Available online on 15.07.2014 at http://jddtonline.info
Journal of Drug Delivery and Therapeutics
Open access to Pharmaceutical and Medical research

RESEARCH ARTICLE

THE HEPATOPROTECTIVE AND ANTIFIBROTIC POTENTIAL OF ROOT EXTRACT OF *ALOCASIA INDICA* LINN. AGAINST CCL_4 INDUCED HEPATIC INJURY AND FIBROSIS IN WISTAR RATS

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ABSTRACT:

The hepatoprotective activity of hydro-alcohol extract of roots of *Alocasia indica* Linn. (AI-E) was evaluated against carbon tetrachloride (CCl_4) induced hepatic damage in rats. The AI-E at dose of 400 mg/kg is administered orally once daily for fourteen days. The substantially elevated serum enzymatic levels of serum glutamate oxaloacetate transaminase (AST), glutamate pyruvate transaminase (ALT), alkaline phosphatase (ALP), total lipoprotein (TP), total cholesterol (TC), triglyceride (TG), albumin (ALB), hepatic malondialdehyde (MDA) content, superoxide dismutase (SOD), hyaluronic acid (HA) and liver index were restored towards normalization significantly by the AI-E at dose of 400 mg/kg. AI-E 400mg/kg dose exhibited significant hepatoprotective activity against carbon tetrachloride induced hepatotoxicity in rats. The biochemical observations were supplemented with histopathological examination of rat liver sections. The results of this study strongly indicate that roots of *Alocasia indica* have potent hepatoprotective activity against carbon tetrachloride induced hepatic damage in experimental animals. This study suggests that possible mechanism of this activity may be due to the presence of flavonoids and phenolics compound in the AI-E which may be responsible to hepatoprotective activity.

Keyword: Hepatoprotective, *Alocasia indica*, Carbon tetrachloride, Roots.

INTRODUCTION

The medicinal plants were a common link between modern and traditional medical sciences as they were the main source of drugs and medicaments[1]. The liver is a vital organ of paramount importance involved in the maintenance of metabolic functions and detoxification from the exogenous and endogenous challenges, like xenobiotics, drugs, viral infections and chronic alcoholism. If during all such exposures to the above mentioned challenges the natural protective mechanisms of the liver are overpowered, the result is hepatic injury[2]. Liver diseases remain one of the serious health problems[3]. In spite of tremendous strides in the modern medicine, there are not much drugs available for the treatment of liver diseases. There are a number of medicinal preparations recommended in the Indian traditional system of medicine ‘‘Ayurveda” for the treatment of liver diseases. There are scientific claims to offer significant relief as hepatoprotective[4].

The *Alocasia indica* Linn. (Family- Araceae) commonly known as Giant Taro is a perennial herb found throughout greater part of India. According to ayurvedic literature survey, different parts of this plant are traditionally used in jaundice, antioxidant, analgesic, antiarthritic, disease of abdomen, spleen inflammation[5]. It has also reported to use in the treatment of piles[6]. The leaves juice is used as digestive, astringent, laxative, diuretic and rheumatic arthritis and antifungal properties[7,8]. This plant contains flavonoids, cynogenetic glycosides, ascorbic acid, gallic acid, mallic acid, oxalic acid, alocasin, amino acids, succinic acid, and ß-lectines.

Silymarin, one of these compounds, was used as a standard reference and exhibited significant hepatoprotective and antioxidant activity against CCl_4-induced haptotoxicity in rat models[9,10]. Carbon tetrachloride accumulates in hepatic parenchyma cells and is metabolised to $C{\cdot}Cl_3$ by liver cytochrome P450-dependent monooxygenases[11]. One of the principal causes of CCl_4- induced liver injury is lipid peroxidation induced and accelerated by free radical derivatives of CCl_4 [12].

The roots of *Alocasia indica* are used for the treatment of jaundice in traditional system of medicine. However, there is lack of scientific report regarding the hepatoprotective activity of *Alocasia indica*. The present investigation is an endeavor to validate the scientific use of hydro-ethanolic extract of the *Alocasia indica* (AI-E) against carbon tetrachloride induced hepatic damage in experimental animals.

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 ISSN: 2250-1177 CODEN (USA): JDDTAO

MATERIALS AND METHODS

Plant material

The fresh roots of *Alocasia indica* Linn. used in the present study were collected from local areas of Ibrahimpatnam, Hyderabad, India in the month of February (winter) 2013. The plant species was authenticated by Prof. B. Amarendhar Reddy, M.sc (Botany), Sai Gouthami College, Ibrahimpatnam, R.R. District, India and the voucher herbarium specimen was deposited in the institute's herbarium. The fresh roots of *Alocasia indica* Linn. were separated from plant, washed under running tap water and then with isopropyl alcohol (5%) followed by distilled water. Roots were cut into small pieces and allowed it to shed dry (temperature 30^0C, relative humidity 45 -55%) for 15 days and then homogenized to get a coarse powder. This powder was stored in an air tight container and used for further successive extraction.

Preparation of Extract: Hydro-alcoholic extract *(by cold maceration method)*

About 250 g of the powder was extracted with hydro-alcohol (ethanol- 95% and water in 1:1 proportion) at room temperature by cold maceration method[13]. The filtrate was collected and concentrated on heating mantle at 45°C till a syrupy mass was obtained. Then the extract was again dried by using rotary evaporator under controlled condition of temperature and pressure. The extract thus obtained was preserved at - 4°C. The percentage yield was found to be 6.14 g.

Chemicals

Silymarin was used as the reference drug (positive control); carbon tetrachloride (CCl_4), purchased from Merck Specialities Pvt. Ltd. Mumbai, (India); total cholesterol (TC), triglyceride (TG), total lipoprotein (TP), serum albumin (SA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), hepatic malondialdehyde (MDA), and superoxide dismutase (SOD) commercial assay kits were purchased from Erba Diagonostics Mannheim GmbH, Mumbai, (India); total hyaluronic acid (HA), obtained from Span Diagnostics Ltd. Mumbai (India), all kits were for animals; all chemicals and reagents, unless specified otherwise, were of analytical grade and were not purified, dried or pretreated, and purchased from commercial sources.

Animals and Experimental procedure:

Male Wistar rats (10 weeks old, 250-300g) were obtained from animal centre of Pretox Research Centre, Sachin, Surat, Gujarat, India. The rats were housed in plastic cages under identical conditions (12/ 12 h light /dark cycle), in environmentally controlled rooms (22 ± 3^0C temperature, 50±20% relative humidity), including free access to both food (standard rat chow) and tap water. Animal care and treatment were conducted in compliance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines. The experimental protocol was approved by the institutional animal ethics committee (IAEC protocol no.PRC/AFC/2012/26) of Pretox Research Centre, Sachin, Surat, Gujarat, India They were allowed for acclimation under climate-controlled conditions for a week before use. We compared survival in all groups throughout the treatment. All animals were carefully monitored, and the number of dead rats was recorded every day.

Acute toxicity study:

Acute toxicity study was carried out according to the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals, TG425[14], 10 female Wister rats were randomly divided into two groups of five animals. The AI-E at a single dose of 2000mg/kg body weight was given orally to one at a time, at a minimum of 48-hour intervals in test group, if the animal dies; conduct the main test to determine the LD_{50}. If the animal survives, dose four additional animals sequentially so that a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. The LD_{50} is greater than 2000mg/kg if three or more animals survive. The control group received vehicle. Body weight, signs of toxicity and mortality were observed after the administration at the first, second, fourth and sixth hour and once daily for 14 days. On the day 15, all rats were fasted for 12-16h, and then sacrificed by an intraperitoneal injection (Overdose 60mg/kg) Ketamine hydrochloride for necropsy examination. The internal organs were excised and weighed. The gross pathological observations of the tissues were performed.

Animal model and drug treatment:

The rats were treated by subcutaneous injection of CCl_4 (with an initial dose of 4 ml/kg followed by 30 ml/kg doses) twice a week (40%, diluted in olive oil) for 14 weeks (from the end of tenth-week, 2 rats were selected every week for pathological observation, until model control rats were established) with the exception of normal control group which were treated with olive oil only[15]. Rats were randomly divided into four groups (n = 10): (a) normal control rats receiving distilled water, (b) CCl_4 rats receiving distilled water, (c) CCl_4 rats receiving Silymarin (100 mg/kg, mixed with distilled water),[2] and (d) CCl_4 rats receiving AI-E (400mg/kg), each given by gavages once daily for 8 weeks starting from the 14 week onset of CCl_4 treatment rats. The model control group and the normal control group were given equal volume of distilled water. The rats were killed; liver in fasted status (12-16h) after 22 weeks. The liver was immediately removed and weighed after blood was collected from the abdominal aorta. Liver samples were fixed in neutralized formalin for pathological examination. Blood samples were centrifuged at 3000 rpm for 10 min at 4^0C to obtain blood serum.

Measurement of liver and biochemical assay:

Liver index were calculated as liver weight divided by body weight. The activities of AST, ALT, and ALP the contents of HA, TC, TG, TP, ALB the levels of MDA and SOD, were determined using analysis kits.

Histopathological observations:

The liver specimens were fixed with 10% neutral formalin and embedded in paraffin. The paraffin

 ISSN: 2250-1177 CODEN (USA): JDDTAO

embedded liver tissues were sliced into 4 µm pieces and stained with Hematoxylin–Eosin (H-E) and Masson–Trichrome (M-T), respectively, for photomicroscopic assessment. A numerical scoring system for histologically assessing the extent of fibrosis was adapted from the formula of Scheuer[16], with minor modification[8].

Briefly, fibrosis was staged as:

Stage 0: no fibrosis; Stage 1: enlarged, fibrous portal tracts; Stage 2: periportal or portal-portal septa, but intact architecture; Stage 3: fibrosis with architectural distortion; stage 4: probable or definite cirrhosis.

Additionally, hepatocyte necrosis or degeneration severity was also graded as:

Grade 0: no hepatocyte necrosis or degeneration; Grade 1: focal necrosis or degeneration of hepatocytes (mild, lesion <3); Grade 2: multifocal necrosis or degeneration of hepatocytes (moderate, lesion >3); Grade 3, locally extensive or diffuse necrosis or degeneration of hepatocytes (severe). The liver scoring examination was performed by a pathologist who was blinded to rats' treatment assignment. Fibrosis and hepatocyte scores were given after the pathologist had examined throughout three different areas in the tissue slide for each rat.

STATISTICAL ANALYSIS

All values were expressed as the means ±S.E.M. (standard error of means). Significant differences between the groups were statistically analyzed using an one-way analysis of variance (ANOVA), followed by Tukey's Multiple Comparison Test, while liver histopathologic examination data was evaluated using SPSS 17.0 package, $P < 0.01$ and < 0.001 considered statistically significant.

RESULTS

Acute toxicity study of AI-E:

In the acute toxicity study, the behavioural signs of toxicity observed in the rats at 2000 mg extract/kg body weight were rubbing of nose and mouth on the floor of the cage and restlessness. Gross pathological study showed no abnormality in all the organs examined. Absence of death in test dose group showed that the LD_{50} of the AI-roots extract is greater than 2000 mg extract/ kg body weight.

Protective effects of AI-E on serum index of CCl_4-injured liver fibrotic rats:

The activities of AST, ALT and ALP in serum were significantly reduced by AI-E, the contents of MDA and HA in serum were significantly reduced by AI-E, and meanwhile the levels of TP and ALB in serum were also increased by AI-E. The activities of ALT, the contents of HA and TC in serum were significantly reduced by Silymarin, and meanwhile the levels of TP and SOD in serum were also increased by Silymarin (Table 1).

Table 1: Effects of AI-E on serum index in CCl_4 injured liver fibrotic rats.

	Normal	CCl_4	Silymarin	AI-E
AST	144.73 ± 7.86	394.92 ± 16.40$^{\#\#\#}$	186.87 ± 9.27$^{\#\#\#***}$	197.43± 11.52$^{\#\#\#***}$
ALT	56.33 ± 2.09	282.85 ± 6.04$^{\#\#\#}$	67.26 ± 7.99$^{\#\#\#***}$	59.64 ± 5.19***
ALP	147.66±9.57	268±17.23$^{\#\#\#}$	164±13.75***	159±14.86***
TP	6.59 ± 0.19	3.27 ± 0.21$^{\#\#\#}$	5.49 ± 0.14$^{\#\#\#***}$	5.26 ± 0.22$^{\#\#\#***}$
TC	85.73 ± 1.71	159.36 ± 3.91$^{\#\#\#}$	90.66 ± 2.80$^{\#\#***}$	105.28 ± 2.80$^{\#\#\#***}$
ALB	3.58 ± 0.10	2.65 ± 0.11$^{\#\#\#}$	3.41 ± 0.08$^{\#\#***}$	3.33 ± 0.09$^{\#\#\#***}$
MDA	1.69 ± 0.03	2.97 ± 0.13$^{\#\#\#}$	2.18 ± 0.09$^{\#\#\#***}$	1.57 ± 0.07$^{\#***}$
SOD	93.60 ± 0.45	92.70 ± 0.37	93.1 ± 1.39	95.30 ± 1.54$^{\#***}$
HA	54.30 ± 2.51	87.20 ± 1.83$^{\#\#\#}$	63.30 ± 2.51$^{\#\#\#***}$	51.40 ± 2.79**
LI	3.35 ± 0.08	3.88 ± 0.05$^{\#\#\#}$	3.52 ± 0.06$^{\#\#\#***}$	3.39 ± 0.05***
LV	3.56 ± 0.08	4.02 ± 0.08$^{\#\#\#}$	3.74 ± 0.04$^{\#\#\#***}$	3.43 ± 0.05$^{\#\#\#***}$

\# Represents statistical significance vs. Normal control.
* Represents statistical significance vs. CCl_4.
**P < 0.01, ***P < 0.001
#P<0.05, ## P<0.01, ###P<0.001.

Effect of AI-E on liver homogenate index:

Results showed that liver AST activities and MDA production were obviously decreased by AI-E (400 mg/kg) treatment ($P < 0.01$), liver ALT production and the content of TC ($P < 0.001$) were markedly decreased by AI-E (400 mg/kg) treatment as compared with model control group. Whereas liver SOD, TG activity and LI were no significant difference when compared with model control group (Table 2).

Table 2: Effects of AI-E on liver indicators in CCl_4 injured liver fibrotic rats.

	Normal	CCl_4	Silymarin	AI-E
AST	44.07 ± 5.67	78.01 ± 6.19$^{###}$	55.11 ± 4.68$^{###***}$	58.38 ± 6.81**
ALT	32.10 ± 7.34	44.83 ± 5.46$^{###}$	25.18 ± 5.87***	20.39 ± 5.15$^{###***}$
TC	85.73 ± 1.71	159.36 ± 3.91$^{###}$	90.66 ± 2.80$^{###***}$	105.28 ± 2.80$^{###***}$
TG	0.12± 0.03	0.27±0.07$^{###}$	0.26±0.07$^{###}$	0.24±0.07$^{###}$
MDA	8.77 ± 1.45	10.28 ± 2.82	8.88 ± 1.34	6.96 ± 1.13**
SOD	11.49 ± 0.87	11.16 ± 0.79	11.19 ± 0.64	12.16 ± 1.54
LI	2.14 ± 0.06	3.21 ± 0.07$^{###}$	2.86 ± 0.06$^{###***}$	3.26 ± 0.06$^{###}$

Represents statistical significance vs. Normal control. * Represents statistical significance vs. CCl_4.
**P < 0.01, ***P < 0.001 ## P<0.01, ###P<0.001.

Effects of AI-E on pathological examination

Liver histopathological examination showed no histological abnormalities in normal control liver, portal areas were clear, the hepatic lobular architecture was normal, did not see connective tissue proliferation (Fig. 1A and B); liver tissue in untreated CCl_4 injured rats had more steatosis, cell necrosis and inflammatory infiltration than those in normal control rats. Histological abnormalities in model rat livers also showed apparent formation of fibrotic septa, encompassing regenerated hepatocytes into pseudolobules, the surface of liver was unsmooth, lobular structure was damaged, and accompanied by higher collagen content in the liver, which fulfilled the diagnostic standard for chronic hepatitis (Fig. 1C and D); AI-E (400 mg/kg) treatment markedly alleviated the degree of liver fibrosis and significantly lowered collagen deposited (Table 3 and Fig. 1E and F).

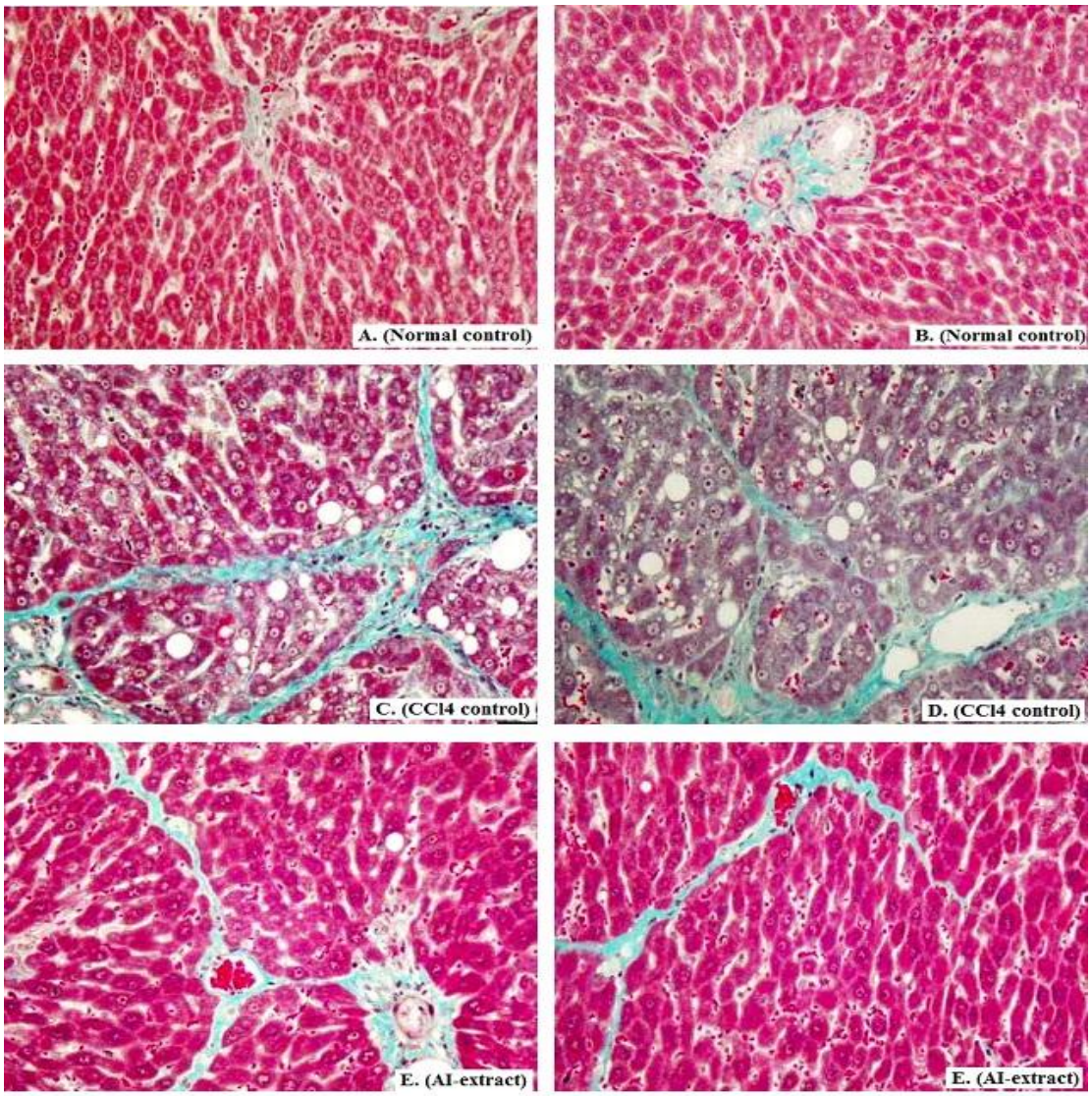

Figure 1: Histological image of liver tissues. The normal lobular architecture with central veins and radiating hepatic cords in normal control rat (A and B). Fatty degeneration, necrosis, infiltration of inflammatory cells and apparent formation of fibrotic septa in the CCl_4 model rat (C and D). The degree of liver damage and fibrosis were significantly reduced in the AI-E (400 mg/kg) treatment rat (E and F). Magnification of microscope is 200X.

Table 3: Effects of AI-E on the pathological grading of CCl_4 injured liver fibrotic rats.

Group	Animal number	Normal	Mild	Moderate	Severe	P
Normal	10	10	0	0	0	
CCL_4	10	0	0	5	6	
Silymarin	10	0	4	4	0	0.002
AI-E	10	0	4	4	0	0.002

The P values were compared with CCL_4 treatment group.

DISCUSSION AND CONCLUSION

In this present study, AI-E was evaluated for the hepatoprotective activity using CCl_4 induced hepatotoxicity in rat. CCl_4 is being used extensively to investigate hepatoprotective activity on various experimental animals[17,18]. The changes associated with CCl_4 induced liver damage are similar to that of acute viral hepatitis, due to preferred as the experimental model[19,20]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects[21]. The hepatotoxicity induced by CCl_4 is due to its metabolite CCl_3, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage[22]. Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood[23]. The increased levels of AST, ALT, ALP and serum bilirubin are conventional indicators of liver injury[24,25].

Many cases it was found that oxidative damage is a substrate for hepatic fibrogenesis. Oxidative stress has been considered as a major molecular mechanism involved in CCl_4toxicity[26]. Previous reports have shown that oxidative stresses play an important role for the inactivation of Kupffer cells in the initial CCl_4-induced rat liver fibrosis[27,28].

Hepatic fibrosis is usually initiated by hepatocyte damage. Biologic factors (such as hepatitis virus, bile duct obstruction, cholesterol overload, schistosomiasis, etc.) or chemical factors (such as CCl_4 administration, alcohol intake, etc.) were known to contribute to liver fibrosis. The incidence of chronic fibrosis is high, but there have been no satisfactory agents with ascertained effectiveness and few side effects. So, finding effective ways to inhibit liver fibrosis and prevent the development of cirrhosis are of great significance.

In this study, CCl_4-induced liver fibrosis model was established to investigate the anti-fibrotic effects of AI-E in vivo. The results have shown that the rats receiving CCl_4 caused a significant elevation of liver index, serum ALT, AST, and HA, while after treatment with AI-E these indexes were markedly reduced. Moreover, the degrees of pathological changes followed chronic intoxication with CCl_4 which were also ameliorated remarkably by AI-E treatment, indicating AI-E have positive antifibrotic effects.

In this present study, CCl_4-induced increased serum AST and ALT were significantly suppressed by treating with AI-E. In chronic liver diseases such as alcoholic hepatitis, hepatic fibrosis, hepatic cirrhosis, the serum ALB and TP levels were reduced due to protein synthesis disorder in hepatocytes[29]. As Table 1 showed, treatment with AI-E elevated the ALB and TP levels revealed the ability of enhancement of liver cells regeneration.

In conclusions, the hydro-alcoholic extracts of root of *Alocasia indica* Linn. exhibited protective effect against CCl_4-induced hepatotoxicity and possess antifibrotic activities. The result supports the use of the plant as described in folk medicine, that the root of plant can be used to treat liver and gastric disorders. Further studies are required to isolate the active constituents involved in the antifibrotic and hepatoprotective activity of the plant.

CONFLICT OF INTEREST:

The author declare that there is no conflict of interest

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 ISSN: 2250-1177 CODEN (USA): JDDTAO