Research Article

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HEPATOPROTECTIVE ACTIVITY OF LINK LIVECARE™ IN CARBON TETRACHLORIDE AND D-GALACTOSAMINE INDUCED HEPATOTOXICITY IN ICR MICE

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ABSTRACT

The liver is the most important organ concerned with metabolic activities in the human body. The human body is constantly bombarded with xenobiotics in the form of drugs and environmental toxins which can damage the liver. There is much current interest in herbal formulations claiming to be hepatoprotective agents. The hepatoprotective activity of an isopropanol:water (70:30 v/v) extract of the proprietary mixture of the 14 plants, *Andrographis paniculata, Eclipta alba, Phyllanthus amarus, Phyllanthus emblica , Terminalia chebula, Terminalia bellerica, Tinospora codifolia, Curcuma longa, Glycyrrhiza glabra, Boerrhavia diffusa, Osbekia octandra, Tephrosia purpurea, Piper longum* and *Vernonia cinerea*, used in the preparation of the Ayurvedic formulation, LINK LIVECARE™ was studied against carbon tetrachloride (0.08 ml/kg) and galactosamine (800 mg/kg) intoxication in ICR male mice. The animals were pretreated for seven days prior to intoxication. Both biochemical and histopathological changes of the liver were investigated. Significant ($p < 0.05$) hepatoprotective effects were observed for the extract tested at doses ranging from 40 mg/kg to 280 mg/kg. It showed optimum protection at the dose of 80 mg/kg against carbon tetrachloride induced hepatotoxicity lowering the increase of AST, ALT, ALP and TB by 46,46,83 and 64% respectively, compared with silymarin at a dose of 50 mg/kg which lowered the increase of the same parameters by 60,67,80 and 89% respectively. The extract also significantly lowered the increase of AST, ALP and TB when the toxicant was galactosamine. However, there was no effect on ALT levels. Pretreatment reduced the extent of liver necrosis caused by both toxicants.

Keywords: LINK LIVECARE™, polyherbal formulation, hepatoprotection, carbon tetrachloride, galactosamine

INTRODUCTION

The liver is the most important organ concerned with metabolic activities in the human body. It plays a critical role in carbohydrate, fat and protein metabolism. It also synthesizes many molecules needed for homeostasis and detoxifies xenobiotics. The human body is constantly bombarded with xenobiotics in the form of drugs and environmental toxins which can damage the liver. Liver disease has serious adverse effects on the health and wellbeing of an individual[1].

Hence, an agent which could protect the liver from such damage and prevent liver disease would be a useful addition to the therapeutic agents currently available. In the absence of any such agent in modern medicine there is a growing focus on the scientific evaluation of traditional herbal medicines which are claimed to possess hepatoprotective activity[2]. The use of herbal resources for the treatment of liver diseases is well documented in traditional medicine, and there are over 100 ayurvedic formulations used as hepatoprotective drugs[1].

Herbal preparations used in traditional medicine contain a large number of compounds which are considered to interact with several different receptors at the same time[3] .On the basis of knowledge available from the indigenous system of medicine in Sri Lanka and Ayurveda, a hepatoprotective formulation Link Livecare™ (LLC) has been formulated by Link Natural Products (Pvt.) Limited, Sri Lanka. It is a polyherbal formulation consisting of the extract of a proprietary mixture of fourteen medicinal plants: *Andrographis paniculata, Eclipta alba, Phyllanthus amarus, Phyllanthus emblica, Terminalia chebula, Terminalia bellerica, Tinospora codifolia, Curcuma longa, Glycyrrhiza glabra, Boerrhavia diffusa, Osbekia octandra, Tephrosia purpurea, Piper longum* and *Vernonia cinerea.*

The hepatoprotective effects of the individual plants of the formulation in various animal models of hepatotoxicity have been reported[4-27]. However, the rationale for the particular formula of LLC is based on Ayurveda pharmacology. Thus, according to Ayurveda, the combination of plants used in LLC is expected to have the following effects on the body: support and regulate the normal functions of the liver including the secretion and excretion of bile, reduce hepatic inflammation, increase metabolism and digestion, detoxify the body and purify the blood, clear obstructions and clean the body channels and rejuvenate the tissues[28]. It is of interest to note that of the 126 hepatoprotective herbal formulations listed by Handa[1], none contain either *Osbekia octandra* or *Vernonia cinerea* found in LLC as ingredients. However, these two plants are used widely in the indigenous system of medicine in Sri Lanka to treat liver disorders.

In the present study the isopropanol: water (70:30 v/v) extract of the proprietary mixture of powdered plant materials used in the formulation of LLC was evaluated for its protective effect against carbon tetrachloride (CCl_4) and galactosamine (GalN) induced hepatotoxicity in ICR male mice.

MATERIALS AND METHODS

Drugs and chemicals

The standardized proprietary mixture of powdered plant materials used in the formulation of LLC was provided by Link Natural Products (Pvt) Limited, Sri Lanka. All the chemicals used were of analytical grade.

Animals

Institute of cancer research (ICR) male mice (8 -12 weeks old) purchased from the Medical Research Institute, Sri Lanka were used. The mice were acclimatized for one week at the animal house of the University of Sri Jayewardenepura before the study. Animals were housed in groups of four under standard laboratory conditions with free access to standard pellet diet and water *ad libitum*. The experimental protocol was approved by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (No. 545/11).

Preparation of the extract of the proprietary mixture of plant material (LLC plant extract)

The proprietary mixture of plant materials (107.5 g) obtained as a dry powder, was extracted by maceration for 24 h with 500 ml of isopropanol: water (70:30 v/v). The residue was macerated twice more with another two portions of 500 ml of isopropanol: water (70:30 v/v). The extracts were combined, filtered and evaporated under reduced pressure at a temperature below 60°C to obtain a brownish solid (19.1 g). The appropriate amount of the solid was suspended in 0.25% carboxymethyl cellulose (CMC) for oral administration to the animals.

Hepatoprotective activity against CCl_4 induced hepatotoxicity

Mice were divided into nine groups of eight animals each and treated orally once daily for seven consecutive days as given below. The administered volume was kept constant at 1 ml.

Group 1: 0.25% CMC (normal control)
Group 2: 0.25% CMC (pathological control)
Group 3: LLC plant extract in 0.25% CMC (40 mg/kg)
Group 4: LLC plant extract in 0.25% CMC (80 mg/kg)
Group 5: LLC plant extract in 0.25% CMC (160 mg/kg)
Group 6: LLC plant extract in 0.25% CMC (200 mg/kg)
Group 7: LLC plant extract in 0.25% CMC (240 mg/kg)
Group 8: LLC plant extract in 0.25% CMC (280 mg/kg)
Group 9: Silymarin in 0.25% CMC (50 mg/kg) (positive control)

One hour after the last treatment on day seven Group 1 received a single dose of olive oil (0.2 ml) and Groups 2-9 received a single dose of carbon tetrachloride (0.08 ml/kg body weight) in olive oil, intraperitoneally.

Animals were sacrificed under light ether anesthesia 24 h after CCl_4 administration and blood samples and liver samples were collected for evaluation.

Hepatoprotective activity against GalN induced hepatotoxicity

Mice were divided into five groups of eight animals each and treated orally once daily for seven consecutive days as given below. The administered volume was kept constant at 1 ml.

Group 1: 0.25% CMC (normal control)
Group 2: 0.25% CMC (pathological control)
Group 3: LLC plant extract in 0.25% CMC (80 mg/kg)
Group 4: LLC plant extract in 0.25% CMC (160 mg/kg)
Group 5: Silymarin (50 mg/kg) (positive control)

One hour after the last treatment on day seven Group 1 received a single dose of saline (0.2 ml) and Groups 2-5 a single dose of GalN (800 mg/kg bodyweight) in saline, intraperitoneally.

Animals were sacrificed 24 h after GalN administration and blood samples and liver samples were collected for evaluation.

Analysis of blood for assessment of liver functions

Blood samples collected by heart puncture were allowed to clot for 45 min at room temperature and serum was separated by centrifugation at 3,000 rpm for 10 min. The serum samples were kept at -20 °C until analysis. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and total bilirubin (TB) were determined using auto analyzer (Konelab 20XT) and commercially available test kits (Biolabo reagents, France).

The hepatoprotective activity of the tested extract was calculated by the following formula and expressed as the percent protection for each parameter:

% Protection = a-b / a-c x 100

Where, a = mean value of animals not treated with the extract but given the toxin; b = mean value of animals treated with the extract and given the toxin; c = mean value of animals not treated with the extract nor given the toxin.

Histopathological studies

Liver samples were fixed in 10% formalin and processed routinely for embedding in paraffin Tissue sections of 4 µm thickness were stained with hematoxylin and eosin (H&E) and observed for pathological changes. The extent of liver cell necrosis was quantified by microscopic (Olympus, FSX 100) examination. The necrotic extent was expressed as the percentage of the total necrotic area within the field observed. Three fields were observed in each specimen randomly.

Statistical analysis

The results are expressed as mean ± SEM. Statistical significance was evaluated by one-way ANOVA followed by two sample t –test using Minitab 14. $P < 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

In this study both biochemical parameters (AST, ALT, ALP and TB) and histopathology were used to assess the protection given by the LLC plant extract against the hepatotoxicants CCl_4 and GalN.

The obvious sign of hepatic injury due to a toxicant is the leakage of the intracellular enzymes AST and ALT in to the serum after hepatocyte damage or necrosis resulting in elevation of their serum concentrations[29]. Serum ALP is excreted normally via bile by the liver. In liver injury, due to a hepatotoxin, high levels of ALP are associated with biliary tract damage and inflammation[30]. Bilirubin is formed from the heme of hemoglobin, myoglobin and other heme proteins. An elevated serum TB reflects increased production, reduced hepatic uptake and/or conjugation, impaired transport of bilirubin esters into bile (with parenchymal liver diseases), or their regurgitation into the blood from biliary canaliculi (with biliary obstruction)[31].

In both humans and mice, the liver is a regulatory center for nutrient processing, protein production, energy homeostasis, and detoxification. Although the physiological functions are similar there are differences in the structure of the mice liver from that of the human liver. The equivalent of mice hepatic lobes are the liver segments in human. Mice have less connective tissue than humans. Further, the human liver often exhibits distinct hepatocyte cords one cell thick from portal triad to centrilobular vein, whereas in mice hepatocytes may present in a homogeneous field particularly in midzonal and centrilobular areas. Despite these differences, there are sufficient similarities in overall structure and function between the two species to make the mouse an applicable model to study human hepatobiliary disease[32].

Table 1: Hepatoprotective activity of LLC plant extract against CCl_4 induced hepatotoxicity in ICR mice

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	TB (mg/dL)	Percentage necrosis
Normal control Group	20.5 ± 5	120 ± 38	115 ± 65	4 ± 1.1	0 %
Pathological control Group	7659 ± 402^{a}	2510 ± 177^{a}	283 ± 23^{a}	8.74 ± 0.76^{a}	23-31 %
Treated Group (40 mg/kg)	5425 ± 996^{a,b} (29)	1881 ± 346^{a,b} (26)	246 ± 29^{a} (22)	5.93 ± 0.43^{a,b} (59)	17-24 %
Treated Group (80 mg/kg)	4172 ± 506^{a,b} (46)	1407 ± 171^{a,b} (46)	143 ± 12^{b} (83)	5.73 ± 0.43^{a,b} (64)	3-9 %
Treated Group (160 mg/kg)	4502 ± 537^{a,b} (41)	1732 ± 243^{a,b} (33)	225 ± 13^{a,b} (34)	6.53 ± 0.44^{a,b} (47)	11-14 %
Treated Group (200 mg/kg)	5271 ± 817^{a,b} (31)	2150 ± 315^{a} (15)	203 ± 17^{a,b} (47)	5.73 ± 0.32^{a,b} (64)	17-25 %
Treated Group (240 mg/kg)	5780 ± 529^{a,b} (24)	1978 ± 274^{a} (22)	181 ± 11^{b} (60)	6.84 ± 0.62^{a,b} (40)	19-25 %
Treated Group (280 mg/kg)	5667 ± 38^{a,b} (26)	1444 ± 115^{a,b} (22)	168 ± 27^{b} (68)	4.53 ± 0.63^{b} (89)	18-25 %
Positive control Group (Silymarin,50 mg/kg)	3032 ± 424^{a,b} (60)	924 ± 160^{a,b} (67)	149 ± 41^{b} (80)	4.54 ± 0.6^{b} (89)	3-7 %

Values for biochemical parameters are expressed as mean ± SE (n = 8). Values within parentheses represent percentage protection.
The histopathological parameter is expressed as a percentage necrosis value range across each treatment group.
a = significantly different when compared with normal control group, b = significantly different when compared with pathological control group ($p < 0.05$).

Table 2: Hepatoprotective activity of LLC plant extract against GalN induced hepatotoxicity in ICR mice

Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TB (mg/dL)	Percentage necrosis
Normal control Group	139 ± 13	31 ± 4	183± 21	3.4 ± 0.2	0 %
Pathological control Group	287 ± 17^{a}	125 ± 30^{a}	251± 21^{a}	4.8 ± 0.5^{a}	0.35-0.1 %
Treated Group (80 mg/ kg bw)	171 ± 10^{a,b} (68 %)	88 ± 14^{a} (39 %)	182 ± 19^{b} (100%)^{c}	3.3 ± 0.3^{b} (100%)^{c}	0.2-0.05 %
Treated Group (160 mg/ kg bw)	169 ± 17^{b} (79 %)	97 ± 14^{a} (29 %)	192 ± 8^{b} (86%)	4.0 ± 0.3^{a,b} (57%)	0.06-0.1 %
Positive control Group (50 mg/kg bw)	165 ± 14^{b} (82 %)	112 ± 24^{a} (14 %)	165 ± 11^{b} (100%)^{c}	4.2 ± 0.1^{a} (42%)	0.07-0.09 %

Values for biochemical parameters are expressed as mean ± SE (n = 8). Values within parentheses represent percentage protection.
The histopathological parameter is expressed as a percentage necrosis value range across each treatment group.
a = significantly different when compared with normal control group, b = significantly different when compared with pathological control group ($p < 0.05$). c = calculated value >100%.

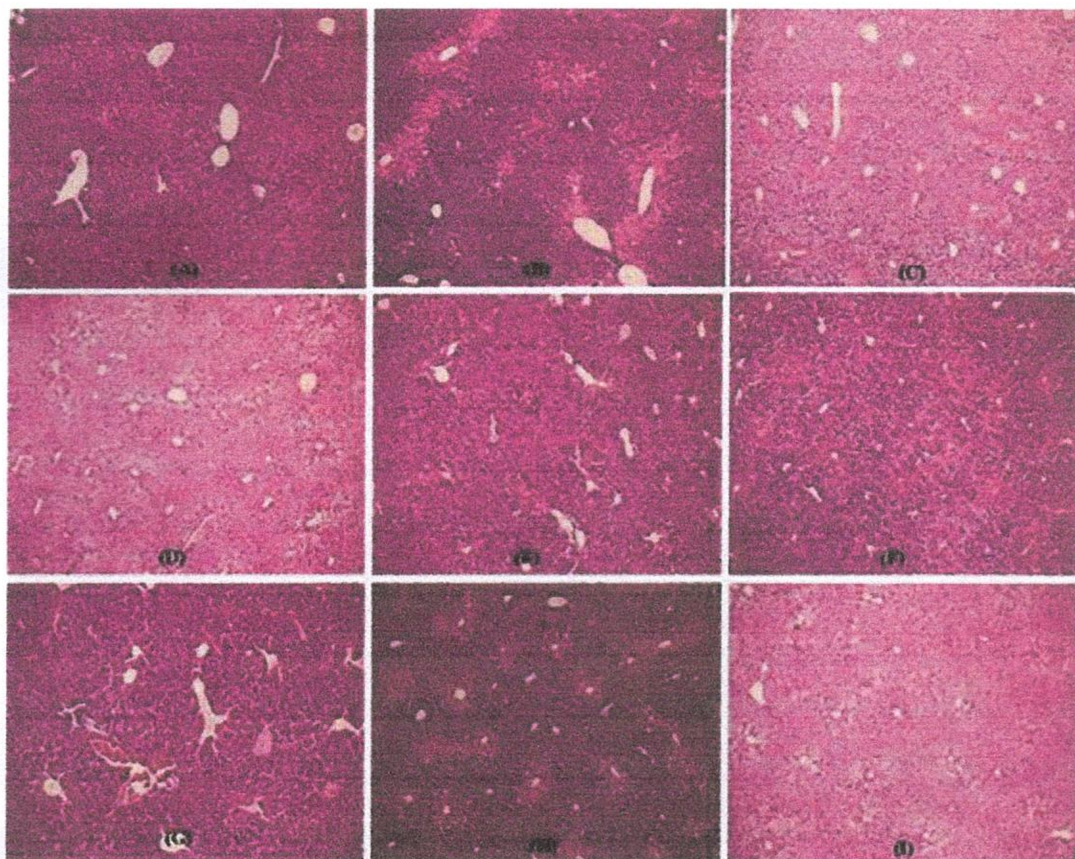

Figure 1: Liver section of different groups of animals used in assessing the hepatoprotective activity of LLC plant extract against CCl_4 induced hepatotoxicity. (A) Vehicle control group. (B) Pathological control group. (C) Treated group (40 mg/kg). (D) Treated group (80 mg/kg). (E) Treated group (160 mg/kg). (F) Treated group (200 mg/kg). (G) Treated group (240 mg/kg). (H) Treated group (280 mg/kg). (I) Positive control group (50 mg/kg). All sections were stained with H & E (40 x 4.2).

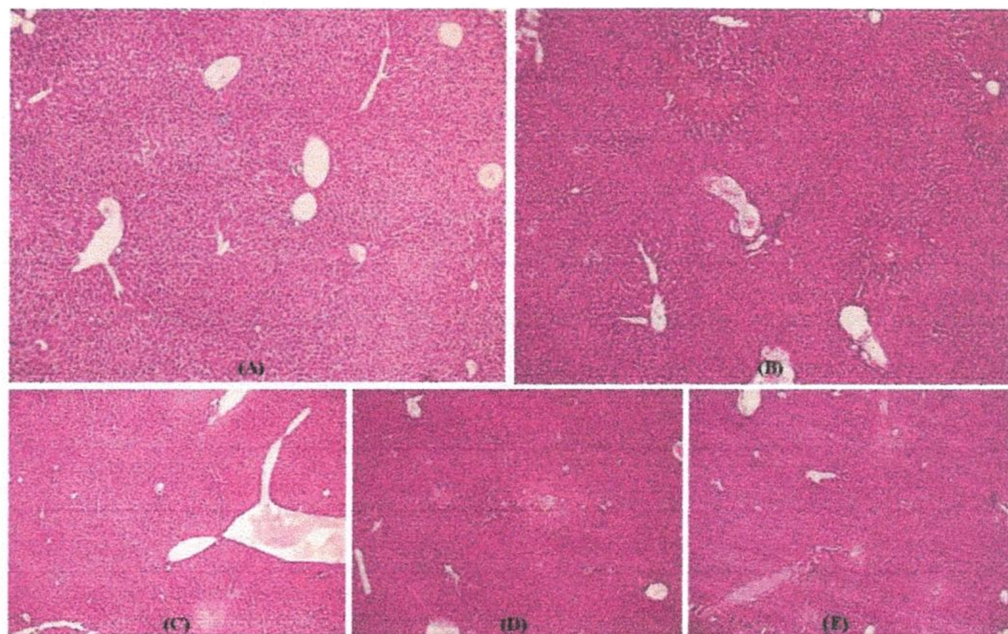

Figure 2: Liver section of different groups of animals used in assessing the hepatoprotective activity of LLC plant extract against GLaN induced hepatotoxicity. (A) Vehicle control group. (B) Pathological control group. (C) Treated group (80 mg/kg). (D) Treated group (160 mg/kg). (E) Positive control group (50 mg/kg). All sections were stained with H & E (40 x 4.2).

Protection against carbon tetrachloride induced toxicity

CCl_4 toxicity is due to its bioactivation, especially by cytochrome P450 to produce the free radical CCl_3 [33,34] which links covalently to membrane lipids determining their peroxidation and altering the physico-chemical properties of cell membranes. The free radical CCl_3 resulting from CCl_4 metabolism can also bind to the cell proteins, damaging the normal functionality of the cells[35].

Table 1 gives AST, ALT, ALP and TB levels and the percentage of liver necrosis in all groups of animals used in the evaluation for protection against CCL_4. The calculated percentage protection provided by the LLC plant extract with respect to each parameter is also indicated. A single intraperitoneal injection of CCl_4 (0.08 ml/kg) causes severe acute liver damage in mice, demonstrated by a marked elevation of serum AST (20 fold) and ALT (380 fold) in the pathological control group compared to the normal control group.

The LLC plant extract at each dose level significantly decreased the serum AST and ALT levels raised by CCl_4. The maximum degree of protection (46%) was observed with the dose of 80 mg/kg body weight of the extract with respect to the levels of both AST and ALT, compared to 67% and 60% respectively by the positive control silymarin at a dose of 50 mg/kg body weight. The salient histopathological abnormality of the liver following administration of CCl_4 was centrilobular necrosis (Fig. 1). Pre-treatment with LLC plant extract reduced the extent of hepatic lesions with maximum protection at a dose of 80 mg/kg body weight in conformity with the results for AST and ALT. These results suggest the possibility of the LLC plant extract to stabilize the structural integrity of hepatocytes.

The increase in ALP and TB levels following intraperitonial injection of CCl_4 suggestive of biliary dysfunction was not as marked as the increase in AST and ALT levels. The dose response for protection against biliary dysfunction was erratic. However, it should be noted that high levels of protection similar to that afforded by silymarin was observed at doses of 80 mg/kg body weight (ALP) and 280 mg/kg body weight (TB).

Protection against GalN induced hepatotoxicity

GalN is also a well-established hepatotoxicant, inducing liver injury which closely resembling human viral hepatitis in its morphologic and functional features[36]. It is therefore useful for the evaluation of hepatoprotection. GalN has a high liver specificity because hepatocytes have high levels galactokinase and galactose-1- uridyltransferase and it disrupts the synthesis of essential uridylate nucleotides. Depletion of these nucleotides ultimately impairs the synthesis of protein and glycoprotein, leads to progressive damage of cellular membranes resulting in a change in permeability of the cellular membrane, and finally with enzyme leakage from the cells[36, 37].

Table 2 gives AST, ALT, ALP and TB levels and the percentage of liver necrosis in all groups of animals used in the evaluation for protection against GalN. The calculated percentage protection provided by the LLC plant extract with respect to each parameter is also indicated. A single intraperitoneal injection of GalN significantly elevated serum levels of AST, ALT, ALP and TB indicating hepatocellular damage and biliary dysfunction. While 7-days pretreatment with LLC at 80 and 160 mg/kg body weight significantly reduced the increased levels of serum AST compared to the pathological control group, there was no significant effect on the increase of ALT levels. Pretreatment with the LLC plant extract significantly reduced the degree of elevation of ALP and TB levels and brought them down to normal levels. It is noteworthy that while silymarin at a dose level of 50 mg/kg body weight also normalizes the ALP level, it had no significant effect on the elevation of TB.

Biochemical changes induced by GalN lead to spotty necrosis of the liver. The cellular damage provokes an inflammatory response closely resembling viral hepatitis[38]. GalN intoxicated mice liver sections showed vacuolization of hepatocytes, sinosidal dilation and congestion, infiltration of cells, loss of boundaries and ballooning degeneration, loss of architecture and cell necrosis. Pre-treatment with LLC plant extract reduced the extent of histopathological changes and reduced the area of spotty necrosis (Figure 2).

CONCLUSION

An extract of the proprietary mixture of plants used in the preparation of the Ayurvedic formulation LLC exhibited prophylactic hepatoprotective activity against carbon tetrachloride and *d*-galactosamine induced hepatotoxicity in mice. The optimum protective effects were shown at a dose of 80 mg/kg body weight.

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