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Hepatoprotective effect of combined extracts of *Andrographis paniculata, Boerhavia diffusa, Eclipta alba* and *Picrorhiza kurroa* on carbon tetrachloride and paracetamol-induced hepatotoxicity in rats

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Extracts obtained from *Andrographis paniculata, Boerhavia diffusa, Eclipta alba* and *Picrorhiza kurroa* were mixed in an equal ratio and the combined extract was evaluated for the hepatoprotective activity against carbon tetrachloride and paracetamol-induced hepatotoxicity in rats. The hepatotoxicants increased the levels of transaminases, total bilirubin, triglycerides and decreased the level of albumin in the serum of rat. Liver homogenate of the rats showed an increase in the lipid peroxidation level and reduction in the levels of glutathione. The rats administered with the combined extract reversed the biochemical parameters reflecting the liver function. The combined extract showed significant ($p<0.05$) dose-dependent hepatoprotective activity against the toxicants as it was evident from the values of biochemical parameters when compared to silymarin administered animals. The results showed that the recovery exhibited by the combined extract at 400 mg/kg dose level was highest among other doses tested and was well comparable to silymarin-treated rats.

Keywords: Carbon tetrachloride, Extract, Hepatoprotective, Paracetamol, Transaminases

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The liver plays an essential role in the maintenance and performance of the body including the metabolism of carbohydrates, proteins and fats, detoxification and secretion of bile[1]. The maintenance of a healthy liver is therefore important to overall health. Unfortunately, alcohol, medications and a numerous environmental toxins are introduced to the liver that placed a burden on the organ[2]. The overloaded liver can impair detoxification and the dysfunctional liver cannot properly perform its tasks. Therefore, the body may be subjected to toxicity. Plenty of herbal medicine practices have been utilised to prevent and cure a variety of diseases including liver disorders. Plants traditionally used for the alleviation of liver dysfunction can be a valuable source of new hepatoprotective agents[3-5]. Natural products in the form of traditional medicine are used for the management of liver disorders and these are the potential sources for new therapeutic agents. Phytochemicals such as diterpene lactones, triterpenes, alkaloids, carotenoids, saponins, flavonoids and polyphenolic compounds have been proven as hepatoprotective agents in experimental studies on cell lines and animal models[6-8]. Herbal preparations from multiple herbs consists of a complex mixture of several chemical compounds and these multi component mixtures might be helpful in the prevention or treatment of liver ailments due to their synergistic effect[9,10]. *Andrographis paniculata* is used traditionally in Asia for various ailments such as dysentery, malaria and liver disorders[11]. *Eclipta alba* is widely used in different regions of India for the treatment of jaundice, fever, hair loss skin and respiratory problems[12]. Different parts of *Boerhaavia diffusa* are used as folk medicine to treat inflammations, heart diseases, asthma, jaundice and kidney problems[13]. *Picrorhiza kurroa* is traditionally used in India for the treatment of liver and respiratory disorders[14]. Aerial parts of *Andrographis paniculata* and *Eclipta alba*, roots of *Boerhavia diffusa* and root and rhizomes of *Picrorhiza kurroa* have been reported to possess hepatoprotective activities[15-18] and exhibiting different mechanism of actions[19-21]. These plants contain various active constituents of different chemical nature and the combination of the extracts from these herbs might show better activity than the individual plants in the prevention of liver diseases due

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to their different mechanism of actions. Hence, the combined extract was assessed for the hepatoprotective effect against carbon tetrachloride and paracetamol-induced experimental hepatotoxicity in rats.

Materials and Methods

Authentication of plants

Andrographis paniculata (Burm. F) Nees (Acanthaceae) and *Picrorhiza kurroa* Royle ex Benth (Plantaginaceae) were procured from local market at Ludhiana, India and *Boerhavia diffusa* L (Nyctaginaceae) and *Eclipta alba* (L.) Hassk. (Asteraceae) were collected from Ludhiana. The plants were authenticated by a taxonomist of Indian Institute of Integrative Medicine, Jammu, India. Shade dried, chopped, and powdered plant materials were used for extraction.

Extraction of plant materials

The powdered plant material of *Andrographis paniculata* (200 g) was extracted twice with methanol (2x1000 mL) by maceration in a round bottom flask for 24 h and filtered. The filtrates were collected, combined and concentrated in a rotary evaporator to obtain methanol extract. The root powder of *Boerhavia diffusa* (200 g) was extracted twice with 50% ethanol (2x1000 mL). The solvent was removed in a rotary evaporator to obtain 50% ethanol extract. Similarly, 80% ethanol extract of *Eclipta alba* and *Picrorhiza kurroa* were prepared by maceration.

Hepatoprotective study

Chemicals, animals, hepatotoxicants and test extract

Analytical grade chemicals were used in the study and were obtained from Sigma Chemicals Co. U.S.A., Merck India Ltd., Mumbai and Loba Chemie Pvt. Ltd., Mumbai. *Charles foster* rats (*Ratus norvegicus*), 12 to 16 weeks old of either sex, weighing 150 – 175 g, bred in the Institute's animal house (Indian Institute of Integrative Medicine, Jammu) were used. Animals were maintained in optimal laboratory conditions (23±2°C, relative humidity 60–70%, 12-12 h light/dark cycle) with free access to water and fed a regular rodent pellet diet (Lipton India Ltd., Mumbai). The animals were divided into groups as per requirement and each group consisted of six animals. The institute's animal ethical committee approved the experimental protocol. Paracetamol overdose is the most common cause of drug-induced hepatotoxicity worldwide[22]. Carbon tetrachloride (CCl_4) is a commonly used hepatotoxicant to induce hepatotoxicity in animals and humans[23]. Hence, these two hepatotoxicants were used to assess the hepatoprotective activity. The extracts of *Andrographis paniculata, Boerhavia diffusa, Eclipta alba* and *Picrorhiza kurroa* were combined in the proportions of 1:1:1:1 and referred as combined extract (CE). The different doses of (100, 200 and 400 mg/kg, p.o) of the combined extract and silymarin (50 mg/kg, p.o) were prepared in 0.2% gum acacia solution.

Experimental protocol of carbon tetrachloride-induced hepatotoxicity

Six groups were employed in the current experiment and each group comprised of six rats. The combined extract and standard drug were freshly prepared in gum acacia solution and carbon tetrachloride was freshly prepared in liquid paraffin. The combined extract was administered through oral route to the animals at the dose of 100, 200 and 400 mg/kg per day for three consecutive days. On day four, the animals were administered with the combined extract followed by the administration of toxicant (CCl_4) after 2 h. Six hours after CCl_4 administration, once again the combined extract was administered. On day five, 24 h after toxicant administration, the rats were sacrificed to collect the blood and liver samples for analysis. CCl_4 (1 mL/kg) and silymarin (50 mg/kg) were administered by oral route to the animals. A vehicle control (normal control) was also maintained in the study.

The experimental protocol is shown below.

Group I: Distilled water was administered through oral route to the rats and this group served as vehicle control.

Group II: Carbon tetrachloride (1 ml/kg) mixed with liquid paraffin was administered to the rats, orally.

Group III, IV and V: Combined extract was administered through oral route to the rats at a dose of 100, 200 and 400 mg/kg respectively as mentioned above.

Group VI: Silymarin (50 mg/kg) was administered through oral route to the rats and this group served as standard control.

Experimental protocol of paracetamol-induced hepatotoxicity

Six groups of animals were employed to investigate the hepatoprotective activity of the combined extract against paracetamol-induced hepatotoxicity. The experimental protocol was adopted as mentioned in the protocol for CCl_4-induced hepatotoxicity. Paracetamol (500 mg/kg) was administered to the animals by oral route to induce hepatotoxicity. The combined extract was administered through oral route to the animals at a dose of 100, 200 and 400 mg/kg/day for three

consecutive days. On day four, the animals were administered with the combined extract followed by the administration of paracetamol after 2 h. Six hours after paracetamol administration; once again the combined extract was administered. On day five, 24 h after toxicant administration, the animals were sacrificed; the liver samples were isolated for the analysis of lipid peroxidation and glutathione estimation. A vehicle control (normal control) was also maintained in the study.

The experimental protocol is shown below;

Group I: Vehicle was administered through oral route to the rats (normal control)

Group II: Paracetamol (500 mg/kg) was administered to the rats orally.

Group III, IV and V: Combined extract was administered through oral route to the rats at a dose of 100, 200 and 400 mg/kg respectively as mentioned above.

Group VI: Silymarin (50 mg/kg) was administered through oral route to the rats (standard control).

Blood and tissue biochemistry

Blood samples were obtained from animals' retro-orbital sinus puncture in Eppendorf tubes. The collected blood samples were allowed to clot completely at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min to obtain clear serum for the biochemical analysis. All the experimental rats were sacrificed by decapitation and liver sections free from any adhering tissues were rapidly removed, washed with normal saline and immediately transferred on ice. Biochemical parameters were analyzed from the serum and liver samples. The levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated by standardised methods[24]. Serum bilirubin was measured using the method by Malloy and Evelyn[25]. Triglycerides were measured colorimetrically using the method by Neri and Frings[26]. The albumin was estimated using a kit provided by Bayer Diagnostic India Ltd. Gujarat, India. Hepatic Glutathione was determined after deproteination reaction with DTNB by the method of Ellman[27]. Lipid peroxidation was measured by the method by Buege and Aust[28].

Statistical analysis

All the data obtained were expressed as mean±standard error mean (SEM). One way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to assess statistical significance. $p<0.05$ was considered significant.

Results

Carbon tetrachloride-induced hepatotoxicity

Carbon tetrachloride administered rats have shown significant increase in serum alanine transferase, aspartate transferase, bilirubin and triglyceride levels and a significant decrease in serum albumin level in comparison to normal control group. Further, these rats have shown an increase in lipid peroxidation and a decrease in glutathione in liver homogenate in comparison to normal control group. These parameters were significantly reversed in a dose-dependent manner after treatment with the combined extract at different doses such as 100, 200 and 400 mg/kg. A marked decrease in serum alanine transferase, aspartate transferase, bilirubin and triglyceride levels was observed in rats treated with combined extract at a dose of 400 mg/kg. A significant increase in albumin level was observed in comparison to CCl_4 control group (Table 1). Moreover, this group also showed a significant increase in glutathione level and a decrease in lipid peroxidation level in comparison to CCl_4 control

Table 1 Effect of combined extract on transaminases, total bilirubin, albumin and triglycerides against carbon tetrachloride-induced hepatotoxicity

Treatment and dose	ALT (U/L)	AST (U/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	Triglycerides (mg/dL)
Normal control	31.33±2.51	51.57±3.22	0.34±0.04	3.35±0.20	103.34±6.14
Vehicle + CCl_4 (1 ml)	132.84±9.63^{C}	162.22±6.82^{C}	1.12±0.09^{C}	2.35±0.10^{B}	147.29±8.27^{B}
CE + CCl_4 (100 mg/kg)	85.29±6.15^{b}	120.24±5.41^{c}	0.79±0.05^{a}	2.57±0.13^{a}	131.09±3.44^{a}
CE + CCl_4 (200 mg/kg)	75.71±6.04^{b}	104.68±6.24^{c}	0.75±0.05^{b}	2.70±0.11^{a}	129.23±3.00^{a}
CE + CCl_4 (400 mg/kg)	69.76±3.88^{c}	96.43±3.40^{c}	0.70±0.06^{b}	2.86±0.19^{a}	125.14±2.76^{a}
Silymarin + CCl_4 (50 mg/kg)	63.05±5.02^{c}	94.02±4.38^{c}	0.70±0.04^{b}	2.86±0.19^{a}	124.35±5.02^{b}

(Values as Mean ± SE, n=6)

p values carbon tetrachloride vs normal control- b<0.01, C<0.001

p values treatment vs carbon tetrachloride control- a<0.05, b< 0.01, c<0.001

group (Fig. 1 and 2). A similar result was also noticed in lower doses. Recovery exhibited by the combined extract at 400 mg/kg dose level was highest among other doses and well comparable to silymarin.

Paracetamol-induced hepatotoxicity

Paracetamol-administered rats have shown increase in serum alanine transferase, aspartate transferase, bilirubin and triglycerides levels. Compared to the control group, serum albumin levels were significantly lower. There was a significant increase in lipid peroxidation and a decrease in glutathione in liver homogenate when compared to vehicle control group. Treatment with combined extract at different doses of 100, 200 and 400 mg/kg, p.o reversed these alterations significantly. Rats that were treated with the combined extract at a dose of 400 mg/kg showed a significant decrease in serum alanine transferase, aspartate transferase, total bilirubin, and triglyceride levels. In comparison to the paracetamol-treated group, there was a significant rise in serum albumin levels. In the liver homogenate of treated animals, the combined extract administered group reported a considerable rise in glutathione and a reduction in lipid peroxidation (Fig. 3 and 4). The combined extract at a dose of 100, 200 and 400 mg/kg showed dose dependent recovery in the values of all the serum and hepatic biochemical parameters towards normal, however, the higher dose (400 mg/kg) exhibited hepatoprotection almost nearer to silymarin (Table 2).

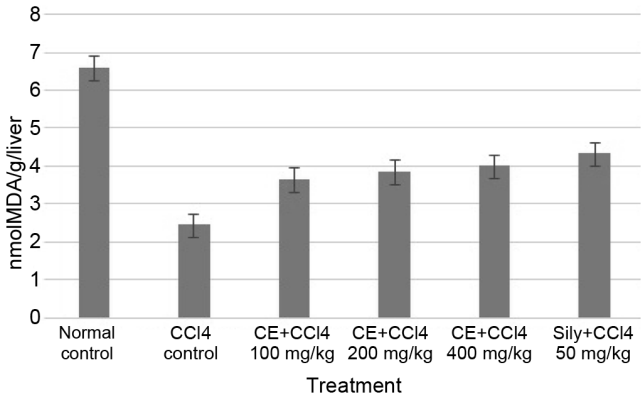

Fig. 1 — Effect of combined extract on glutathione in liver homogenate of rat against CCl_4-induced hepatotoxicity

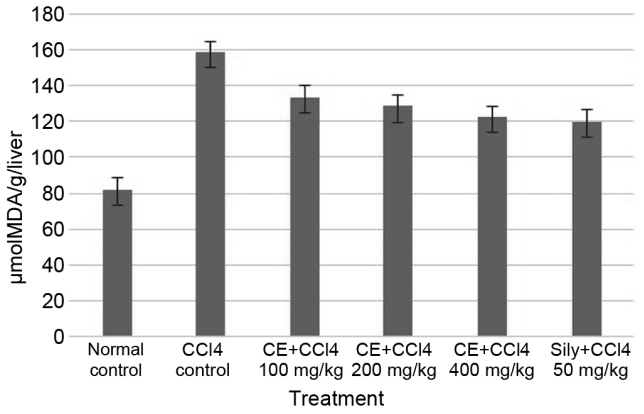

Fig. 2 — Effect of combined extract on lipid peroxidation in liver homogenate of rat against CCl_4-induced hepatotoxicity

Discussion

The plants, *Andrographis paniculata, Boerhavia diffusa, Eclipta alba* and *Picrorhiza kurroa* are used traditionally for treating liver disorders and many other diseases. In addition, hepatoprotective activities of these plants were reported in animals against various hepatotoxicants. In recent years, many attempts have been made to develop herbal formulations that can be useful in liver diseases. The multiple herbs in the herbal formulations are anticipated to provide better hepatoprotection due to their synergistic activity. A study on combination of two herbs reported synergistic hepatoprotective activity[29]. So, in the present study, two mechanistically different hepatotoxicants, namely,

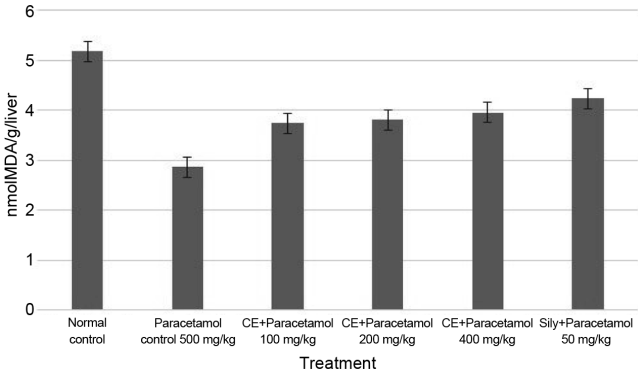

Fig. 3 — Effect of combined extract on glutathione in liver homogenate of rat against paracetamol-induced hepatotoxicity

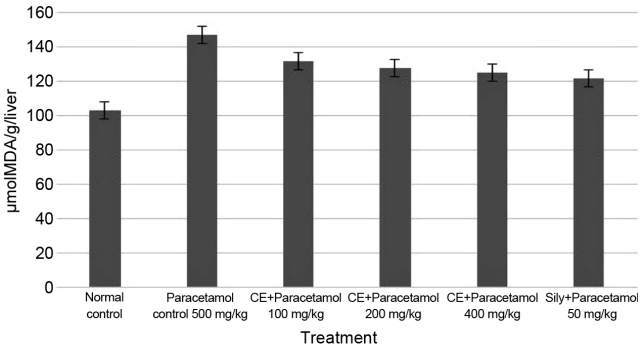

Fig. 4 — Effect of combined extract on lipid peroxidation in liver homogenate of rat against paracetamol-induced hepatotoxicity

Table 2 — Effect of combined extract on transaminases, total bilirubin, albumin and triglycerides against paracetamol-induced hepatotoxicity

Treatment and dose	ALT (U/L)	AST (U/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	Triglycerides (mg/dL)
Normal control	33.67±1.53	54.09±3.10	0.30±0.06	3.06±0.03	96.21±4.68
Vehicle + paracetamol (500 mg/kg)	164.47±10.59^{A}	181.81±9.43^{B}	1.04±0.05^{C}	2.47±0.13^{A}	129.66±9.63^{A}
CE + paracetamol (100 mg/kg)	105.05±9.91^{a}	108.13±4.42^{b}	0.73±0.07	2.65±0.08^{b}	119.58±4.52^{a}
CE + paracetamol (200 mg/kg)	72.76±8.86^{b}	104.64±6.25^{b}	0.70±0.03^{a}	2.70±0.90^{b}	117.97±5.76^{a}
CE + paracetamol (400 mg/kg)	66.29±6.90^{c}	90.83±5.78^{c}	0.71±0.07^{a}	2.72±0.07^{b}	115.19±10.36^{a}
Silymarin+paracetamol (50 mg/kg)	54.23±3.96^{c}	86.65±4.87^{c}	0.69±0.08^{a}	2.78±0.07^{b}	111.68±9.67^{a}

(Values as Mean ± SE, N= 6)
p values paracetamol vs normal control- a <0.05, B<0.01
p values treatment vs paracetamol- a<0.05, b< 0.01, c<0.001

CCl_4 and paracetamol were used for investigations to evaluate the combined extract against these hepatotoxicants in rats. CCl_4 is one of the most potent necrosis causing hepatotoxins leading to biochemical changes similar to those of acute viral hepatitis[30]. It has been assumed that the cytochrome P_{450} system bio transforms CCl_4 to produce the free radical, CCl_3 and to form a trichloromethyl peroxyl radical that can attack lipids[31]. Therefore, CCl_4-induced liver cell injury is severe and importantly rapid onset. Paracetamol (acetaminophen) is a therapeutic agent widely used as an analgesic and antipyretic drug[32]. Chronic doses of paracetamol cause centrilobular hepatic necrosis in animals[33]. The toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) can bind covalently to intracellular target proteins and produce cellular dysfunctions including mitochondrial damage and oxidant stress[34]. Serum ALT and AST are reliable markers of liver function and the measurement of these transferases provides valuable information on the severity of liver damage. In the present study, administration of CCl_4 and paracetamol resulted in marked elevation of both the enzymes because of cell lysis indicating severe hepatic cell necrosis. The increased serum enzyme levels indicate their release from the impaired hepatic cells to bloodstream, thus confirming liver damage. Treatment with the combined extract reversed the transaminases activity and restored it to normal, showing a marked protective effect. Bilirubin content reflects the pathophysiology of liver thus, hyperbilirubinemia is one of the most valuable clues to the severity of necrosis and hepatic damage[35]. Treatment with the combined extract significantly reduced the total bilirubin levels to near normal levels in comparison with the hepatotoxicant administered animals. The animals administered with high dose level showed almost similar results to standard control group, indicating hepatoprotective activity of the combined extract.

In the present study, the treatment with the combined extract effectively diminished lipid peroxidation induced by CCl_4 and paracetamol thus maintaining hepatic GSH levels. The results indicate that the combined extract was effective against CCl_4 and paracetamol-induced hepatotoxicity. The accumulation of triglycerides triggered by a reduction in apoprotein synthesis is the key cause of hepatotoxins-induced fatty liver[36]. Treatment with the combined extract significantly reversed CCl_4- and paracetamol-induced serum and hepatic increase in triglycerides demonstrating protective role of the combined extract against fatty liver disease. The combined extract at the dose of 400 mg/kg-p.o. showed maximum protection against triglycerides accumulation, which is well comparable to silymarin. Thus, dose-dependent investigation of the combined extract for their hepatoprotective activity on different biochemical parameters confirmed its hepatoprotective potentials against CCl_4- and paracetamol-induced hepatotoxicity. Investigation on a polyherbal formulation for hepatoprotective activity against carbon tetrachloride hepatotoxicity showed similar results[37] which further supports the hepatoprotective activity of the combined extract.

Eventually, the mechanisms by which these two hepatotoxicants cause liver damage differ greatly. CCl_4- and paracetamol-induced models, on the other hand, depend on the cytochrome P450 system to generate reactive metabolites such as CCl_3 and NAPQI. As a result, the combined extract's hepatoprotective action may be attributed to their antioxidant activity, as shown by a decrease in lipid peroxidation and an increase in glutathione levels.

The other biochemical parameters support the combined extract's protective activity by demonstrating the structural and functional integrity of the cells. Hence, the combined extract containing *Andrographis paniculata, Boerhavia diffusa, Eclipta alba* and *Picrorhiza kurroa* may be developed as a suitable hepatoprotective poly herbal formulation.

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Conflict of Interest

Authors declare that they do not have any conflict of interest

Authors' Contributions

Conceptualization-N.M. & M.G.; Methodology-N.M. & M.G.; Computational methods-V.R. & N.A.; writing & original draft preparation-K.C.; writing-review and editing-all authors. Further, all authors have read and approved the manuscript.

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