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Hepatoprotective and Antioxidant Activity of Ethanol Extract of Whole Plant of *Aristolochia krisagathra* Sivarajan and Pradeep against CCl_4 - Induced Liver Injury in Rats

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Abstract

The present study was aimed to evaluate the hepatoprotective and antioxidant activity of ethanol extract of whole plant of *Aristolochia krisagathra* Hepatotoxicity was induced in Wistar albino rats by administration of carbon tetrachloride (CCl_4). The ethanol extract of whole plant of *A. krisagathra* at a dose of 250 and 500 mg/kg body weight was administrated at single dose per day to CCl_4 induced hepatotoxic rats for a period of 14 days. The effect of ethanol extract of *A. krisagathra* whole plant on SGOT, SGPT, ALP, serum bilirubin, GGT, lipid peroxidation and antioxidant enzymes like superoxidase (SOD), catalase (CAT) glutathione peroxidase (GPx), reduced glutathione (GSH) and glutathione reductase (GRD) were measured.Elevated serum marker enzymes of SGOT, SGPT, ALP and serum bilirubin were significantly reduced to near normal level in whole plant ethanol extract of *A. krisagathra* treated rats. Lipid peroxidation level was decreased significantly in whole plant ethanol extract of *A. krisagathra* 250 and 500 mg/kg doses; similarly it increased the antioxidant enzyme levels of GPx, GRD, SOD, CAT and GSH. Thus, the results suggest that *A. krisagathra* extract acts as a potent hepatoprotective agent against CCl_4 induced hepatotoxicity in rats.

Keywords: *Aristolochia krisagathra*, carbon tetrachloride, GGT, lipid perioxidation.

INTRODUCTION

The liver demonstrates a major role in metabolism of xenobiotics by regulating the synthesis, secretion and metabolism of xenobiotics. Various physiochemical functions of the body including oxidation, reduction, hydroxylation, hydrolysis, conjugation, sulfation, acetylation, etc. are well balanced by the liver alone. Injury to liver and damage to the hepatic parenchyma are always proved to be associated with distortion of different metabolic functions of liver [1, 2, 3]. Liver disease has become a global concern worldwide. Liver is often abused by environmental toxins, poor eating habits, alcohol and over the counter drug use, that damage and weaken the liver leading to important public health problems like hepatitis, cirrhosis and alcoholic liver diseases [4].

Recent research in free radical biology also suggested the pathophysiological role of free radicals and oxidative stress in liver damage and injury. Revealing the mechanism of actions of potent hepatotoxin such as CCl_4, paracetamol, etc indicated the role of oxidative stress and free radicals in the pathophysiology of hepatic injury [5].

The conventional drugs used on the treatment of liver diseases viz., corticosteroids, antiviral and immunosuppressant agents are sometimes inadequate and may lead to serious adverse effects. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedicinal practices and in traditional system of medicine in India [6]. Some of these plants are evaluated for their hepatoprotective actions against hepatotoxins.

The genus *Aristolochia* finds a prominent place in different Indian Systems of Medicine. The different ethnic communities in India have used different species of *Aristolochia* in the treatment of various human ailments [7]. Kanikkar tribals of Kalakad – Mundanthurai Tiger Reserve Sanctuary, Tamil Nadu, boiled equal quantity of fresh root and leaves of *Aristolochia krisagathra* in coconut oil for about 15 – 20 minutes over a flow flame. The oil is filtered after cooling and applied on the head once in a day to treat rheumatism. This therapy is used to reduce excessive heat of the body [8]. However, no data are available in the literature on the hepatoprotective activity of whole plant of *A. krisagathra.* This study was therefore undertaken to evaluate the effect of ethanol extract of the whole plant of *A.krisagathra* on hepatoprotective activity in CCl_4 induced liver injury in rats.

MATERIALS AND METHODS

Collection of Plant Sample

The whole plant of *Aristolochia krisagathra* Sivarajan and Pradeep was collected from Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant was identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of Plant Extract for Phytochemical Screening and Hepatoprotective Studies

The whole plant of *A.krisagathra* was shade dried at room temperature and the dried whole plant were powdered in a Wiley mill. Hundred grams of powdered A.*krisagathra*

whole plant was packed in a Soxhlet apparatus and extracted with ethanol. The extracts were subjected to qualitative test for the identification of various phytochemical constituents as per the standard procedures [9,10,11]. The ethanol extracts was concentrated in a rotary evaporator. The concentrated ethanol extracts was used for hepatoprotective studies.

Animals

Normal healthy male Wistar albino rats (180-240g) were used for the present investigation. Animals were housed under standard environmental conditions at room temperature (25±2°C) and light and dark (12:12h). Rats were fed with standard pellet diet (Goldmohur brand, MS Hindustan lever Ltd., Mumbai, India) and water *ad libitum.* The experimental protocol was approved by the Institutional Animal Ethical Committee of Kongunadu Arts and Science College (Reg. No: 659/02/a CPCESEA) Coimbatore, India.

Acute Toxicity Studies

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study [12]. The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5mg/kg body weight by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 upto 2000 mg/kg body weight.

Experimental Design

In this investigation, a total of 25 rats (20 CCl_4 hepatic toxicity induced rats and 5 normal rats) were taken and divided into five groups of 5 rats each. Group I: Rats received normal saline was served as a normal control. Group II: CCl_4 hepatic toxicity induced control: Rats received 2.5ml/kg body weight of CCl_4 for 14 days. Group III: Liver injured rats received ethanol extract of whole plant of *A.krisagathra* at the dose of 250mg/kg body weight for 14 days. Group IV: Liver injured rats received ethanol extract of whole plant of *A.krisagathra* at the dose of 500mg/kg body weight for 14 days. Group V: Liver injured rats received standard drug silymarin at the dose of 100mg/kg body weight for 14 days.

Biochemical Analysis

The animals were sacrificed at the end of experimental period of 14 days by decapitation. Blood was collected, sera separated by centrifugation at 3000 g for 10 minutes. Serum protein [13] and serum albumins were determined quantitatively by colorimetric method using bromocresol green. The total protein minus the albumin gives the globulin. Serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) was measured spectrophotometrically by using the method of Reitman and Frankel [14]. Serum alkaline phosphatase (ALP) was measured by the method of King and Armstrong [15]. Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw [16]. The unconjugated bilirubin concentrations were calculated as the difference between total and conjugated bilirubin concentrations. Gamma glutamyl transferase (GGT) was estimated by the method of Szasz [17]. Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in plasma by the method of Satoh [18]. Enzymatic antioxidants, superoxide dismutase (SOD) [19], Catalase (CAT) [20], glutathione reductase (GRD) [21], reduced glutathione (GSH) [22] and glutathione peroxidase (GPx) [23] were also assayed in erythrocytes.

Statistical Analysis

The data were expressed as the mean ± S.E.M. The difference among the means has been analyzed by one-way ANOVA. $p<0.01$ and $p<0.05$ were considered as statistical significance using SPSS Software.

RESULTS

Phytochemical screening

Phytochemical screening of *A.krisagathra* whole plant reveals the presence of alkaloids, coumarins, flavonoids, quinones, phenols, saponins, steroids, tannins, terpenoids, sugars, glycosides and xanthoproteins.

Acute Oral toxicity study

Ethanol extract of *A.krisagathra* whole plant did not cause any mortality upto 2000 mg/kg dose level. Hence $1/8^{th}$ and $1/4^{th}$ of the maximum dose (ie 250 and 500 mg/kg p.o) were selected for the present study.

Effect on body weight

The effect of ethanol extract of *A.krisagathra* whole plant on body weight of the normal control, CCl_4 intoxicated control and plant extract treated rat groups are shown in table 1. An increase in body weight was noticed in all groups except group II liver damaged control rats when compared with normal control. The body weight loss was higher in liver damaged control rats.

Effects on serum protein and serum enzymes

Table 2 shows the effect of ethanol extract of whole plant of *A.krisagathra* on serum total proteins, albumin, globulin, A/G ratio, serum transaminases and alkaline phosphatases in CCl_4 intoxicated rats. There was a significant ($p<0.01$) increase in serum GOT, GPT and ALP levels in CCL_4 intoxicated control group (Group II) when compared to the normal control group (Group I). The total protein level was significantly ($p<0.05$) decreased from 8.14 mg/dl in normal control to 6.74 mg/dl in CCl_4 intoxicated control. The whole plant ethanol extract of *A.krisagathra*, at a dose of 500 mg/kg, decreased the elevated serum marker enzymes significantly. Treatments with silymarin, the standard drug and plant extract reversed the altered total protein and albumin to almost near normal level.

Table 1: Effect of the whole plant ethanol extract of *A.krisagathra* on the body weight of the normal, liver damaged and drug treated rats

Treatment Groups	Dose	Initial Body weight (g)	Final Body weight (g)	Mean weight Gain (+)/ Loss (-) (g)	Percentage of Difference
Group I	0.9% Saline	206.31±4.94	218.33±3.14	+12.02	5.83
Group II	0.9% Saline	201.54±6.84	192.16±4.31**	-9.38	4.65
Group III	250 (mg/kg)	192.16±4.36	198.56±3.16*	+6.40	3.33
Group IV	500 (mg/kg)	204.56±9.16	214.38±8.16^{aa}	+9.82	4.80
Group V	100 (mg/kg)	192.34±5.16	208.27±8.36^{a}	+15.93	8.28

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test * $p < 0.05$; ** $p < 0.01$ Compared normal control vs liver injured rats ^{a} $p < 0.05$; ^{aa} $p < 0.01$ Compared liver injured rats vs drug treated.

Table 2: Effect of the whole plant ethanol extract of *A. krisagathra* on the serum protein, albumin, Globulin concentration and serum GOT, GPT and ALP enzyme activity in the normal, liver damaged and drug treated rats

Treatment Groups	Parameters							
	Dose (mg/kg BW)	T. Protein (mg/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
Group I	0.9% Saline	8.14±0.74	4.85±0.27	3.29±0.38	1.5:1	18.36±0.8	21.31±0.67	138.36±1.22
Group II	0.9% Saline	6.74±0.13*	3.91±0.23	2.83±0.12*	1.4:1	92.16±7.84**	104.16±2.19**	198.24±6.29**
Group III	250 (mg/kg)	7.92±0.34ns	4.36±0.12	3.56±0.11	1.2:1	38.16±0.93*^{a}	36.13±1.18^{a}	141.18±1.34^{aa}
Group IV	500 (mg/kg)	8.13±0.25^{a}	4.71±0.22^{a}	3.42±0.74	1.3:1	24.18±1.13^{aa}	21.98±1.36^{aa}	118.48±1.98^{a}
Group V	100 (mg/kg)	8.09±0.13	4.31±0.73	3.78±0.32	1.1:1	20.16±0.93^{aa}	23.16±0.83^{aa}	126.22±4.86^{aa}

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test * $p < 0.05$; ** $p < 0.01$ Compared normal control vs liver injured rats a $p < 0.05$; aa $p < 0.01$ Compared liver injured rats vs drug treated; ns – not significant

Table 3: Effect of the whole plant ethanol extract of *A. krisagathra* on the serum Total bilirubin, conjugated and unconjugated bilirubin and GGTP levels in the normal control, liver injured control and drug treated rats

Treatment Groups	Parameters				
	Dose (mg/kg BW)	Total Bilirubin (µmol/L)	Conjugated Bilirubin (µmol/L)	Unconjugated Bilirubin (µmol/L)	GGTP (U/L)
Group I	0.9% Saline	0.93±0.041	0.23±0.068	0.70±0.026	7.34±0.64
Group II	0.9% Saline	4.08±0.36**	1.97±0.05**	2.11±0.36**	19.28±1.13**
Group III	250 (mg/kg)	1.04±0.05^{a}	0.32±0.04^{aa}	0.72±0.02^{ns}	13.46±0.83*
Group IV	500 (mg/kg)	0.83±0.07^{aa}	0.26±0.03^{aa}	0.57±0.07^{aa}	8.13±0.21^{a}
Group V	100 (mg/kg)	0.88±0.04^{aa}	0.26±0.02^{aa}	0.62±0.07^{aa}	8.13±0.31^{aa}

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test * $p < 0.05$; ** $p < 0.01$ Compared normal control vs liver injured rats a $p < 0.05$; aa $p < 0.01$ Compared liver injured rats vs drug treated; ns – not significant

Table 4: Effect of the whole plant ethanol extract of *A. krisagathra* on the serum LPO, GPX, GRD, SOD, CAT and GSH activity in the normal control, liver injured control and drug treated rats

Treatment Groups	Parameters						
	Dose	LPO (n mole of MDA/mg protein)	GPx (u/mg Protein)	GRD (u/mg)	SOD (u/mg)	CAT (u/mg)	GSH (u/mg)
Group I	0.9% Saline	1.843±0.013	4.318±0.164	0.402±0.054	0.273±0.034	3.743±0.051	33.16±0.14
Group II	0.9% Saline	6.34±0.124**	2.841±0.013**	0.216±0.074**	0.114±0.083**	1.241±0.046**	14.84±0.36**
Group III	250 (mg/kg)	2.93±0.161^{a}	3.784±0.056^{ns}	0.324±0.015*^{a}	0.204±0.056*^{a}	2.316±0.052^{ns}	26.13±0.18^{a}
Group IV	500 (mg/kg)	1.78±0.026^{aa}	4.054±0.018^{a}	0.398±0.023^{aa}	0.264±0.076^{aa}	3.684±0.018^{a}	29.84±0.24^{a}
Group V	100 (mg/kg)	1.69±0.039^{aa}	4.268±0.028^{a}	0.426±0.16^{aa}	0.263±0.059^{aa}	3.691±0.084^{aa}	30.83±0.21^{aa}

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test * $p < 0.05$; ** $p < 0.01$ Compared normal control vs liver injured rats a $p < 0.05$; aa $p < 0.01$ Compared liver injured rats vs drug treated; ns – not significant.

Effects on balirubin and γ-glutamyl transferase

The effect of whole plant ethanol extract of *A.krisagathra* on total, conjugated, unconjugated bilirubin and γ-glutamyl transferase are shown in table 3. There was a significant (P<0.01) elevation of total, conjugated, unconjugated billirubin and γ-glutamyl transferase in the serum of CCl_4 intoxicated group (Group II) when compared to normal control (Group I). Whole plant ethanol extract of *A.krisagathra*, at a dose of 500mg/kg body weight, reduced the levels of total, conjugated and unconjugated bilirubin (Group IV).

Effects on lipid peroxidation and antioxidant enzymes

The hepatoprotective effect of whole plant ethanol extract of *A.krisagathra* on lipid peroxidation (LPO), glutathione peroxidase (GPx), glutathione reductase (GRD), superoxide dismutase (SOD) catalase (CAT) and reduced glutathione (GSH) activities are shown in table 4. When compared to the normal control rats (Group I), there was a significant (p<0.01) increase in the level of lipid peroxidation and a significant decrease in the levels of glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase in CCl_4 intoxicated control rats (Group II). Treatment with whole plant of ethanol extract of *A.krisagathra* at the dose of 500mg/kg body weight, decreased the elevated lipid peroxidation level significantly and restored the altered glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and reduced gluthathione levels towards normal in a dose dependent manner. The results were well comparable with that of silymarin, the standard drug treated rats.

DISCUSSION

Liver is an important organ actively involved in several metabolic functions mainly in detoxification of toxicants [24] CCl_4 has been one of the most intensively studied hepatotoxicants to date and provides a relevant model for other halogenated hydrocarbons that are used widely [25]. A single exposure to CCl_4 can lead to severe centrizonal necrosis and steatosis [26]. The changes associated with CCl_4 – induced liver damage are similar to that of acute viral hepatitis [27]. CCl_4 is transformed by cytochrome P_{450} system to produce trichloromethyl free radical. 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 [28]. Marked increase in release of hepatic enzymes into the blood stream is often associated with massive necrosis of the liver. CCl_4 is known to cause marked elevation of serum enzymes.

In general, liver damage is assessed by histopathological evaluation and levels of hepatic enzymes such as SGOT, SGPT, ALP and also bilirubin release in circulation [29, 30]. The estimation of gamma glutamyl transpeptidase (rGGT) is an important screening test with a high negative predictive value for hepatic disease [31].

Administration of hepatotoxin CCl_4 elevated the serum levels of SGOT, SGPT, ALT, rGGT and bilirubin as well as decreases total protein significantly [32,33]. The rise in serum enzymes level and bilirubin has been attributed to the damaged structural integrity of the liver, because they are cytoplasmic in location and released into circulation after cellular damages [34].

In the present investigation, the biochemical changes were observed after CCl_4 treatment. Thereby, it was found that the animal groups which are pretreated with ethanol extract of *A.krisagathra* whole plant at the dose of 250 and 500mg/kg (Group III and IV) as well as silymarin at the dose of 100 mg/kg (Group V), resulted in significantly decreases the hepatic enzymes such as SGOT, SGPT, ALP and rGGT and also bilirubin; as well as increased the total serum proteins when compared to rats treated only with CCl_4 (Group II). The recovery towards normalization of serum enzymes caused by ethanol extract of *A.krisagathra* whole plant was almost similar to that caused by silymarin in the present study. Silymarin is a known hepatoprotective compound, protecting the plasma membrane of hepatocytes [35]. Similar results have also been reported [36].

CCl_4 induced rats showed increased plasma levels of lipid peroxidation markers such as TBARS and lipid hydroperoxides. The increased peroxidation can result in changes in cellular metabolism of the hepatic and extra hepatic tissues. Increased accumulation of lipid peroxidation products in cells can result in cellular dehydration, whole cell deformity and cell death [37].

Administration of ethanol extract of *A.krisagathra* whole plant showed significantly lowered levels of these lipid peroxidative markers compared to CCl_4 induced rats. Decreased lipid peroxidation with plant extract administration suggests a decreased impact of reactive oxygen species (ROS) on lipid membranes and therefore increased protection against CCl_4 induced liver injury.

The GSH (reduced glutathione), CAT (Catalase) and superoxide dismutase (SOD) stored may be decreased because of the toxic free radicals of CCl_4 [38]. Decrease in SOD production can be attributed to an enhanced superoxide generation and utilization of this enzyme during reactive metabolites detoxification. High amount of superoxide inhibit catalase, which is another important antioxidant enzymes [39]. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [40]. Therefore, reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. GPx works in tandem with CAT to scavenge excess H_2O_2 as well as other free radicals in response to oxidative stress. The equilibrium between these enzymes is important for the effective removal of oxidative stress in intracellular organelles. CCl_4 – induced rats showed the decreased activities of these enzymes; result in the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of cell membrane integrity and membrane function. [41]. Treatment with ethanol extract of *A.krisagathra* whole plant significantly increased SOD, catalase , GPx and GSH activity and thus reactive free radical induced oxidative damage to liver.

CONCLUSION

Investigation on various plant phytochemicals revealed that the presence of flavonoids, phenolic compounds has the potential free radical scavenging activity [42]. In the present study, the phytochemicals like phenols, flavonoids and tannins may be the reason for the hepatoprotective activity through which the antioxidant enzymes may be raised and oxidative stress may be plugged. However, the hepatoprotective, curative and antioxidant qualities of *A.krisagathra* need to be confirmed by characterizing the active ingredient(s) of this plant as well as its mechanism(s) of action.

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