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The Methanolic Extract of Hemidesmus Indicus and Its Fraction Induces Antihepatotoxic Activity in Vivo : Possible Involvement of Antioxidant Action

Jagdish R. Baheti

Kamla Nehru College of Pharmacy, Butibori Nagpur, Maharashtra, India

ABSTRACT

Aim: To evaluate methanolic extract of *Hemidesmus indicus* and its different fractions for their hepatoprotective activity against CCl_4 induced hepatotoxicity and to find out the better efficacious fraction. The study was also aimed to probe possible mechanism behind offered hepatoprotection by extract and fraction.

Method: The methanolic extract was prepared and fractionated using the solvents of varying polarity like toluene, chloroform, ethyl acetate and n-butanol and tested for hepatoprotective activity against CCl_4 induced liver damage in the rats. Extent of hepatic damage was assessed by levels of SGPT, SGOT, ALP, Total Bilirubin and Direct Bilirubin and histopathologic study of liver sections. Probable mechanism was investigated by carrying out free radical scavenging activity of extract and its fractions using different invitro models like DPPH, NO and superoxide scavenging activity.

Result: There was a significant increase in the levels of serum GOT, GPT after administration of CCl_4 (0.7ml/kg) to rats. Methanolic extract (100mg/kg) and (250mg/kg) and toluene fraction 50mg/kg (HM) produced significant reduction in the level of these enzymes. Methanolic extract and its toluene fraction also preserves the structural integrity of the hepatocellular membrane as revealed from histological studies. The methanolic extract and its HM (toluene fraction) exhibited significant antioxidant activity by inhibiting DPPH radical, nitric oxide and supeoxide scavenging activity

Conclusion: The methanolic extract of *H. indicus* roots and HM exhibited antihepatotoxic effect against CCl_4 induced hepatic damage rationalise its ethnopharmacological claim and it appears that the hepatoprotection offered by *H. indicus* may be related to its antioxidant activity.

Keywords: *H. indicus,* Hepatoprotective, DPPH assay, CCl_4, Free radical scavenger

I. INTRODUCTION

Hemidesmus indicus belonging to family Asclepiadaceae, known as *Indian Sarsaparilla* or *Anantmul* used for medicinal purpose in different parts of the world. The plant is distributed throughout India and many parts of the world in plains and low hills[1]. The root is sweet bitter, cooling, aphrodisiac, antipyretic and cures leprosy, leucoderma, asthma, bronchitis and general debility[2,3]. Traditionally it is used as blood purifier, diuretic antirheumatic and antidote for snake bite[4]. Roots are reported to have antimicrobial[5] and anti-inflammatory[6,7] properties. Studies with methanolic extract of bark of *H. indicus* have shown protection against rifampicin and isoniazide induced hepatic damage[8]. Root bark has been reported to possess antioxidant activity[9]. Some important chemical constituents of the root include hemidesmin1, hemidesmin2, amyrins, and lupeol 2-hydroxy 4-methoxy benzoic acid and some triterpenes[10-12]. From aerial parts of the plant, several pregnan steroids and glycosides have been isolated[13-16]. Coumarinolignoids Hemidesmin I, Hemidesmin II

found in *H. indicus* are important class of natural product that have shown enormous and potential biological activities[17].

Liver diseases are among the most serious ailments. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatitis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in fibrosis of the liver). Liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon tetrachloride, chlorinated hydrocarbons, etc.), excess consumption of alcohol, infections and autoimmune/disorders. Most of the hepatotoxic chemicals damage the liver cells mainly by inducing lipid peroxidation and other oxidative damages in the liver[18]. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis[19].

Inspite of the tremendous advances being made in allopathic medicine, no effective hepatoprotective medicine is available. The available therapeutic agents bring about only symptomatic relief without any influence on the curative process, thus, causing the risks of relapses and danger of untoward effects. A large number of populations still suffer from hepatic diseases due to various reasons. The development of hepatoprotetive/anti-hepatotoxic drugs is a major thrust area in the field of natural product research. There are numerous plants and polyherbal formulations claimed to have hepatoprotective activities [20, 21]. But still we do not have readily available satisfactory plant drugs/formulations to treat severe liver disease.

Our previous studies on this plant have shown the remarkable hepatoprotective activity at a dose of 250-500 mg/kg against CCl_4 and paracetamol-induced liver damage in Wistar Albino rats [22]. There is need to carry on follow up studies leading to therapeutically valuable drug development. Reactive oxygen species and free radicals play an important role in the etiology of various diseases such as inflammation, cataract, atherosclerosis, rheumatism, arthritis, ischemia reperfusion injury including liver disorders [23]. It is well documented that carbon tetrachloride triggers hepatic and renal damage in animals and man [24] and hepatotoxicity induced by carbon tetrachloride is attributed to generation of trichloromethyl free radical during metabolism by hepatic microsomes. Therefore, in the present study, the hepatoprotective effect of *H indicus* was evaluated against CCl_4 induced liver damage in the rats and probable mechanism was investigated by carrying out free radical scavenging activity of extract and its fractions using different invitro models like DPPH assay, NO scavenging effect and superoxide scavenging activity.

II. MATERIAL AND METHODS

CCl_4 was procured from E. Merck India Ltd. Mumbai; Silymarin was obtained as gift sample from Cadila Pharma Ltd., India. Standard kit of SGOT, SGPT ALP and bilirubin was obtained from Span Diagnostics Ltd. All other reagents used for the experiments were of analytical grade.

Preparation of extract of H. indicus and its fraction

The *H. indicus* was collected from wildly grown plant and authenticated in our Pharmacognosy Department with the help of Botanist and a voucher specimen KB-PD 08/01 was preserved. It was air dried and powdered to 40 mesh and stored in airtight container till further use. 500 gm of the powder was defatted by petroleum ether and extracted with methanol using Soxhlet apparatus and the solvent was evaporated under reduced pressure. The methanolic extract was subjected fractionation by toluene, ethyl acetate, chloroform and butanol. The percentage yields of each extract was calculated and were then subjected to qualitative chemical examination for various phytoconstituents as per method described by Harborne [25].

Animals

Wistar albino rats of either sex weighing between 150 and 160 gm were used for the hepatoprotective study. The animals were housed in polypropylene cages and maintained at 24±2°C under 12 hr light dark cycle and they were fed *ad libitum* with standard pellet diet (Amrut, India) and had free access to water. They were initially acclimatized for the study and the study protocol was approved by the Institutional Animal Ethics Committee as per the requirements of Committee for the Purpose of Control and Supervision on Animals, CPCSEA, New Delhi.

1) Experimental protocol for hepatoprotective study

CCl_4 induced hepatotoxicity

Rats were divided into eleven groups of five animals each. Group I served as vehicle control and received normal saline 5 ml/kg. Group II was administered with CCl_4 / Olive oil 1:1v/v, 0.7 ml/kg *i.p.*on alternate days [26]. Group III and IV received methanolic extract 100 mg/kg and 250 mg/kg *p.o* respectively daily for seven days simultaneously with toxicant CCl_4/Olive oil. Group V was administered with reference drug, silymarin [27] 100mg/kg *p.o.* simultaneously with toxicant. Group VI to XI received toluene, ethyl acetate, butanol fraction respectively at 25-50 mg/kg simultaneously with toxicant.

Assessment of hepatoprtective activity:

On the seventh day of the start of respective treatment the rats were anaesthetized by light ether anesthesia and the blood was withdrawn by making intracardiac puncture to the rats. It was allowed to coagulate for 30 minutes and serum was separated by centrifugation at 2500 rpm. The serum was used to estimate Serum Glutamate Pyruvate Transaminase, SGPT, Serum Glutamate Oxaloacetate Transaminase, SGOT Alkaline Phosphatase, ALP Total Bilirubin and Direct Bilirubin.

The results of antihepatotoxic activity were presented as the mean±SEM of 5 animals each group. Results were analyzed statistically using analysis of variance ANOVA followed by Tukeys test. Values of $P<0.05$ were considered significant.

Histopathology:

The method for histological studies was as described by Garg. Briefly the procedure used included fixation of the tissue with formalin, embedding in paraffin blocks, sectioning with microtome (0.7 µ thickness) and finally staining by Haemotoxylin and Eosin stain technique.

Haemotoxylin stains nucleus light blue, which turns red in presence of acid. The cell differentiation is achieved by treating the tissue with acid solution the counter staining is performed by using Eosin, which imparts pink colour to cytoplasm.

Free radical scavenging activity

Diphenyl-picryl-hydrazyl (DPPH) assay:

The free radical scavenging capacity of methanolic extract was tested by its ability to bleach the stable 2, 2 diphenyl 2-picryl hydrazyl radical (DPPH) [28]. A stock solution of DPPH (1.5 mg /ml of methanol) was prepared such that 75 µl of it in 3 ml methanol gave initial absorbance of 0.9. This stock solution was used to measure the antiradical activity. Decrease in absorbance in the presence of methanolic extract and/or fractions at different concentration was noted after 15 minutes. IC_{50} was calculated from percentage inhibition. Ascorbic acid was used as reference standard.

Scavenger effect on superoxide radical:

Superoxide anion radicals were estimated by spectrophotometric measurement of the reduction products of nitroblue tetrazolium (NBT) generated in riboflavin-light system according the method of Mccord and Fridovic[29]. The reaction mixture consisted of EDTA (6µM; with 3µg NaCN), riboflavin (2 µm), NBT (50µM), different concentrations of methanolic extract and/or fractions andphosphate buffer (67 mM; pH 7.8) added in a final volume of 3 ml. The tubes

were uniformly illuminated with an incandescent lamp for 15 min, and then the optical density was measured at 530 nm before and after illumination[29]. Ascorbic acid was used as a positive control.

Nitric oxide scavenging activity:

The interaction of methanolic extract (in final concentrations between 0.5 and 10 μg/ml) with nitric oxide was assessed by the nitrite detection method. The chemical source of NO was sodium nitroprusside (10mM) in 0.5 M phosphate buffer, pH 7.4, which spontaneously produces nitric oxide in an aqueous solution. Nitric oxide interacts with oxygen to produce stable products, leading to the production of nitrites. After the incubation of 60 min at 37°C, the Greiss reagent (α-naphthyl-ethylenediamine 0.1% in water and sulfanilic acid 1% in H_3PO_4 5%) was added. The same reaction mixture without the methanolic extract and fractions of sample but with equivalent amount of methanol served as control[30]. Ascorbic acid was used as positive control.

III. RESULT

Effect of H. indicus on carbon tetrachloride induced hepatotoxicity:

There was a significant increase in the levels of serum GOT and GPT after administration of CCl_4 (0.7ml/kg) to rats. Methanolic extract at 100mg/kg and 250mg/kg and HM 50mg/kg produced significant reduction in the level of these enzymes which was comparable to that observed with reference standard silymarin 100mg/kg. Further increase in dose of methanolic extract 500mg/kg did not produce any change in the levels of these enzymes.

ALP level was found to be significantly increased after administration of CCl_4. There was significant decrease in level of ALP after simultaneous administration of CCl_4 and methanolic extract 100 mg/kg to 500mg/kg (Table1). Similar decrease in ALP activity was observed with toluene fraction, HM and standard silymarin (Table 1).

The increased bilirubin (total and direct) observed after CCl_4 administration was found to be significantly decreased in group of rat orally treated with methanolic extract at dose of 100mg/kg-500mg/kg and HM (Table 1).

Examination of liver sections of the control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Plate 1-Fig A). The liver sections of CCl_4 intoxicated group showed complete disarrangement of normal hepatic cells with intense centrilobular necrosis and vacuolization. Fatty degeneration was also observed in areas other than the centrilobular ones with lymphocyte infiltration (Plate 1-Fig B).

Treatment with methanolic extract and HM protected the hepatocyte from damage caused by CCl_4, as evidenced by absence of necrosis and fibrosis, low infiltration on inflammatory cells less vacuole formation and marked regenerative process indicating the presence of normal hepatic cords confirmed its activity (Plate1-Fig D&F).

Free Radical Scavenging Activity

The methanolic extract and its different fractions of root of *H. indicus* were evaluated for its antioxidant activity using several *in vitro* and *ex vivo* models. Out of these various extracts and fractions tested, methanolic extract of *H indicus* and its toluene fraction were found to be most effective in scavenging different radicals in tested models. Both showed marked antioxidant effect by scavenging superoxide, hydroxyl and nitric oxide radicals.

The methanolic extract of *H. indicus* roots, showed significant anti-radical activity with an IC_{50} value of 28.8 μg/ml with maximum activity 74.8%. Toluene

fraction was found to be effective with an IC_{50} value of 96.56µg/ml while other fractions like ethyl acetate and n-butanol were found to be ineffective [Table 2].

The methanolic extract of *H indicus* and its toluene fraction showed nitric oxide scavenging activity with an IC_{50} value of 65.86µg/ml and 47.25µg/ml respectively the maximum obtained inhibition was 87.35% and other tested respective fractions were not found to produce any significant effect [Table 2].

The toluene fraction of the methanolic extract and methanolic extract of *H indicus* showed super oxide scavenging activity with an IC_{50} value of 35.061µg/ml and 28.61µg/ml respectively. The methanolic extract showed maximum inhibition of 98.83 % while toluene fraction showed maximum inhibition of 87.65%. However, other tested fractions such as ethyl acetate, chloroform and butanol were found to be ineffective [Table 2] except that of ethyl acetate fraction, which showed little effectiveness in scavenging superoxide. Methanolic extract was found to be effective at little lesser concentration than its toluene fraction in certain models.

DISCUSSION

In the present study methanolic extract and its different fractions were evaluated for the hepatoprotective activity using hepatotoxicity induced by CCl_4 in rat model and find out the therapeutically better efficacious fraction. An attempt was made to probe possible mechanism behind offered hepatoprotection by extract and fraction by carrying out its free radical scavenging activity. The methanolic extract was fractionated using the solvents of varying polarity like toluene, chloroform, ethyl acetate and n-butanol and undertaken for the present study.

Preventive action in liver damage induced by CCl_4 has widely been used as an indicator of the liver protective activity of drugs in general[26, 32]. The extent of hepatic damage induced by CCl_4 is assessed by the level of released cytoplasmic alkaline phosphatase and transaminases [GOT and GPT] in circulation[33]. The present investigation also revealed that the given dose of CCl_4 [0.7 ml/kg, *i.p.*] produced significant elevation in SGPT, SGOT and alkaline phosphatase levels indicating an impaired liver function. The massive production of reactive species may lead to depletion of protective physiological moieties [glutathione and tocopherols etc.] and ensuing widespread propagation of the alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes. The investigation further reveals that the methanolic extract of *H. indicus* and HM [toluene fraction] had been effective in offering protection, which is comparable to silymarin. The methanolic extract of *H. indicus* roots and HM when administered to the rats exhibited protection against CCl_4 induced liver injuries as manifested by the reduction in toxin mediated rise in serum enzymes. While other fractions such as HM1 (ethyl acetate fraction) and HM2 (butanol fraction) were failed to produce any significant protection.

It is well known that the toxicity of CCl_4 depends on the cleavage of a carbon-chlorine bond to generate a trichloromethyl free radical [$CCl_3^{\bullet}$] by cytochrome P-450 mediated reactions, which is further, converted to a proxy radical, $CCl_3O_2^{\bullet}$[34]. The free radicals CCl_3^{-} and $CCl_3O_2^{\bullet}$ readily react with polyunsaturated fatty acids of the endoplasmic reticulum and other hepatocellular membranes to initiate the formation of lipid peroxides. In the presence of cellular O_2, these organic peroxy radicals in turn can react with other polyunsaturated fatty acids to perpetuate a series of self-propagating chain reactions, a process commonly referred to as 'propagation of lipid peroxidation' [32]. This 'propagation of lipid peroxidation' causes severe membrane alteration this in turn causes leaking of transaminases through damaged membrane and thereby resulting in the elevation of transaminases in plasma or serum [35]. Many compounds exhibit liver protection against CCl_4

induced damage either by decreasing the production of CCl_3 free radicals[36] or by impairment of CCl_4 induced lipid peroxidation[37]. The rise in serum levels of SGPT, SGOT, ALP and bilirubin following CCl_4 administration could also be attributed to the damaged structural integrity of the liver cell membrane [38] causing leakage of cellular enzymes into the blood. Inhibition of CCl_4 bioactivation could reduce this toxic effect of CCl_4 and it is possible that by this way the methanolic extract of *H. indicus* and HM produces reduction in the level of SGPT, SGOT, ALP and bilirubin. The liver sections of CCl_4 intoxicated group showed disarrangement of normal hepatic cells with centrilobular necrosis and vacuolization. Histopathological examination of liver sections of the rats intoxicated with CCl_4 and simultaneously treated with methanolic extract and HM showed marked regenerative activity without any necrosis with little lymphocytic infiltration confirming their hepatoprotective effect against CCl_4 intoxication. The improved histology of liver after treatment with methanolic extract and HM as compared to that seen in animals administered with only CCl_4 indicates the possibility that methanolic extract can stabilize the liver cells and thus reduce the leakage of GPT, GOT and ALP into the blood. Thus we found that methanolic extract and HM not only reduces the levels of various marker enzymes of liver but also preserves the structural integrity of the hepatocellular membrane as revealed from histological studies.

In order to explore probable mechanism of action behind this hepatoprotection, studies were performed to investigate free radical scavenging activity using DPPH, NO and superoxide scavenging reaction. The result of DPPH-scavenging activity of the extracts suggests that it contain a free radical scavenging which could exert a beneficial action against pathological alterations caused by the generated free radical CCl_3. Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation.

Further studies were carried out on superoxide radicals which are formed normally in the body and known to be very harmful to cellular components as a precursor of more reactive oxygen species[39]. The superoxide anion radical has been implicated in several pathophysiological processes, including the ischemia-induced tissue damage, due to its transformation into more reactive species such as the hydroxyl radical. Several enzymes, such as NADPH oxidase and xanthine oxidase, produce superoxide as a reaction product, eventually contributing to tissue injury[40]. The extract and HM was found to be an effective scavenger of superoxide radical generated by *in vitro* riboflavin-NBT-light system and its activity was comparable to that of ascorbic acid. However, ethyl acetate and butanol fraction of methanolic extract of *H. indicus* did not produce any significant antioxidant activity.

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states[41]. NO is produced in various cells including neurons, endothelial cells and neutrophils by three isoforms of nitric oxide synthase enzyme [encoded by a unique gene], from nitrogen of the guanidine group of L-arginine an from molecular oxygen[42]. The interaction NO with other radicals leads to the formation of more hazardous radicals such as peroxynitrite anion and hydroxyl radical. In fact, NO reacts more rapidly with superoxide than the latter does with superoxide dismutase. Methanolic extract and HM significantly decreased, in a dose-dependent fashion, the concentration of nitrite after spontaneous decomposition of sodium nitroprusside, indicating that methanolic extract may contain compounds able to scavenge nitric oxide. However, ethyl acetate and butanol fraction of methanolic extract of *H. indicus* did not produce any significant antioxidant activity.

These active fractions showed effectiveness only at high dose concentrations as compared to that of crude methanolic extract of plant. Our phytochemical

analysis indicated that flavonoids, tannins, saponin as well as coumarinolignans as major constituents in methanolic extract of both the plants. However, toluene fraction of methanolic extract of *H. indicus* showed presence of only flavonoids and phenolics. Similarly butanol fraction showed predominantly saponins only. It is possibility that there is some synergistic effect of flavonoids with saponins, as both were present in methanolic extract of *H. indicus*. The literature has already documented the antioxidant and hepatoprotective value of flavonoid and phenolics[43]. Thus, it appears that the hepatoprotection offered by *H. indicus* extract may be related to its free radical scavenging activity.

It is thus concluded that methanolic extract of *H. indicus* roots and HM exhibited antihepatotoxic effect against CCl_4 induced hepatic damage rationalise and maintain its ethnopharmacological claim. Further studies in progress in our laboratory for isolation and characterization of phytoconstituents may lead to development of lead nucleus for hepatic dysfunction.

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