled water for 7 days and given CCl4 single dose, sc on day 8 followed by a single dose of phenobarbitone, ip on day 9

Groups 17 to 22- Pretreatment with 2.6 ml/kg BW/d of polyherbal formulations for 7 days followed by CCl4 on day 8 and phenobarbitone, ip on day 9.

Groups 23 to 28- Pretreatment with 5.2 ml/kg BW/d of polyherbal formulations for 7 days followed by CCl4 on day 8 and phenobarbitone on day 9.

Biochemical and histopathological parameters (Groups 1-14) — After 24 hr of CCl4 administration, animals were anaesthetized using ether and 1 ml of blood was collected by cardiac puncture. The blood was allowed to clot and centrifuged (Remi, Mumbai) at 2500 rpm for 10 min. The serum was separated and used for assay of alanine transaminases (ALT), aspartate transaminases (AST) and alkaline phosphatase (ALP), by standard methods using enzyme assay kits (Span Diagnostics Ltd, India) adopted to Microlab 200 semiauto analyzer (E. Merck, Germany)\(^7,8\). The animals were sacrificed by cervical dislocation and liver was excised, washed with phosphate buffer and dried with tissue paper. It was weighed and transferred to a 10% formalin fixative solution for 48 hr. The liver tissues were processed for paraffin embedding and sections (5 μm thick) were taken in a microtome. After staining with hematoxylin and eosin, slides were examined under microscope (40,100 and 200 ×) for histopathological changes.

Phenobarbitone-induced sleeping time (Groups 15-28) — After phenobarbitone administration, sleeping time (min) was recorded from onset of sleep to their natural arousal\(^9\). The method was modified from earlier report by using phenobarbitone instead of hexobarbitone\(^10\). The dose selection of phenobarbitone was based on a pilot study conducted in our laboratory. In male Swiss albino mice, varying doses of phenobarbitone were given ranging from 20-60 mg/kg, ip. With the dose of 20 mg/kg, the mice were sedated and did not go in to sleep. On the other hand, with 60 mg/kg dose, the mice went on to a prolonged sleep for more than 6½ hr. Hence, 40 mg/kg, (ip) dose was standardized to study the phenobarbitone-induced sleeping time\(^11\). The normal control sleeping time was compared with CCl4-toxicity group and PLF pretreatment groups.

Statistical analysis — Data were expressed as mean ± SE. The biochemical data were analyzed by one way ANOVA followed by Student- Newman-Keuls test using Graphpad Instat version 3.06. A P value of less than 0.05 was considered as statistically significant.

Results

The pretreatment with six PLFs at the dose of 2.6 ml/kg were found to be ineffective as hepatoprotectants (data is not shown). The effect was found with high dose (5.2 ml/kg BW) of pretreatment. PLFs at this dose appreciably reduced the markers of hepatic damage in CCl4 treated animals, but the difference was found to be statistically significant only with Liv 52 and Livergen.

Effect of CCl4 and PLFs on biochemical parameters of hepatotoxicity — In CCl4-treated group, without any pretreatment, the activity of ALT (677.5± 66.04 IU/L), AST (967.5± 77.82 IU/L) and ALP (350.0± 22.62 IU/L) was significantly higher in comparison to normal control (ALT 33.3± 0.61; AST 89.3± 0.95; ALP 152.17± 11.40 IU/L). Six PLFs namely Liv 52, Livergen, Livokin, Octogen, Stimuliv, and Tefroliv when administered as pretreatment for 7 days prior to CCl4 injection, brought about significant lowering of liver enzyme levels. The effects in Liv 52 and Livergen groups were remarkably high (Fig. 1), and found to be statistically significant.

Effect of CCl4 and PLFs on phenobarbitone-induced sleeping time — The CCl4-induced liver injury led to an increase in the duration of barbiturate-induced sleeping time (from 277.50± 8.04 min in
normal control to 347.50±12.16 min in CCl4-induced hepatotoxicity group; \( P < 0.05 \). A significant restoration of phenobarbitone-induced sleeping time was observed with all the polyherbal drugs, except Stimuliv, when pretreated at higher dose (Table 2).

**Effect of CCl4 and PLFs on microscopic appearance of the liver tissue** — The liver tissues were stained with hematoxylin and eosin and viewed under optical microscope. In healthy controls, the typical architecture of liver tissue was observed with central vein (CV) from which chords of hepatocytes were radiating (Fig. 2). CCl4-treatment produced extensive necrosis of hepatocytes, which was more pronounced in the centrilobular (zone 3) region with scattered fatty changes with inflammatory reaction (Fig. 3). Pretreatment with low dose of Liv 52 (2.6 ml/kg/d) showed partial hepatic protection with minor hepatic necrosis and mild portal inflammation (Fig.4). When administered at higher doses (5.2 ml/kg/d), Liv 52 and Livergen was found to protect the liver from CCl4-induced damage as evidenced by restoration of a near normal architecture of the liver parenchyma (Fig. 5).

**Discussion**

In the present study, six commercial formulations available in Indian market were evaluated and compared for their efficacy as hepatoprotective agents in CCl4-induced hepatotoxicity in mice. The doses of PLFs used in the study were calculated to precisely match with the human doses. The statistically

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**Table 2**—Effect of pretreatment with polyherbal formulations (5.2 ml/kg body wt/day) on phenobarbitone-induced sleeping time of mice in CCl4-induced hepatotoxicity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Phenobarbitone-induced sleeping time (min)</th>
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</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>277.50 ± 8.04</td>
</tr>
<tr>
<td>CCl4</td>
<td>347.5 ± 12.16 ( ^\dagger )</td>
</tr>
<tr>
<td>Liv 52 + CCl4</td>
<td>284.17 ± 9.17 ( ^* )</td>
</tr>
<tr>
<td>Livergen + CCl4</td>
<td>279.17 ± 14.97 ( ^* )</td>
</tr>
<tr>
<td>Livokin + CCl4</td>
<td>285.83 ± 16.70 ( ^* )</td>
</tr>
<tr>
<td>Octogen + CCl4</td>
<td>295.0± 21.83</td>
</tr>
<tr>
<td>Stimuliv + CCl4</td>
<td>235.83± 15.62 ( ^* )</td>
</tr>
<tr>
<td>Tefroliv + CCl4</td>
<td>231.67± 22.01 ( ^* )</td>
</tr>
</tbody>
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\( P \) values: <0.05; compared to \( ^\dagger \) normal control group; \( ^* \) CCl4 group, by One-way ANOVA followed by Student-Newman-Keuls test as post-hoc test.

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**Fig. 1**—Effect of pretreatment with polyherbal formulations (5.2 ml/kg body wt/day) on serum levels of liver enzymes of mice with CCl4-induced hepatotoxicity [Values are mean ± SE from 6 mice per group. \( P \) values: <0.05; compared to \( ^\dagger \) normal control group; \( ^* \) CCl4 group; \( ^\ddagger \) Liv 52-treated group; \( ^\ddagger \) Livergen-treated group; \( ^\ddagger \) Livokin-treated group, by one-way ANOVA followed by Student-Newman-Keuls test as post-hoc test]

**Fig. 2**—Normal appearance of mouse liver showing central vein (CV) with radiating hepatocytes and portal triad [portal vein (PV), hepatic artery and bile duct (BD)]. H&E \( \times 40 \)
significant reduction in serum biochemical markers observed with Liv 52 and Livergen clearly indicate their superior hepatoprotective activity than other PLFs studied.

CCl₄ is a well known hepatotoxin which is widely used to induce toxic liver injury in laboratory animals. Hepatocellular injury due to CCl₄ occurs due to a toxic metabolite, trichloromethyl radical (CCl₃⁺), and

Fig. 3—CCl₄-induced hepatotoxicity; (a): typical centrilobular necrosis. H&E × 40. (b): centrilobular necrotic areas, congested sinusoids, and fatty degeneration. H&E ×100. Fig. 4—Pretreatment with low dose of Liv 52 (2.6 ml/kg/d) showing partial protection of hepatocytes; a and b: tiny focal necrosis with mild portal inflammation. H&E ×100. Fig. 5—Pretreatment with high dose of Livergen (5.2 ml/kg/d); a and b: complete normalization of liver architecture. H&E × 40 and 200.
the drug metabolizing enzymes activate CCl₄ to produce electrophilic metabolites. These potent agents bind covalently to cell proteins and induce necrosis. In addition, metabolites with unpaired electrons are produced by oxidative reaction of Cytochrome P450. These free radicals also damage proteins and unsaturated fatty acids causing lipid peroxidation. The end result is due to depression in \( Ca^{2+} \) homeostasis, membrane pumps and mitochondrial function. CCl₄ may be the result of stabilization of plasma membrane as well as the repair of hepatic tissue damage caused by CCl₄ toxicity. A similar study has reported that polyherbal formulations have hepatoprotective activity like an active compound silymarin, a flavonolignan from *Silybum marianum*. The protective actions of Liv 52 and Livergen as observed in the present study are in agreement with that study. Other formulations also showed appreciable activity, but the magnitude of effect was inferior to Liv 52 and Livergen. The differences observed in the present study with previous may be due to the difference in the route, dose and duration of administration, formulation, and may also due to difference in animal model used. In previous study, the herbal drugs were administered intraperitoneally in rats for 4 days whereas in present study, the drugs were administered orally for 7 days in mice.

Phenobarbitone is a hypnotic drug that is metabolized mostly in liver. When it is given in mice having hepatotoxicity induced by CCl₄, it causes an enhancement of mean duration of sleeping time. This was due to the delay in barbiturate metabolism as a result of hepatic injury and resultant decrease in Cytochrome P450-mediated metabolic functional activity of hepatocytes. As observed in the present study, pretreatment of hepatoprotective PLFs prevented the alteration in phenobarbitone-induced sleeping time in CCl₄-induced hepatotoxicity. This indicates that Cytochrome P450, which is the most important and abundant xenobiotic metabolizing enzyme system in liver, is protected by these PLFs. However, the mechanism how these PLFs protect Cytochrome P450 is worth investigating.

As observed in the present study, CCl₄ treatment produced various histological changes in the hepatocytes including centrilobular necrosis (zone 3), fatty and hydropic changes with congestion of sinusoids, ballooning degeneration, cell inflammation, and infiltration of inflammatory cells. Pretreatment with PLFs, Liv 52 and Livergen prevented CCl₄-induced change in the hepatic architecture and protected the liver tissue from necrotic, fatty and degenerative changes. This may be by preventing the toxic chemical reactions which generate oxidative stress, lipid peroxidation and molecular changes, which ultimately leads to liver tissue necrosis. The hepatoprotective drugs may have a role in the process of regeneration, prevention of fibrosis or formation of nodules which may be expressed in the long term use of the drug. The improvement in histopathological findings with hepatoprotective PLFs were found to be associated with the improvement in biochemical parameters.

Hepatic necrosis is found to be greater in zone 3, where drug metabolizing enzymes are found in highest concentration and where the oxygen tension is lowest in the sinusoidal blood. The present results confirm the centrilobular necrosis by hepatotoxic agents and amelioration of such changes and restoration to normalcy in the centrilobular area by Liv 52 and Livergen. The various active ingredients present in PLFs (like andrographolide) may be helpful in the changes in the membrane, in the mitochondria or at the ionic level like calcium. They may also facilitate protein synthetic and regenerative activity in the liver which makes it more resistant to toxic insults.

The present study has a few limitations as we did not study the safety profile and the hepatoprotective drugs were given as pretreatment. However, it has been reported that pretreatment with andrographolide
proved to be more effective than post treatment in preventing galactosamine and paracetamol induced hepatotoxicity.  

In conclusion, it may be emphasized that the polyherbal hepatoprotective formulations Liv 52 and Livergen were most effective in the study, justifying their use as hepatoprotective agents. However, the efficacy of Livokin, Octogen, Stimuliv, and Tefroliv was low; hence a dose adjustment may be necessary for their justified use in traditional medicine for human liver diseases.

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References