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Research Article

HEPATOPROTECTIVE ACTIVITY OF *LAWSONIA INERMIS LINN*, WARM AQUEOUS EXTRACT IN CARBON TETRACHLORIDE- INDUCED HEPATIC INJURY IN WISTER RATS

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ABSTRACT

Lawsonia inermis Linn is used in tribal medicine of Orissa, the southern most state of India, to treat liver diseases. In the present study, the effect of the warm aqueous extract of *Lawsonia inermis* leaf on Carbon tetrachloride (CCl_4) induced liver damage in healthy Wister albino rats was studied. The results showed that significant hepatoprotective effects were obtained against liver damage induced by CCl_4 as evidenced by decreased levels of serum enzymes, glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SAKP), serum bilirubin (SB). The extracts also showed significant antilipid peroxidant effects *in vitro*, besides exhibiting significant activity in quenching 1, 1-diphenyl -2-picryl hydrazyl (DPPH) radical .indicating its potent antioxidant effects.

Key words: Carbon tetrachloride, DPPH quenching, hepatoprotection, lipid peroxidation.

INTRODUCTION

Role of free radicals in the causation of diseases has been well established [1].Several substances have been known to produce excessive free radicals and thereby produce tissue damage [2, 3]. Since liver is the major organ involved in the detoxification of xenobiotics, it is the main target of tissue injury produced by these chemicals and their metabolites. Reactive oxygen species produce deleterious effect on membrane lipids of the cellular components thereby producing peroxidation of lipids which leads to cell death [4]. An association of reactive oxygen species with collagen synthesis and fibrosis has also been reported [5].

Many of drugs, useful against diseases are known to produce severe side effects. Antitumour drugs are known to produce myelosupression, nephrotoxicity and hepatotoxicity which are mainly caused by free radical generation [6]. Scavengers of free radicals can reduce side effects of these drugs. Plant kingdom posses several non-toxic compounds that can scavenge free radicals and boost the antioxidant defense mechanism in body and have a protective role against tissue damage induced by several chemicals and drugs [7-10].

The Plant *Lawsonia inermis*, commonly known as Mehendi, belongs to the familyof Lythraceae. It is a shrub and cultivated throughout India, Bangladesh and Pakistan. The leaves are greenish brown to brown, about 2.5-5cm long, the margine entire and venation pinnate[11]. Topical application of Henna reduced severity of Hand-foot Syndrome caused by capecitabaine used for cancer treatment.This effect of Henna reported to have anti-inflammatory, antipyretic and analgesics activities[12].

An ointment prepared from the leaves was used to cure ulcers and wounds [13]. The astringent stem bark of *L. inermis* is traditionally used in India for the treatment of jaundice, enlargement of liver and spleen [14]. It has been reported to posses anti-inflammatory [15], antimicrobial [16] and anticancer activity [17]. One of the major active constituents of leaves of *Lawsonia inermis* are flavanoids [18].Hepatoprotective activity of ethanolic extract of leaves of *Lawsonia alba* has been reported [19]. But detailed study of hepatoprotective activity has not been done.The present study has been undertaken with aim to determine the hepatoprotective activity of the warm aqueous leaves extract of *Lawsonia inermis* in animal models by estimating numbers of parameter.

MATAERIALS AND METHODS

Chemicals- Nitro blue tetrazolium (NBT), glutathione (GSH), 5-5'dithibios (2-nitro benzoic acid) (DTNB), were purchased from Sisco Research Laboratories Pvt.Ltd,Mumbai,India. Thiobarbituric acid was purchased from HiMedia Laboratories, Mumbai, India. CCL_4 was purchased from E-Merck, Mumbai, India. Silymarin was obtained from Ranbaxy Pvt.Ltd, Gurgaon, India. The kits for estimating enzyme activities were purchased from Agappe Diagnostics, Ernamkulam. The kits for total protein and bilirubin were purchased from Span Diagnostics, Surat. All other chemicals and reagents used were of analytical grade and obtained locally.

Plant material and preparation of the extract

The leaves of *Lawsonia inermis* were collected from Durgapur, Burdwan district,West Bengal. They were authenticated by Botanical Survey Of India, Shibpur,Howrah district,West Bengal and a voucher specimen(CNH/I-I/274/2008/Tech.II/316, dated17-11-2008) was deposited at the Institutes's Herbarium.

The leaves were washed thoroughly in tap water, shade dried and powdered. The powder (100gm) was dissolved in 1000ml of luke warm distilled water and was allowed to macerate for 48 hours at room temperature with constant stirring. The extract was filtered and the the residue was pressed out through clean linen, and added to the filtrate. The filtrare was evaporated to dryness in a shaker water bath at 42°C.The yield was found to be 2.2gm. This crude extract was referred to as WAELI (warm aqueous extract of *Lawsonia inermis*).It was reconstituted in 0.25% CMC, to desired concentrations and used for experiments.

Animals

Wistar albino rats, males (270-300g) and Swiss albino mice, males (25-30g) were obtained from the Institute's Animal House. They were housed under standard conditions (temperature 24-28°C, relative humidity 60-70% and 12 hr dark-light cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were carried out according to National Institute of Health guidelines, after getting the approval of the Institute's Animal Ethics Committee (Reg No-955/A/06/CPCSEA, 2006).

Toxicities studies

Mice of either sex, weighing 20-25g were used for acute toxicity studies. Acute toxicity study for WAELI was performed by Miller and Tainter method[20] to determine lethal dose (LD_{50}). CMC (0.025%) was used as vehicle to suspend the extracts and the suspension was administered intraperitoneally. One tenth of the LD_{50} was used as a maximum dose of extracts tested for acute toxicity. The dose was selected for evaluation of hepatoprotective activity i.e. 400mg/kg i.p.

Hepatotoxins and test substances

All the test substances including silymarin were suspended in vehicle i.e. 0.025%CMC for administration. CCl_4 at a dose level of 1.25ml/kg diluted in liquid paraffin (1:1) was administered intraperitoneally.

Carbon tetrachloride-induced hepatotoxicity [21]

Rats were divided into 4 groups of 6 each, control, CCl_4, silymarin and test group.The rats of control and CCl_4 group received three doses of 0.025% CMC (1ml/kg, po) at 12 hr intervals (0 hr, 12 hr and 24 hr). The rats of CCl_4 group received a single dose of CCl_4 (1.25ml/kg, ip) diluted in liquid paraffin (1:1) 30 minutes after the administration of first dose of vehicle. The animals in silymarin and test group received three doses of respective test substances (silymarin100mg/kg; WAELI 400mg/kg, i.p.) at 0 hr,12 hr, and 24 hr. CCl_4 (1.25ml/kg i.p.) was administered 30 minutes after the first dose of the respective test substances. After 36 hr of administration of CCl_4, the animals were sacrificed by mild ether anaesthesia. Blood samples were collected and serum was separated at 2500 rpm for 15 min and biochemical investigations were carried out. Liver was dissected out and used for biochemical determinations and liver tissue slices were collected for histopathological studies.

Biochemical and histopathological studies

Biochemical parameters like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SAKP) and serum bilirubin (SB) were assayed according to standard methods. [22, 23, 24]. Animals were sacrificed to remove liver. The liver was fixed in Bouin's solution for12 hr, and then embedded in paraffin using conventional methods[25], cut into 3µm thick sections and stained using haematoxylin-eosin dye. The sections were then observed for histopathological changes.

Assesment of lipid peroxidation

The antilipid peroxidant effect of WAELI was studied in vitro, following modified methods [26, 27]. Briefly, 0.5g of the rat liver tissue was sliced and homogenized with 10ml of 150m*M* KCL-Tris-HCL buffer (*p*H 7.2). The reaction mixture was composed of 0.25 ml of liver homogenate, Tris-HCL buffer (*p*H 7.2), 0.1 m*M* asborbic acid (AA), 4 m*M* FeCl2 and 0.05 ml of various concentrations of WAELI extract. The mixture (in triplicate) was incubated at 37°C for 1 hr in capped tubes. Then, 0.1 N HCL, 0.2 ml of 9.8% sodium dodecyl sulphate (SDS), 0.9 ml of distilled water and 2 ml of 0.6 thiobarbituric acid (TBA) were added to each tube and vigorously shaken. The tubes were placed in a boiling water bath at 100°C for 30 min. After cooling, the flocculant precipitate was removed by adding 5 ml of n-butanol and they were centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured by the spectrophotometric method [28]. To a methanolic solution of DPPH (200µ*M*), 0.05 ml of the test compound dissolved in ethanol were added at different concentrations (100-500µg/ml). An equal amount of ethanol was added to the control. After 20 min, the decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula [29]given below:

$$\text{Inhibition \%} = \frac{(\text{Control-Test})}{\text{Control}} \times 100$$

Behavioural and toxic effects

Seven groups of ten mice were treated with WAELI extract, (500, 1000, 2000, 3000, 4000 and 5000 mg/kg i.p). They were observed continuously for 1 hr for any gross behavioural changes, symptoms of toxicity and mortality if any and intermittently for the next 6 hr and then again, 24 hr after dosing with WAELI extract.

Statistical analysis

The mean values ± SEM were calculated for each parameter. For determining the significant inter group difference one-way analysis of variance[30] was carried out and individual comparisons of the group mean values were done using Dunnett's test[31].

RESULTS

Effect of WAELI on serum hepatic enzyme levels

There was a significant increase in hepatic enzymes SGPT, SGOT, SAKP activity of CCl_4 treated groups when compared with that of normal control (Table 1). Free treatment of *Lawsonia inermis* leaf extract (Group IV and Group V) could significantly reduced SGPT, SGOT and SAKP activities of serum total bilirubin in the group II animals was significantly increased when compared with the group I animals. Pretreatment with *Lawsonia inermis* leaf extract decreased the bilirubin level (Table I) the total protein and glutathione in group II animals was significantly decreased when compared with that of Group I animals. Pretreatment with *Lawsonia inermis* leaf extract increased the total protein and glutathione level (Table 1). The lipid peroxidation in liver tissue of untreated control animals was 471.40± 3.0which was significantly higher when compared with that of normal control level(118±1.68) .This increased level was found to be significantly lowered in the extract treated groups (table1)

Table 2: Inhibitory effect of warm aqueous extract of *Lawsonia inermis* leaf on (WAELI) on $FeCl_2$ –ascorbic acid (AA) induced lipid peroxidation in rat liver homogenate *in vitro* [values are mean ± SD of 6 animals]

Groups	WAELI Concentration (µg/ml)	MDA (nmol/ g wet liver)	MDA Inhibition (%)
Normal control	-	1.32±0.58	-
$FeCl_2$– AA control	-	2.28±0.02	-
$FeCl_2$ – AA+ WAELI	25	1.82± 0.02	22.02
$FeCl_2$–AA+WAELI	50	1.37± 0.02	39.98
$FeCl_2$–AA+WAELI	100	0.84±0.02**	62.54**
$FeCl_2$–AA+WAELI	200	0.90± 0.01**	59.33**

**** P ≤ 0.01, compared to $FeCl_2$ – AA control**

Table 3: Effect of warm aqueous extract of *Lawsonia inermis* leaf on (WAELI) on DPPH radical scavenging activity [values are mean of 3 experiments]

Conc. (µg/ml)	DPPH (% inhibition)
25	40.25
50	62.8 5
100	68.35
400	90.75
800	88.23

Effect of WAELI on in vitro lipid peroxidation

WAELI showed very potent inhibition of $FeCl_2$-AA stimulated rat liver lipid peroxidation in vitro at concentrations of 100 & 200 µg/ml. There was a significant increase of malondialdehyde (MDA) in FeCl2 – AA treated rat liver homogenate, Compared to normal control without $FeCl_2$ –AA. (Table 2).

Effect of HH on DPPH free radical scavenging activity

WAELI showed maximum inhibition (90.75%) of DPPH free radical at 400 ug/ml . 25 µg/ml dose failed to evoke significant response & it was observed that the free radical was scavenged in a concentration dependent manner up to 400 µg/ml.(Table 3)

Toxicity Studies- In the acute toxicity studies, the WAELI extracts was found to be toxic (5/10 mice died) at the dose of 4000 mg/kg, but it was found to be safe up to 2000mg/kg. The LD_{50} of WAELI was therefore 4000mg/kg i.p, in mice (data not shown).

Table1: Effect of warm aqueous extract of *Lawsonia inermis* leaf on (WAELI) on rat serum enzymes after CCl_4 administration [values are mean ± SD of 6 animals]

Groups	SGOT(IU/L)	SGPT(IU/L)	SAKP(KA units/100ml)	SB (IU/L)	TP(IU/L)	Liver wt in (g/100g body weight)	Lipid peroxides (µ*M* of MDA/ mgof protein	GSH(µg / mg of protein)
GroupI Normal control(0.025% CMC,1ml/kg)	97.60±1.67	65.68± 1.47	24.57 ± 1.91	2.35± 0.20	7.61 ± 0.40	2.17± 0.22	118± 1.68	314± 1.9
GroupII CCl_4 treated (1.25 ml/kg)	181.25± 0.95	181.25± 0.95	68.59± 2.26	3.04± 0.04	3.63± 0.35	5.49± 0.39	471.4 ± 3.0	220± 4.03
GroupIII CCl_4 + Silymarin (100mg /kg)	107.13±0.85**	65.93±1.48**	26.80± 1.80**	1.35±0.01**	6.90±0.45**	3.99± 0.39**	135.7± 1.28**	335.60±2.95**
GroupIV CCl_4 +WAELI (400mg /kg)	141.5±1.42**	76.77±1.95**	36.13± 1.31**	1.83±0.02**	6.20± 0.27 **	4.08 ± 0.24**	290.6± 2.60 **	333± 3.0**

****significance P ≤ 0.01, compared to CCl_4 control**

DISCUSSION

In recent years, many studies have been undertaken with traditional medicines, in an attempt to develop new drugs for hepatitis[32]. In the present study, we used animal models including CCl_4 for liver damage induction to investigate whether the plant extract WAELI could decrease efficiently the toxicity produced by these hepatotoxicants.

CCl_4 has been one of the most intensively studied hepatotxicants to date & provides a relevant model for other halogenated hydrocarbons that are used widely [33]. A single exposure of CCl4 can lead to severe centizonal necrosis and steatosis [34]. The changes associated with CCl_4- induced liver damage are similar to that of acute viral hepatitis [35]. CCl_4 is transformed by Cytocrome P-450 system to produce trichloromethyl free radicals. These free radicals may again react with oxygen to form trichloromethyl peroxyl radicals which may attack lipids on the membrane of endoplasmic reticulum to elicit lipid peroxidation, finally resulting in cell necrosis and consequent cell death. [36].

Marked increase in release of hepatic enzymes into the blood stream is often associated with massive necrosis of liver. CCl_4 is known to cause marked elevation of serum enzymes. In the present study, a significant increase activity of SGOT, SGPT, SAKP, SB, Lipid peroxides, Liver weight and decrease content of TP and Glutathione was observed within 24 hr of exposure to CCl_4 indicating considerable hepatocellular injury. Our results indicated that WAELI at 400mg/kg doses tested significantly prevented the increased serum enzyme activity and decreased TP and Glutathione content induced by CCl4, indicating improvement of the functional status of the liver by this herb. $FeCl_2$ – ascorbic acid mixture is known to stimulate lipid peroxidation in microsomes of rat liver in vitro[37]. In the present study, WAELI prevented the rise of lipid peroxides (MDA production), showing its significant anti lipid peroxidant effects.

DPPH radical is widely used as the model system to investigate the scavenging activities of several natural compound[38]. DPPH is scavenged by antioxidants through the donation of proton forming the reduced DPPH which can be quantified by its decrease of absorbance. WAELI significantly quenched DPPH radicals indicating its potent free radical scavenging activity, in the present study.

The result also revealed the non-toxic nature of WAELI up to 4000mg/kg in mice and was not surprising as it is extensively used in tribal medicine of Orissa to treat various ailments. Major active constituents of henna leaf are flavonoids [18] which have been shown to possess various biological properties related to antioxidant mechanisms[39-40]. Perhaps the triterpenes and flavonoids present in WAELI extracts are responsible for its hepatoprotective effects. The findings of the present study therefore support the reported therapeutic use of *Lawsonia inermis* in tribal medicine of Orissa to treat liver ailments. The probable mechanism of action of *Lawsonia inermis* appears to be its effect as a free radical scavenger & inhibitor of lipid peroxidation of the liver plasma membrane. Further studies are in progress in our laboratory to explore its chemical constituents & detailed mechanism of action.

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