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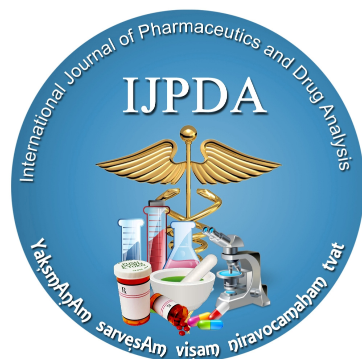

Hepatoprotective and antioxidant activities of *Amaranthus gangeticus* leaves against carbontetrachloride induced hepatic damage in albino rats

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Abstract:
Amaranthus gangeticus is a medicinal plant. Our aim is to investigate its organ protection property. Ethanolic extract of *Amaranthus gangeticus* leaves was taken and the parameters studied were serum glutamate pyruvate transaminase, serum glutamate oxaloacetate transaminase, total bilirubin, direct bilirubin, total cholesterol, high density lipoproteins and alkaline phosphatase activities. Bio-chemical studies of blood samples of carbontetrachloride treated animals showed significant increase in the levels of serum markers and decrease in high density lipoproteins reflecting the liver injury caused by carbontetrachloride. Whereas, the animals treated with ethanolic extract of *Amaranthus gangeticus* showed significant dose dependent decrease in the elevated levels of serum markers and increase in high density lipoproteins indicating the protection of hepatic cells. Results were analyzed by one-way analysis of variance by Dunnet's 't' test. Therefore ethanolic extract afford significant protection against carbontetrachloride induced hepatocellular injury and remarkable rejuvenation of these tissues found in histopathological studies which may be due to polyphenols and its antioxidant activities.

Keywords: *Amaranthus gangeticus* leaves, carbontetrachloride, serum markers and polyphenols.

Introduction

Liver is an organ of importance in the body. It is the key organ of metabolism and excretion. During its normal physiological functioning it metabolizes various endogenous and exogenously administered chemicals so as to terminate or inactivate these agents. Hence due to this function, it protects the whole body from the various environmental and chemical challenges. In addition to this liver has got an inbuilt mechanism to protect itself and to regenerate on several occasions many of these hepatotoxic challenges overpower inbuilt protective mechanism and cause hepatotoxicity resulting in the hepatic necrosis and hepatitis.

Amaranthus gangeticus is one such edible plant used as vegetable which is being used by native practitioner as hepatoprotective in treating various types of jaundice. The leaves of this plant contain polyphenolic compounds like tannins and flavonoids. These polyphenolic compounds have antioxidant property and anti-oxidants have known to possess hepatoprotective activity. Keeping the native knowledge and the above mentioned literature information[1], this plant was selected for present study to screen the leaves of this edible plant for the presence of phytoconstituents, antioxidant and hepatoprotective activities.

This study was carried out by using 70% ethanolic extract of *Amaranthus gangeticus* (AG) as hepatoprotectant and CCl_4 as hepatotoxicant.

MATERIALS AND METHODS

Collection and identification of plant: The plant was collected from Kusnoor village (Gulbarga district), Karnataka in the month of March and was authenticated by Dr. Srinath Rao, chairman, P.G. Department of Studies and Research in Botany, Gulbarga University, Gulbarga, Karnataka. The plant was thoroughly cleaned to remove adherent soil and other impurities, the leaves were shade dried and made into a coarse powder by rubbing in the palms.

Extraction

250gms of shade dried leaf powder of AG were extracted in Soxhlet's apparatus using petroleum ether for defatting and then it was extracted with 70% ethanol. The solvent evaporated on a water bath at a low temperature (50°C) and finally the residue was obtained.

Materials used

CCl_4 (central drug house, New Delhi), Silymarin (SD fine chemicals, Mumbai), Ready to use diagnostic kits (Aspen Labs Pvt. Ltd., Delhi-India), 70% ethanolic extract of AG, standard quercetin and standard naringin (sigma, Germany). All chemicals and reagents used were of analytical grade.

Animals used

Wistar albino rats of either sex weighing between 150-200 gms were housed in polypropylene cages and were maintained at 27° ± 2°C with 12:12 hr, light/dark cycle. They were fed with commercial diet (VRK Nutritional Laboratory, Sangli) and water *ab libitum* during the experiment. The study was permitted by Institutional Animal Ethical Committee (Reg. No. 342).

I.Evaluation of hepatoprotective activity in CCl_4-induced hepatotoxicity:

The method of Suja SR, et. al., was followed[2]. In the dose response experiment, albino rats were randomly assigned into 5 groups of 6 animals in each. Group-I animals (-ve control) were administered with 1 ml of distill water p.o., for 5 days. Group-II animals (+ve control) were administered with 1 ml of distill water p.o., for 5 days. Group-III animals were administered with silymarin 100mg/kg p.o., for 5 days. Group-IV animals were administered with 70% ethanolic extract 200mg/kg p.o., for 5 days. Group-V animals were administered with 70% ethanolic extract 400mg/kg p.o., for 5 days.

Then group-I received liquid paraffin (1ml/kg) S.C. on 2nd and 3rd day, Group-II, III, IV and V received CCl_4 : liquid paraffin (1:1) at a dose of 2ml/kg S.C., on 2nd and 3rd day, after 30 min of vehicle, 100mg/kg silymarin, 200mg/kg 70% ethanolic extract and 400mg/kg 70% ethanolic extract of *Amaranthus gangeticus* Linn leaves were administered. On 6th day, the blood samples were collected from the animals of each group through carotoid artery puncture under mild ether anaesthesia for evaluating the serum biochemical parameters by using Aspen diagnostic kits and liver was dissected out, the blood was blotted off, washed with saline and stored in 10% formalin and proceeded for histopathology to evaluate the details of hepatic architecture in each group microscopically. The results are shown in table-1.

II. *In-vitro* antioxidant activities

The following *in-vitro* models were carried out to evaluate antioxidant activity.

a. Reducing power

b. DPPH (1,1-Diphenyl-2-Picryl-hydrazyl) free radical scavenging activity.

a. Reducing power

The reducing power of 70% ethanolic extract of AG leaves were determined according to the method of Oyaizu (Oyaizu, 1986)[3].

Procedure

Different doses of 70% ethanolic extract of AG leaves were mixed in 1 ml of distilled water so as to get 10 µg, 20µg, 25µg, 50µg and 100µg concentrations. This was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%) and the absorbance (OD) was measured at 700 nm in double beam spectrophotometer. Increased absorbance of the reaction mixture indicates increase in reducing power. The % reducing power was calculated by using the formula.

% increase in absorbance =

$$\frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

The results are complied in table -2.

b.DPPH (1,1-Diphenyl-2-Picryl-hydrazyl) free radical scavenging activity[4].

DPPH radical scavenging activity of AG (70% ethanolic extract) was measured by the method described by Sabir et. al. Different concentrations of this extract (10, 20, 25, 50, 100 µg/ml) were added to a 0.5 ml solution of DPPH (0.25 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm in a double beam spectrophotometer using DPPH solution as blank.

Percentage inhibition was calculated from the control.

Vitamin C (25 µg) was used as a standard compound in the DPPH assay. The results are complied in table-2.

III. Total flavonoid estimation[5]

1. Extraction of flavonoids from *Amaranthus gangeticus* leaves[6]Method

In this process, 10 gms of shade dried, leaf powder of *Amaranthus gangeticus* is placed with the whole of the menstrum (250 ml) in a closed vessel for 2 days. During this period shaking is done occasionally. After 2 days, the liquid is strained and marc is pressed. The expressed liquid is mixed with strained liquid. It is then filtered to make a clear liquid. The final volume is not adjusted. It is evaporated on a water-bath at low temperature not exceeding (50°C) and preserved.

Plant extracts were prepared using three different extracting solvents :
AG_{CW} : *Amaranthus gangeticus* (AG) shade dried leaf powder macerated for 48 hrs with cold water (distilled water stored at room temperature). AG_{HW} :AG shade dried leaf powder macerated for 48 hrs with hot water (50°C). AG_{m15} :AG shade dried leaf powder macerated with methanol for 15 days.

2. Aluminium chloride colorimetric method

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino[7]. Quercetin was used to make the calibration curve. 10 milligrams of quercetin was dissolved in 80% ethanol and then diluted to 0.125, 0.25, 0.5, 0.75, 1.00, 1.25 and 1.5 %mg/100 ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm by using UV spectrophotometer. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of various extracts of AG (1000 µg/ml) were reacted with aluminium chloride for determination of flavonoid content as described above.

3. 2,4-Dinitrophenylhydrazine Colorimetric Method

The current method was modified from the procedure described by Nagy and Grancai[8]. Naringin was used as the reference standard. 20 milligrams of naringin was dissolved in methanol and then diluted to 125, 250, 500, 1000, 2000 ppm. One milliliter of each of the diluted standard solutions was separately reacted with 2 ml of 1% 2,4-dinitrophenylhydrazine reagent and 2 ml of methanol at 50°C for 50 min. After cooling to room temperature, the reaction mixture was mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged at 1,000 rpm for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm. The various extracts of AG (1000 µg/ml) were similarly reacted with 2,4-dinitrophenylhydrazine for determination of flavonoid content as described above.
The total flavonoid contents are shown in table-3.

Statistical analysis

The data presented in table-1 (n=6), table 2 and 3 (n=3) were expressed as mean ± standard error of mean ((SEM) (n = 6). Significant difference among the mean were calculated at the level of $p < 0.001$ and analyzed by one-way analysis of variance by Dunnet's 't' test. A value of $p < 0.05$ was defined as significant.

RESULTS

There is a marked increase in serum biomarkers which were observed in CCl_4 treated group. But, these levels were reversed to near normal levels with the treatment of 200mg/kg and 400mg/kg of ethanolic extract of AG. Whereas, the standard silymarin has restored the serum biomarker levels significantly which is depicted in table-1.

It is observed that 70% of ethanolic extract of AG have demonstrated dose dependent increase in the reducing property. Whereas 25 µg of sodium metabisulphate (standard) has 240% reducing property. But this extract at 50 µg has more reducing property than compared to standard. However 100 µg of this extract has shown maximum reducing power i.e., 580%. The results are shown in table-2.

DPPH is an unstable nitrogen centered free radical that accepts an electron or hydrogen radical from suitable antioxidants and gets reduced to stable diamagnetic molecule along with stiochiometric loss of colour. This phenomenon has been widely used by researchers as a quick and reliable parameter to assess the *in-vitro* antioxidant activity of crude extracts. From the DPPH radical scavenging activity of this extract is shown in table-2. It is clear that this extract has shown a dose dependent activity and this extract claims that it is more efficient in scavenging DPPH radical.

Results showed that, among the three different extracts of AG, AG_{m15} contained the lowest level of total flavonoids, while AG_{HW} contained the highest level of total flavonoids. The observations are recorded in table-3.

Table-1
Effect of 70 % ethanolic extract of *Amaranthus Gangeticus* leaves on hepatic enzymes in CCl_4 induced hepatotoxicity

Treatment	Biochemical parameters Mean ± SEM						
	SGPT IU/L	SGOT IU/L	Total Bilirubin mg/dl	Direct Bilirubin mg/dl	Total Cholesterol mg/dl	HDL mg/dl	ALP IU/L
Negative control (1ml dist. Water p.o. + 1ml/ kg liquid paraffin s.c.)	52.26 ± 0.50	67.36 ± 0.55	0.96 ± 0.003	0.24 ±0.006	109.20 ±0.15	7.62 ±0.02	124.2 ±1.17
CCl_4 treated (positive control) (2ml/kg s.c. CCl4	312.7 ± 1.94	233.20 ± 0.26	4.52 ± 0.05	1.42± 0.06	164.6 ±0.59	4.74 ±0.006	215.4 ±1.50
CCl_4 + Silymarin (2ml/kg s.c. + 100mg/kg, p.o.)	69.67 ± 1.02***	97.06 ± 0.67***	1.35 ± 0.03***	0.38± 0.005***	113.6± 0.74***	6.62± 0.005***	126.3± 1.13***
CCl_4 + 70% ethanolic extract (2ml/kg s.c. + 200 mg/kg, p.o)	156.8 ± 0.83***	165.7 ± 0.69***	2.92± 0.02***	0.78± 0.005***	138.6± 0.37***	5.35± 0.005***	150.0± 1.03***
CCl_4 + 70% ethanolic extract (2ml/kg s.c. + 400 mg/kg, p.o)	92.17 ± 2.77***	102.50 ± 0.63***	1.42± 0.04***	0.53± 0.005***	114.8± 0.68***	6.44± 0.005***	131.1± 0.88***

Values are the mean ± S.E.M. of six rats/treatment. *** $P<0.001$ Significance compared to CCl_4 treatment.

Table-2
Reducing power and DPPH radical scavenging activity of 70 % ethanolic extract of *Amaranthus Gangeticus* leaves (AGEE)

Reducing power activity			DPPH radical scavenging activity	
Groups	**Absorbance Mean ± SEM**	**% Increase**	**Absorbance Mean ± SEM**	**% Inhibition**
Control	0.056±0.003	---	0.540±0.010	---
Control + Std. 25 µg	0.176±0.003***	240	0.390±0.005***	27.777
Control + AGEE 10 µg	0.100±0.005***	100	0.220±0.011***	59.259
Control + AGEE 20 µg	0.113±0.008**	120	0.196±0.003***	63.703
Control + AGEE 25 µg	0.150±0.005*	200	0.170±0.005***	68.518
Control + AGEE 50 µg	0.180±0.015	260	0.116±0.003***	78.518
Control+AGEE 100 µg	0.343±0.021**	580	0.103±0.003***	80.925

Values are the mean ± S.E.M., n=3
Significance ***P<0.001, **P<0.01, *P<0.05, compared to standard.
Std: Sodium metabisulphate for Reducing power and Ascorbic acid (Vitamin C) for DPPH radical scavenging activites.

Table-3
Total flavonoid contents of *Amaranthus gangeticus* leaves determined by aluminium chloride and 2,4-dinitrophenylhydrazine colorimetric methods

Sl. No.	Name of the sample	Total flavonoid contents (%)[a]		Total (µg/ml)
		$AlCl_3$[b] (µg)	2-4-D[c] (µg)	
1	AG_{CW}	40.16 ± 0.36	16.52 ± 0.55	56.68 ± 0.21
2.	AG_{HW}	44.58 ± 0.35	29.85 ± 2.78	74.44 ± 2.72
3.	AG_{M15}	32.20 ± 0.24	16.24 ± 0.28	48.45 ± 0.10

a : Results are presented as mean ± SEM (n = 3); b: Levels calculated as quercetin equivalents; c : Levels calculated as naringin equivalents

DISCUSSIONS

The model selected to asses organ protection, the ethanolic extract of AG was first tested against CCl_4 induced hepatotoxicity in rats. The CCl_4 treated group exhibited extensive fatty changes, congestion of sinsusoids, necrosis etc. upon histopathological observations. But treatment with AG, the serum levels of biochemical markers of hepatocellular damage like SGPT, SGOT, bilirubin (total and direct), ALP, cholesterol were increased as a mark of fatty change, congestion, inflammation etc.

The animals treated with ethanolic extract of AG for 5 days showed remarkable rejuvenation of hepatocellular architecture, the AG reversed the elevated serum markers of liver damage proportionate to the doses employed. Protection was offered by silymarin, (100mg/kg), 200mg/kg and 400mg/kg of AG respectively (compared to positive control w.r.t. biochemical parameters).

The hepatoprotection offered by AG may be attributed due to the presence of antioxidant phyto-constituents like flavonoids phytosterols and other polyphenolic constituents, by the virtue of which the extract nullified the powerful hepatotoxic radicals generated by CCl_4 (i.e, CCl_3 ad CCl_3OO) before they could initiate fatal consequences. These findings add strength to our claim.

The flavonols formed complexes only with aluminium chloride, but flavanones strongly reacted only with 2,4-dinitrophenylhydrazine. The content determined by the two methods were added upto evaluate the total content of flavonoids.

CONCLUSIONS

Ethanolic extract of AG has a powerful organ protection property and significantly it has a good *in-vitro* antioxidant properties which are attributed due to presence of antioxidant phyto-constituents. Therefore the above findings revels that the use of *Amaranthus gangeticus* leaves in our food protects our liver.

SCOPE FOR FUTURE STUDY

As it is a medicinal plant, hence isolation of its phytoconstituents are needed to screen various organ protective potentials.

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