

Research Article

Hepatoprotective Effect of *Adenema hyssopifolium* G. Don (Gentianaceae) in Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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Abstract

Purpose: The effects of oral administration of ethyl acetate, ethanol and aqueous extracts of *Adenema hyssopifolium* G. Don (Gentianaceae) on carbon tetrachloride-induced liver disorders were investigated.

Methods: Rats were individually treated daily with 300 and 600 mg/kg dose of either ethyl acetate, ethanol or aqueous extracts of *A. hyssopifolium*, respectively, following induction of liver damage with the hepatotoxin, carbon tetrachloride. The hepatoprotective activity of the extracts was assessed by estimating the levels of serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP) and total bilirubin (TBL) in the rats. Silymarin was used as the reference hepatoprotective agent. Acute toxicity test on the extracts in male mice was also carried out.

Results: At doses of 300 and 600 mg/kg p.o., the ethyl acetate and ethanol extracts showed significant ($p < 0.001$ and $p < 0.01$) dose-dependent hepatoprotective activity, showing decreases in serum levels of ASAT, ALAT, ALP and TBL. The aqueous extract, however, did not exert any significant effect on hepatoprotective activity. All three extracts, up to a dose of 3000 mg/kg p.o. each, did not cause mortality in the acute toxicity test.

Conclusion: The ethyl acetate and ethanol extracts showed significant hepatoprotective activity when compared to untreated (normal) control group while the aqueous extract did not. The active extracts could find future use in countering hepatic damage.

Keywords: Hepatoprotection; Iridoid glycosides; Hepatotoxicity.

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INTRODUCTION

Adenema hyssopifolium G. Don (Gentiana-ceae) is a slender perennial herb of great medicinal value in India [1]. It is widely used as a substitute for *Swertia chirayita* Ham. Ex Wall and is also used in folk medicine for the treatment of diabetes mellitus in Western and Southern India [2]. Ethnomedical studies in north Gujarat (India) also reveal the use of the hot aqueous extract of the entire plant of *Adenema hyssopifolium* by tribal inhabitants for the treatment of diabetes, fever, stomach ache, dyspepsia and for malaria in the interior part of Gujarat [3]. The extracts of the plant are used in Indian Medicine to treat cardiac dropsy, rheumatism and certain mental disorders.

Seven flavonoids have been isolated from the ethanol extract of the entire plant, including apigenin, genkwanin, isovitexin, swertisin and 5-O- β -D-glucoside [4]. The iridoid glycoside, swertiamarin, was reported to produce central nervous system depressant [5]. A monoterpene alkaloid was reported in this species [6]. Gentiocrucine was shown to be a mixture of three compounds (I, II and III) and erythrocentaurin [7]. Betuline, a triterpene saponin, was isolated by earlier workers [8].

The methanol extract of the entire plant has been reported to show protective activity against Dalton's ascitic lymphoma in Swiss albino mice [9]. To the best of our knowledge, no work on the hepatoprotective activity of the ethyl acetate, ethanol and aqueous extracts of *Adenema hyssopifolium* in carbon tetrachloride-induced liver damage in animals has been reported. Therefore, the present study was undertaken to evaluate the hepatoprotective ability of *Adenema hyssopifolium* in rats.

EXPERIMENTAL

Collection, identification and extraction of the plant material

The entire plant of *Adenema hyssopifolium* G. Don was collected in September 2004 from in and around Madurai, Tamilnadu, India. The plant was identified and authenticated by Dr D Stephen, a taxonomist at American College, Madurai, India. A voucher specimen ((R.A. no.11/04) was preserved at the herbarium of the Department of Pharmacognosy, Arulmigu Kalasalingam College of Pharmacy, Coimbatore, India for future reference. The entire plant, including the roots, were washed well with water, air-dried for 10 days under controlled temperature ($37\pm 1^\circ\text{C}$), pulverized, passed through a $420\ \mu$ sieve and stored in an air-tight container until further use.

A quantity (1.3 kg) was then subjected to maceration with 3500 ml of either ethyl acetate or 90% ethanol at room temperature. The extracts were collected and evaporated to dryness under reduced pressure in a rotary evaporator (Eyela, Japan) at $40 - 45^\circ\text{C}$ to give yields of ethyl acetate and ethanol extracts of 10.36 and 14.72% w/w, respectively. Also, the coarse material (600 g) of the entire plant was soaked in sufficient warm water for 30 min in a clay pot. Thereafter, the contents were boiled for 45 minutes while constantly stirring the contents and then strained to obtain a decoction [10].

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures, as described by Kokate [11] and Harborne [12].

Experimental animals

Swiss male mice (20 - 25 g) and albino adult Wistar male rats (150 - 200 g) were obtained from the animal house of Pharmacology Department, Arulmigu Kalasalingam College

of Pharmacy Tamilnadu, India. The study protocol was approved by the institutional animal ethics committee, Committee for the Purpose of Control and Supervision on Animals (CPCSEA), New Delhi, India, as per approval no. 509/01/C/CPCSEA of 10th July 2002. The animals were housed in plastic cages (47×34×18 cm) in an air-conditioned environment with 10/cage and 6/cage for mice and rats, respectively. The floor of the cages was lined with saw dust which was renewed every 48 h, and the animals were fed standard pellet diet from Kamadenu Enterprises, Bangalore, India. The animals also had access to water *ad libitum*.

Acute toxicity test

Male mice were divided into twenty two groups of ten animals each. The control group received 0.5 ml of 0.5% w/v sodium carboxymethylcellulose orally. The other groups received either 100, 200, 400, 800, 1000, 2000 or 3000 mg/kg of either ethyl acetate or ethanol extract in 0.5% sodium carboxymethylcellulose orally. Immediately after dosing, the animals were observed continuously for the first 4 h for behavior, occasionally up to the 6th h, and then kept for up to 14 days post-treatment in order to observe for any toxic symptoms and mortality.

Hepatoprotective test

The animals were divided into nine groups of six animals each and labeled (Groups F₁ to F₉). Group I (F₁) served as normal control and received sterile olive oil (vehicle) 1ml/kg (*p.o.*). Group II (F₂) animals constituted the hepatotoxic group, and received CCl₄ suspended in sterile olive oil (1:1 v/v, 2 ml/kg, *i.p.*) every 72 h for 10 days. Group III (F₃) received standard drug silymarin 100 mg/kg (*p.o.*) for 10 days and CCl₄ suspended in sterile olive oil (1:1 v/v, 2 ml/kg, *ip*). Groups IV - IX (F₄ - F₉) received ethyl acetate, ethanol or aqueous extract (300 or 600 mg/kg/day) suspended in 0.5 % sodium carboxymethylcellulose for 10 days and CCl₄

suspended in sterile olive oil (1:1 v/v, 2 ml/kg, *ip*).

At the end of the experimental period, the rats were fasted overnight and sacrificed by ether anesthesia. Blood and liver samples were collected for biochemical and histological studies.

Assessment of liver functions

The biochemical parameters, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), were analyzed using ASAT and ALAT kits (Span Diagnostics Private Ltd, Surat, Ltd), respectively [13]. Alkaline phosphatase (ALP) and total bilirubin (TBL) were analyzed using a commercial enzyme kit (Crest Biosystems, Goa, India) [14]. The mean values (\pm SEM) were computed for each parameter.

Histopathological studies

Paraffin sections (7 μ m thick) of buffered formalin-fixed liver samples were stained with hematoxylin-eosin (which stains the nuclei blue and the cytoplasm pink) to study the liver histological structure of the control and treated rats.

Statistical analysis

For determination of significant inter-group differences, each parameter was analyzed separately and one way analysis of variance was carried out. Dunnet's test was used for individual comparisons. The $p < 0.05$ or less considered statistically significant.

RESULTS

Preliminary phytochemical screening revealed the presence of flavonoids, aminoacids, iridoid glycosides, steroids and phenolic compounds in ethyl acetate extract. Sugars, iridoid glycosides, flavonoids, phenolic compounds, amino acids and tannins were present in ethanol extract. Aqueous extract showed the presence of

sugars, trace amounts of phenolic compounds and tannins (Table 1). All three extracts of *A. hyssopifolium*, at a dose of 3000 mg/kg *p.o.*, did not produce any mortality. Consequently, 1/10th and 1/5th of this dose (i.e., 300 mg/kg and 600 mg/kg, *p.o.*, respectively) were used for the pharmacological screening [15]. Among the various extracts tested for hepatoprotective activity, ethyl acetate extract (F₄ and F₅) and ethanol extract (F₆ - F₇) exhibited a marked protective effect while the decoction (aqueous extract) showed less effects. The effect of the ethyl acetate extract (600 mg/kg) was comparable to that of the standard drug, silymarin. As shown in Table 2, treatment with the ethyl acetate extract produced significant decreases in serum levels of ASAT, ALAT, ALP and TBL (P < 0.001), compared with the group that was treated only with carbon tetrachloride.

Table 1: Preliminary phytochemical analysis of entire plant extract of *Adenema hyssopifolium*

Constituents	Ethyl acetate extract	Ethanol extract	Aqueous extract
Alkaloids	0	0	0
Amino acids	+	+	0
Anthraquinone glycosides	0	0	0
Coumarins	0	0	0
Flavonoids	++	++	±
Oils	0	0	0
Phenolic groups	+	++	±
Iridoid glycosides	++	++	+
Saponins	0	±	±
Steroids	±	0	0
Sugars	0	++	++
Tannins	0	+	±
Triterpens	0	0	0
Cardiac glycosides	0	0	0

+ = Present, ++ = abundant, ± = traces, 0 = absent

DISCUSSION

Administration of CCl₄ caused liver damage due to the free radical CCl₃· formed from

CCl₄ by the activation of NADPH Cyt-P450 system of liver endoplasmic reticulum.[16]. This leads to functional and morphologic changes in the cell membrane, resulting in leakage of hepatic enzymes. Compared to other toxins, CCl₄ is known to cause hepatic damage with a marked increase in blood serum transaminases and phosphatase activity [17]. CCl₄, an extensively studied liver toxicant, and its metabolites, such as trichloromethyl peroxy radical (CCl₃*O₂⁻), are known to be involved in the pathogenesis of liver damage. The oxidation of fatty acids by free trichloromethyl radical (CCl₃*₃) liberates lipid peroxides. [18].

The current study also confirmed these effects of CCl₄ toxicity, as indicated by marked increases in serum hepatic enzymes. Indeed, the levels of all the marker enzymes increased significantly in Group II (F₂) rats after CCl₄ administration (p < 0.001) but ethyl acetate extract treatment (600 mg/kg) caused significant decreases in the activities of all these enzymes. Thus, our findings support the reported therapeutic use of this plant in traditional medicine for liver ailments and jaundice.

Various flavonoids and iridoid glycosides are reported to be present in the whole plant of *Adenema hyssopifolium* G. Don and they probably play a contributory role in hepatoprotective action and the plant's antioxidant potential [19]. Another important constituent, swertiamarin, which is an iridoid glycoside, may also play an important role in this respect. Furthermore, it has been suggested that iridoid glycoside in certain plants is a critical compound in hepatoprotective activity [20]. The protective activity of aucubin, an iridoid glycoside, against carbon tetrachloride-induced liver damage in mice has been reported [21]. The iridoid glycoside, kutkin, and its glycoside mixtures have also been found to exert hepatoprotective activity. Aucubin derived from traditional medicine was said to possess liver protective activity [22,23].

Table 2: Effect of the extracts of *Adenema hyssopifolium* on hepatotoxicity induced by carbon tetrachloride

Group	Group/treatment	ALAT (IU/L)	ASAT (IU/L)	ALP (KA units)	Total bilirubin (mg dL ⁻¹)
F1	Na CMC (Control)	61.8 ± 1.2	44.0 ± 1.0	37.7 ± 0.6	0.8 ± 0.0
F2	CCl ₄ - treated	93.0 ± 1.7	101.0 ± 0.8	83.3 ± 1.1	9.3 ± 0.2
F3	Silymarin 100 mg/kg	62.5 ± 2.8 ^a	51.5 ± 3.2 ^a	42.0 ± 2.8 ^a	1.12 ± 0.0 ^a
F4	Ethyl acetate extract 300 mg/kg	78.8 ± 1.8 ^a	90.6 ± 2.1 ^a	59.3 ± 0.8 ^a	5.78 ± 0.1 ^b
F5	Ethyl acetate extract 600 mg/kg	64.0 ± 3.7 ^a	57.1 ± 2.5 ^a	43.8 ± 1.7 ^a	3.71 ± 0.0 ^a
F6	Ethanol extract 300 mg/kg	84.5 ± 1.5 ^b	73.1 ± 1.7 ^b	69.3 ± 1.0 ^b	6.2 ± 0.3 ^b
F7	Ethanol extract 600 mg/kg	80.1 ± 3.2 ^a	67.6 ± 1.2 ^a	62.3 ± 1.3 ^a	5.83 ± 0.1 ^a
F8	Aqueous extract 300 mg/kg	88.2 ± 2.8 ^c	88.6 ± 1.1 ^c	71.6 ± 1.1 ^c	7.06 ± 0.1 ^c
F9	Aqueous extract 600 mg/kg	84.1 ± 1.4 ^b	83.5 ± 1.0 ^b	73.0 ± 2.7 ^b	6.46 ± 0.1 ^b

Values are mean ± SE; n= 6 in each group ; ^a p < 0.001 compared to the corresponding value for carbon tetrachloride group; ^b p < 0.01 compared to the corresponding value for carbon tetrachloride group; ^c p < 0.1, not significant.

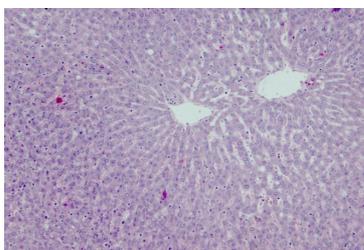


Fig 1(a): Normal healthy central vein and normal liver, normal parenchymal cells

Sections showing structure of liver with sheets of hepatocytes arranged around central vein. Sinusoids in between the rows of hepatocytes appear normal.

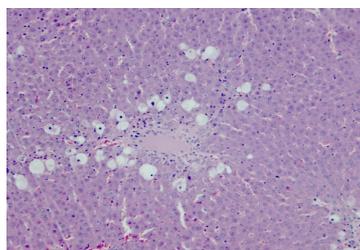


Fig 1(c): Silymarin-treated liver

Section showing liver parenchyma with sheets of hepatocytes, around central vein; portal tract showing mild inflammatory infiltrate; occasional hepatocytes showing fatty change

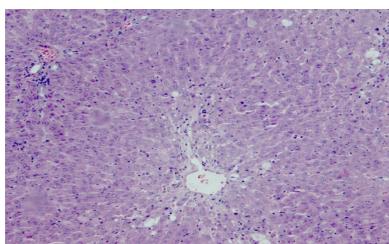


Fig 1(b): CCl₄-induced liver damage

Section showing liver parenchyma with central vein and many hepatocytes showing extensive fatty change. Portal tract showing inflammatory infiltrate.

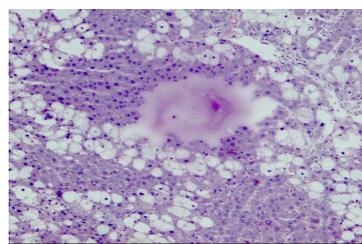


Fig 1(d): Liver-treated with ethyl acetate (600 mg/kg) of *Adenema hyssopifolium*

Section showing portal tract with inflammatory infiltrate and fatty change

CONCLUSION

The results of this investigation indicate that the ethanol and ethyl acetate extracts of *Adenema hyssopifolium* contain pharmacologically active substances with hepatoprotective properties. These attributes provide the rationale for the use of *Adenema hyssopifolium* in liver disorders by traditional healers in India. Further research is needed to fractionate the extracts and to isolate the molecule(s) responsible for the hepatoprotective activity.

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