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Protective effect of DHC-1, a Polyherbal formulation, against CCl_4 induced Liver damage.

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Abstract

Plan: *DHC-1, a standardized polyherbal formulation, was investigated for its hepatoprotective and antioxidant effects against CCl_4-induced liver damage in rats.*
Methodology: *DHC-1 at various doses (125, 250, 500 and 1000 mg/kg, p.o.) was studied for its effect on the levels of SGPT, SGOT, alkaline phosphatase and total bilirubin. The drug was also evaluated for its effect on markers of tissue oxidative stress, namely extent of lipid peroxidation (MDA), levels of enzymatic antioxidants (SOD and catalase), non-enzymatic antioxidant (GSH) and membrane bound enzymes (Ca^{2+}ATPase, Mg^{2+}ATPase and Na^+K^+ATPase).*
Outcome: *Reduction in the levels of serum markers of liver damage; and decrease in tissue MDA levels and increase in SOD, catalase, GSH and membrane bound enzymes indicated the hepatoprotective and antioxidant property of DHC-1. Thus it is evident that DHC-1, at least partly by virtue of its antioxidant activity, elicited protective effects on the liver against the oxidative damage induced by CCl_4.*
Keywords: *Polyherbal formulation; Hepatoprotective; CCl_4; lipid peroxidation, superoxide dismutase, catalase, reduced glutathione.*

1. Introduction

Liver is an organ of paramount importance as it plays an essential role in maintaining the biological equilibrium of vertebrates. It is the "alchemical wizard" of the body, transforming toxins into harmless chemicals for excretion, and digestively absorbed nutrients into the proper biochemical forms which the cells can use to function. Yet the liver is probably the organ most assaulted by toxic modern lifestyles, full of pollution, stress, junk foods, drugs, etc. About five percent of all deaths worldwide are the result of liver diseases. It ranks ninth in overall causes of death in the U.S.[1] and is the fifth 'big killer' in England & Wales.

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Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The effectiveness of treatments such as interferon, colchicine, penicillamine and corticosteroids are inconsistent at best and the incidence of side effects is profound. All too often the treatment is worse than the disease.[2] Physicians and patients are thus in need of effective therapeutic agents with a low incidence of side effects. Plants potentially constitute such a group; and thus there is a worldwide trend to go back to traditional medicinal plants. Many natural products of herbal origin are in use for the treatment of liver ailments.[3-5]

The pathogenesis of hepatic diseases as well as the role of oxidative stress and inflammation therein is well recognized,[6] and consequently, blocking or retarding the chain reactions of oxidation and inflammation development could be promising therapeutic strategies for the prevention and treatment of liver injury. Search for crude drugs of plant origin with antioxidant activity has thus become a central focus for study of hepatoprotection today.

The hepatoprotective effects of *Bacopa monnieri*,[7] *Emblica officinalis*,[8,9] *Glycyrrhiza glabra*,[10,11] *Mangifera indica*[12] and *Syzygium aromaticum*[13,14] have been mentioned. Also, the antioxidant properties of these plants, namely *Bacopa monnieri*,[15,16] *Emblica officinalis*,[17-19] *Glycyrrhiza glabra*,[20,21] *Mangifera indica*,[22-24] and *Syzygium aromaticum*[25,26] have been investigated earlier and were found to possess free radical scavenging properties.

The antioxidant activity[27] and tissue protective effects of DHC-1, a formulation containing the above mentioned plants, have already been proved in different oxidative stress-induced disease conditions like pylorus-ligation and ethanol-induced ulcers,[28] cisplatin-induced nephrotoxicity and isoproterenol-induced myocardial infarction[29] in rats.

The present study was thus aimed to further test the efficacy of DHC-1 against hepatic injury induced by CCl_4 in rats to determine the possible use of this formulation in preventing hepatic damage and to justify whether the formulation exerts hepatoprotective effects by means of its antioxidant activity.

2. Materials and Methods

2.1 Plant Material

Bacopa monnieri, *Emblica officinalis*, *Glycyrrhiza glabra*, *Mangifera indica* and *Syzygium aromaticum* were procured from a local supplier and identified by Dr. Kannan, Ph.D., Botanist, The Himalaya Drug Company, Bangalore. Samples were retained for reference purpose at the R & D herbarium. The HPTLC fingerprint analysis of the individual ingredients and the formulation (DHC-1) have already been reported[27].

2.2. Composition

Each gram of DHC-1 contains extracts of:

Herbs	*Voucher code*	*Part used*	*Qty.*
Bacopa monnieri Linn. (Scrophulariaceae)	HDHB-157	Whole plant	200 mg
Emblica officinalis Gaertn. (Euphorbiaceae)	HDHB-143	Fruit	200 mg
Glycyrrhiza glabra Linn. (Papilionaceae)	HDHB-174	Roots	200 mg
Mangifera indica Linn. (Anacardiaceae)	HDHB-17	Bark	200 mg
Syzygium aromaticum Linn. (Myrtaceae)	HDHB-208	Flower bud	200 mg

2.3. Animals:

Albino rats of Wistar strain weighing 150-180g were housed in polypropylene cages under standard light/dark cycle, with food and water provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) and conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Forests and Environment, Govt. of India, New Delhi (India).

2.4. Experimental Procedure:

The animals were divided into six groups each consisting of six rats. Group 1 represented the Normal control group, which received 5 ml/kg of vehicle (1% gum acacia) orally for a period of 15 days. Group 2 served as the Negative control and received the vehicle orally (1% gum acacia; 5ml/kg) for 15 days followed by administration of CCl_4 (2.5 ml/kg, p.o.) in olive oil (1:1). Groups 3-6 received DHC-1 orally at the doses of 125, 250, 500 and 1000 mg/kg, respectively for 15 days followed by CCl_4 administration. After 24 hours of CCl_4 administration, blood was collected and serum was separated for estimations of SGPT, SGOT, alkaline phosphatase and total bilirubin using standard diagnostic kits [Span Diagnostics Ltd., Surat, India]. The animals were then sacrificed and the liver was dissected out, weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were then centrifuged at 10,000 x g at 0^oC for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was then used for the assays of lipid peroxidation (MDA content), endogenous antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT), and reduced glutathione (GSH). The sediment was resuspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase, and Mg^{2+}ATPase) and proteins.

2.5. Biochemical estimations:

Superoxide dismutase was determined by the method of Mishra and Fridovich.[30] Catalase was estimated by the method of Hugo Aebi as given by Colowick et al.[31] Reduced glutathione was determined by the method of Moron et al.[32] Lipid peroxidation or malondialdehyde formation was estimated by the method of Slater and Sawyer.[33]

Membrane bound enzymes namely Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were assayed according to the methods of Bonting,[34] Hjerken and Pan,[35] and Ohinishi et al.,[36] respectively. The inorganic phosphorus was estimated by the method of Fiske and Subarow.[37] Total proteins were determined by the method of Lowry et al.[38]

2.6. Statistical Analysis of Data:

Results of all the above estimations have been indicated in terms of Mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons test, with the level of significance set at $p < 0.05$.

Table 1: Effect of DHC-1 on the serum levels of SGPT, SGOT, alkaline phosphatase and total bilirubin in CCl_4-induced hepatotoxicity in rats.

Groups	*SGPT (U/ml)*	*SGOT (U/ml)*	*AlkP (mg/dl)*	*T.Bil. (mg/dl)*
Group 1	47.88 ± 2.33	77.64 ± 2.85	62.50 ± 2.57	0.103 ± 0.009
Group 2	257.90 ± 4.85***	314.33 ± 6.66***	248.12 ± 23.29***	1.420 ± 0.208***
Group 3	224.12 ± 8.83^{NS}	222.06 ± 21.87^{NS}	163.37 ± 7.55**	0.807 ± 0.099**
Group 4	194.66 ± 23.49^{NS}	221.47 ± 41.43^{NS}	141.89 ± 5.66**	0.350 ± 0.068***
Group 5	166.94 ± 22.66*	209.26 ± 11.15*	139.51 ± 10.61***	0.143 ± 0.009***
Group 6	105.32 ± 21.68***	201.43 ± 20.71*	139.07 ± 17.09***	0.110 ± 0.010***
F value	22.127	12.310	20.429	28.942
P value	<0.0001	0.0002	<0.0001	<0.0001

Values are expressed as Mean ± SEM. ,Group 2 was compared with Group 1..Groups 3, 4, 5 and 6 were compared with Group 2.
*p<0.05; **p<0.01; ***p<0.001; NS = Non Significant

Table 2: Effect of DHC-1 on the levels of lipid peroxidation (MDA content), reduced glutathione, superoxide dismutase and catalase in liver of rats in CCl_4-induced hepatotoxicity model.

Groups	*Lipid Peroxidation (nmoles of MDA/mg protein)*	*Reduced Glutathione (µg of GSH/mg protein)*	*Superoxide Dismutase (Units/mg protein)*	*Catalase (µmoles of H_2O_2 consumed/min/mg protein)*
Group 1	1.530 ± 0.052	3.760 ± 0.095	3.103 ± 0.085	3.757 ± 0.200
Group 2	5.331 ± 0.102***	0.474 ± 0.035***	1.497 ± 0.084***	1.520 ± 0.161***
Group 3	4.336 ± 0.496^{NS}	1.854 ± 0.177^{NS}	1.833 ± 0.074 ^{NS}	1.630 ± 0.129 ^{NS}
Group 4	4.029 ± 0.154*	3.074 ± 0.590**	2.050 ± 0.110*	1.883 ± 0.074 ^{NS}
Group 5	2.555 ± 0.166***	4.291 ± 0.386***	2.177 ± 0.077**	2.260 ± 0.055*
Group 6	1.587 ± 0.311***	4.474 ± 0.397***	2.507 ± 0.112***	2.627 ± 0.057***
F value	36.297	20.897	37.224	43.980
P value	<0.0001	<0.0001	<0.0001	<0.0001

Values are expressed as Mean ± SEM. , Group 2 was compared with Group 1. , Groups 3, 4, 5 and 6 were compared with Group 2.
*p<0.05; **p<0.01; ***p<0.001; NS = Non Significant

Table 3: Effect of DHC-1 on the levels of Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in liver of rats in CCl_4-induced hepatotoxicity model.

Groups	*Na^+K^+ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)*	*Ca^{2+}ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)*	*Mg^{2+}ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)*
Group 1	4.683 ± 0.137	3.263 ± 0.061	3.130 ± 0.070
Group 2	$2.500 \pm 0.085^{***}$	$1.555 \pm 0.039^{***}$	$2.166 \pm 0.082^{***}$
Group 3	2.637 ± 0.090^{NS}	1.704 ± 0.098^{NS}	2.326 ± 0.037^{NS}
Group 4	2.877 ± 0.107^{NS}	$2.136 \pm 0.091^{*}$	2.458 ± 0.120^{NS}
Group 5	$3.270 \pm 0.201^{**}$	$2.477 \pm 0.065^{***}$	2.577 ± 0.108^{NS}
Group 6	$3.977 \pm 0.067^{***}$	$3.019 \pm 0.170^{***}$	$2.837 \pm 0.081^{**}$
F value	48.174	51.031	16.290
P value	<0.0001	<0.0001	<0.0001

Values are expressed as Mean ± SEM. , Group 2 was compared with Group 1. ,Groups 3, 4, 5 and 6 were compared with Group 2.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; NS = Non Significant

3. Results

Effect of DHC-1 on Serum Parameters:

Administration of CCl_4 in Group 2 animals (Negative control) resulted in a significant (p<0.001) elevation in SGPT, SGOT, alkaline phosphatase and total bilirubin levels, as compared to the Normal control group (Group 1).

The administration of DHC-1 significantly decreased the levels of SGPT and SGOT at the doses of 500 and 1000 mg/kg; whereas the alkaline phosphatase and total bilirubin were reduced at all the doses of DHC-1 (Table 1) as compared to the Negative control group (Group 2).

Effect of DHC-1 on Tissue Antioxidant Parameters:

In the liver of animals of Negative control group (Group 2), significant (p<0.001) reduction in the levels of SOD, catalase and reduced glutathione, and significant (p<0.001) increase in lipid peroxidation was observed when compared to Group 1 (Normal control) animals. Administration of DHC-1 at the doses of 250, 500 and 1000 mg/kg to animals of Groups 4, 5 and 6, respectively significantly increased the levels of SOD and GSH and decreased the levels of lipid peroxidation as compared to Group 2. A significant increase in the catalase level was observed at the doses of 500 mg/kg (Group 5) and 1000 mg/kg (Group 6) of DHC-1 as compared to Group 2 (Table 2).

In the negative control group animals (Group 2) significant ($p<0.001$) decrease in the activities of membrane bound enzymes, namely $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were observed as compared to the Normal control group (Group 1). The drug, DHC-1 was found to increase the activity of all the ATPases, namely $Na^{+}K^{+}$ATPase (500 and 1000 mg/kg), Ca^{2+}ATPase (250, 500 and 1000 mg/kg) and Mg^{2+}ATPase activity (1000 mg/kg) at different doses (Table 3) as compared to the Negative control (Group 2).

4. Discussion

The presence of enzymes of the electron transport systems and high amounts of unsaturated fatty acids in the liver makes it vulnerable to peroxidative attack.[39]

The hepatotoxin generally used to study the liver protective effect of drugs is CCl_4 because CCl_4-induced liver dysfunction in rats simulates liver cirrhosis in man.[40,41] It has been stated that one of the principal causes of carbon tetrachloride (CCl_4)-induced hepatopathy is lipid peroxidation by $CCl_3^{\bullet}$, a free radical derivative of the toxin.

Ko et al.[42] have examined the impairment in hepatic antioxidant status during the development of CCl_4-induced hepatotoxicity in rats and the protection of such tissue injury by pretreatment with vitamin E, herbal extracts or herbal preparations known to possess antioxidant activities. This generalized impairment in hepatic antioxidant defense mechanism was paralleled by an elevation in SGPT and SGOT activity, an indication of hepatocellular damage.[43]

In liver injury due to hepatotoxin, there is a defective excretion of bile by the liver, which is reflected in the increased levels of alkaline phosphatase in serum.[44] Hyperbilirubinaemia is a very sensitive test to substantiate the functional integrity of the liver and severity of necrosis which increases the binding, conjugating and excretory capacity of hepatocytes that is proportional to the erythrocyte degeneration rate.[45]

The stimulation of lipid peroxidation in either artificial membrane of liposomes or in subcellular organelles has been shown to increase membrane rigidity. In addition to the changes in fluidity, lipid peroxidation causes an increase in the ionic permeability and affects the surface potentials of the membranes. It has also been reported that administration of CCl_4 resulted in decrease in the activities of membrane bound enzymes[46-48] ($Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase), thus leading to oxidative stress.

Administration of CCl_4 alone resulted in a significant elevation in SGPT, SGOT, alkaline phosphatase and total bilirubin levels. It also increased the levels of lipid peroxidation, and reduced the levels of both, endogenous antioxidants (SOD, catalase and GSH) and membrane bound enzymes ($Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase).

Depletion of elevated bilirubin level together with the suppression of the activities of SGPT, SGOT and ALP in the serum of rats treated with DHC-1, suggests the possibility of the herbal product to stabilize biliary dysfunction of rat liver during chronic injury with CCl_4. Reduction in lipid peroxidation (MDA) and enhancement in the levels of endogenous antioxidants (glutathione, catalase and SOD) proves the efficacy of DHC-1 in preventing free-radical induced damage in liver by CCl_4. Pretreatment with DHC-1 also increased the activity of all the ATPases indicating its membrane stabilizing action.

Thus the results prove that DHC-1 protected the liver from the damaging effects of CCl_4 by its antioxidant mechanism of action and can thus be used as a hepatoprotectant against such chemical insults.

The hepatoprotective effect of DHC-1 can be due to the ingredients, *Bacopa monnieri*,[7] *Emblica officinalis*,[8,9] *Glycyrrhiza glabra*,[10,11] *Mangifera indica*[12] and *Syzygium aromaticum*[13,14] which have individually been shown to protect the liver from the toxic effects of various hepatotoxicants.

As regards to the antioxidant activity of the constituents present in DHC-1, various reports are available. The *in vitro* antioxidant activity of *B. monniera,* one of the ingredients of DHC-1 was evaluated earlier by $FeSO_4$ induced lipid peroxidation in rat brain homogenate and the mechanism of action was thought to be through metal chelation at the initiation level and also as a chain breaker.[15] The active tannoid principles of *Emblica officinalis* (amla), another ingredient of DHC-1, were found to induce an increase in both frontal cortical and striatal concentrations of the oxidative free radical scavenging enzymes, SOD, catalase and glutathione peroxidase and concomitant decrease in lipid peroxidation in these brain areas.[17] Wang and Han[10] suggested that the anti-lipid peroxidation effect of Glycyrrhiza flavonoids contributed to its protective action against carbon tetrachloride-induced hepatotoxicity. The extract of *Mangifera indica* reduces ischemia-induced neuronal loss and oxidative damage in the gerbil brain most probably due to the antioxidant activity of the extract.[23] The antioxidant activity of the extract was also studied on hydroxyl-mediated oxidation of bovine serum albumin (BSA) and in a hepatic microsome system and was found to reduce the oxidation of BSA and inhibited lipid peroxidation, which was, initiated enzymatically by NADPH.

The results suggested that the extract has an antioxidant activity probably due to its ability to scavenge free radicals involved in microsome lipid peroxidation. In addition, the extract's antioxidant profile *in vitro* was probably similar to its principal polyphenolic component, mangiferin, a glycosylated xanthone.[24]

In another study[22], Vimang, an aqueous extract of *M.indica* was found to provide significant protection against 12-O-tetra-decanoyl-phorbol-13-acetate (TPA)-induced oxidative damage in serum, liver, brain as well as against hyperproduction of ROS by peritoneal macrophages.

Thus Vimang could be useful to prevent the production of ROS and the oxidative tissue damages *in vivo*. The aroma extracts and aroma components isolated from *Syzygium aromaticum* (clove) were found to inhibit malondialdehyde formation from blood plasma oxidised with Fenton's reagent.[49]

5. Conclusion

The results obtained from this study indicate that DHC-1 pretreatment offers significant protection to liver (hepatoprotective effect) and reduces the risk of CCl_4-induced liver damage by inhibiting lipid peroxidation and activating antioxidant defense system in the organ. The formulation may thus occupy a promising place in the market as a hepatoprotectant along with other popular herbal preparations like Liv 52, Tefroliv and Stimuliv, providing clinical studies also prove the same.

Earlier studies have proved the tissue protective effects of DHC-1 in other organs like heart, kidneys and gastric tissue and thus can be marketed as an overall good antioxidant formulation for defense against oxidative stress induced disease conditions.

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