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INVESTIGATION OF HEPATOPROTECTIVE ACTIVITY IN LEAVES OF DESMODIUM GANGETICUM AGAINST DICLOFENAC INDUCED-LIVER DAMAGE IN RATS

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ABSTRACT

The aim of the present study was carried out with the objective of phytochemical screening and to evaluate the hepatoprotective activity of aqueous extract of Desmodium gangeticum using drug induced hepatotoxicity models like Diclofenac induced methods. The rats were divided into five groups with six rats in each. Group I (Control) served as normal and received the vehicle alone (Sterile distilled water, 10 ml/kg, p.o.) for 5 days. Group II (Toxin control) animals on the 3rd and 4th day. Group III and IV were treated with DGL at a dose level of 400 mg/kg and 600 mg/kg body weight p.o. per day respectively for 5 days and on the 3rd and 4th day with hepatotoxic drugs was given 1 h after the treatment of the extract. Group V (Standard) was treated with standard drug silymarin (100 mg/kg p.o.) for 5 days and on the 3rd and 4th day hepatotoxic drugs was given 1h after the treatment of the drug. In hepatoprotective studies, the induced Diclofenac toxicity elevated levels of serum marker enzymes ALT, AST, ALP and the level of BUN along with the decrease in total protein and albumin levels. It also increased the relative liver weight and decreased the level of liver total protein and GSH. The activity of catalase and GPx significantly decreased in Diclofenac intoxicated animals. The pre-treatment of methanol extract of Desmodium gangeticum leaves at dose levels of 400 and 600 mg/kg had restored the ALT, AST, ALP and BUN levels towards normalization and the effects were comparable with standard drug (Silymarin 100 mg/kg). The data obtained from animal experiments are expressed as mean Â± SEM (standard error of mean). For statistical analysis data were subjected to analysis of variance (ANOVA) followed by Student t-test. Values are considered statistically significant at $p < 0.01$ for ANOVA and $P < 0.05$ for t-test

Keywords: Desmodium gangeticum, hepatoprotective activity, serum, total protein, albumin, Blood Urea Nitrogen, Alkaline Phosphate.

1. INTRODUCTION

Liver is the largest organ in the body which serves as a gland also. It plays an important role in the maintenance of internal environment through its multiple and diverse functions. Any damage to the liver or impairment of its functions leads to injurious effects. Liver diseases (like jaundice) are the common ailments affecting mankind, though no remedy is available in allopathic at present. In the recent past years many medicinal plants are screened for their hepatoprotective activity and quite a few of them are already successful in entering the market. Desmodium gangeticum DC (Leguminosae, SubfamilyPapilionaceae) is an erect or suberect undershrub, distributed throughout central and eastern Himalayas, South India and Sri Lanka. The leaves are used as a substitute for tea by hill tribes in upper Assam. Preliminary phytochemical investigations on the D. gangeticum revealed the presence of flavonoids, glycosides, steroids, saponins, phenolic compounds, amino acids and fixed oils [4]. It was also reported that the whole plant is boiled and used against the treatment of liver parasite by Lao people [5]. Although the leaves were used traditionally for varied ailments, no scientific study has been reported. Hence a systematic study has been undertaken to evaluate the hepatoprotective and antioxidant activities of ethanol extract of leaves of D. gangeticum against Diclofenac induced hepatotoxicity in rats.

2. MATERIALS AND METHODS

2.1. Collection and Identification of Plant Material

The fresh flowers of Desmodium gangeticum leaves were collected.

2.2. Preparation of the Extract

The flowers were washed under tap water, air dried, homogenized to fine powder and stored in airtight bottles. Ten grams of dried powder was first defatted with petroleum ether and then extracted with methanol by using Soxhlet apparatus (Lin et al., 1999).

The solvent was evaporated to dryness and the dried crude extract was stored in air tight bottle at 4°C. The percentage yield of methanol extract was 36%. The methanol extract of Desmodium gangeticum Leaves was used for the entire study.

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2.3. Phytochemical Analysis

Preliminary chemical tests were carried out for methanolic extract to identify different phyto-constituents**.**

2.4. Animals

Wistar albino rats of both sexes (180-220 g) were used for the study. All the rats were kept in standard plastic rat cages with stainless steel coverlids and wheat straw was used as bedding material. The animals were kept at the animal house of Department of Pharmacology. The animals were facilitated with standard environmental condition of photoperiod (12:12 h dark: light cycle) and temperature (25 ± 2°C). They were provided with commercial rat and mice feed (Pranav Agro Industries Ltd., Baroda. Amruth Brand rat & mice pellet feed) and water given ad libitum. The use of these animals and the study protocols were approved by CPCSEA recognized local ethical committee.

2.5. Selection of the doses for animal study

The dose considered for the experiment on rats was obtained from conversion of human dose of Desmodium gangeticum (3-5 g/kg). The conversion factor of human dose (per 200 g body weight) is 0.018 for rats (Ghosh 1984). Hence the calculated dose for the rats (considering human dose 5 g/kg) is 450 mg/kg. Thus, hepatoprotective activity was done at two different doses 400 and 600 mg/kg body weight. Acute toxicity was done at three different doses 450, 1800, and 3600 mg/kg body weight.

2.6. Hepatoprotective Studies

2.6.1. Diclofenac induced hepatotoxicity

The rats were divided into five groups with six rats in each. Group I (Control) served as normal and received the vehicle alone (Sterile distilled water, 10 ml/kg, p.o.) for 5 days. Group II (Toxin control) animals received diclofenac (50 mg/kg i.p.) on the 3rd and 4th day. Group III (DGL-400) and IV (DGL-600) were treated with DGL at a dose level of 400 mg/kg and 600 mg/kg body weight p.o. per day respectively for 5 days and on the 3rd and 4th day diclofenac (50 mg/kg i.p.) was given 1 h after the treatment of the extract. Group V (Standard) was treated with standard drug silymarin (100 mg/kg p.o.) for 5 days and on the 3rd and 4th day diclofenac (50 mg/kg i.p.) was given 1 h after the treatment of the drug. The animals were sacrificed 48 h after the last injection of diclofenac under mild ether anesthesia. The blood was collected and allowed to stand for 30 min at 37°C and then centrifuged to separate the serum to estimate various biochemical parameters.

2.6.2 Preparation of liver homogenate

The liver was quickly removed and perfused immediately with ice-cold saline (0.9% NaCl). A portion of the liver was homogenized in chilled Tris HCl buffer (0.025 M, pH 7.4) using a homogenizer. The homogenate obtained was centrifuged at 5,000 rpm for 10 min, supernatant was collected and used for analysis.

2.6.3 Biochemical analysis from serum

The absorbance of all the biochemical parameters was measured in a UV–VIS Spectrophotometer - 1601.

The serum total protein was estimated by modified Biuret method using the total protein test kit.

Procedure

3.0 ml of Reagent I added to all the test tubes. Thereafter, 0.03 ml serum was added for the test + 0.03 ml Reagent II was added for the standard, while in blank 0.03 ml of purified water was added. They were then mixed well and incubated at 37ºC for 5 minutes. The absorbance was read at 578 nm.

The serum albumin was estimated by the method given by Corcoran and Durnan (1977) using readymade albumin test kit.

Procedure

3.0 ml of albumin reagent (Reagent I) was added to all the test tubes. Thereafter, 0.03 ml serum was added for the test and 0.03 ml Reagent II was added for the standard, while in blank 0.03 ml of purified water was added. They were then mixed well and incubated at room t emperature for 1min. The absorbance was read at 630 nm.

Estimation of blood urea nitrogen (BUN) content

The serum blood urea nitrogen was estimated by Enzymatic Urease (Berthelot) method (Fawcett and Scott, 1960) using Urea Berthelot test kit .

Procedure

1.5 ml Solution I was added to clean test tubes. 0.01 ml serum was added for the test and 0.01 ml Reagent III was added for the standard. It was then mixed well and incubated at 37ºC for 3 min; then 1.5 ml of Solution II was added. It was then mixed well and incubated at 37ºC for 5 min. The absorbance was read at 578 nm against reagent blank.

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Estimation of alkaline phosphatase (ALP) activity

Alkaline phosphatase activity was estimated by the method of Kind and King (1954) using ALP test kit.

Procedure All the test tubes were marked properly as blank (B), standard (S), control (C), and test (T). 0.5 ml of working buffered substrate was added in clean tubes. 1.5 ml of purified water was added in all the tubes. They were mixed well and incubated at 37°C for 3 min.

0.05 ml of serum was added in test (T), 0.05 ml of reagent III (Phenol standard) was added in standard (S) and 0.05 ml of purified water was added in blank (B) tubes. All the tubes were mixed well and incubated at 37°C for 15 min. 1 ml of reagent II was added in all the tubes. 0.05 ml of serum was added in control (C). All the tubes were mixed well and absorbance was read at 510 nm. Serum alkaline phosphatase activity is expressed as KA units.

Estimation of aspartate aminotransferase (AST) activity

The serum aspartate aminotransferase was estimated by the method of Reitman and Frankel (1957) using AST test kit.

Procedure

0.25 ml of Reagent I was added in clean test tubes and incubated at 37ºC for 5 minutes. 0.05 ml of serum was added in the test, 0.05 ml Reagent IV was added in standard and 0.05 ml distilled water was added in the blank. They were mixed well and incubated at 37ºC for 60 minutes.

Thereafter, 0.25 ml of Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature for 20 min. Then 2.5 ml of Solution I was added to all the tubes mixed well and allowed to stand at room temperature for 10 min. The absorbance of blank, standard and test were read at505 nm.

2.6.4. Acute Toxicity Study

Acute oral toxicity (Ryu et al., 2004), study was performed as per OECD-423 guidelines (1987). 10 rats/group (5 males and 5 females) were used for the study.

Group 1 was control group , Group III and Group IV were that of WFM at different doses (400, 600 mg/kg body weight). Single dose of the extract was administrated orally to each animal. Signs of toxicity, body weight, feed and water consumption of each animal was observed every day for 14 days.

2.6.5. Statistical Analysis

The data obtained from animal experiments are expressed as mean ± SEM (standard error of mean). For statistical analysis data were subjected to analysis of variance (ANOVA) followed by Student's t-test. Values are considered statistically significant at $p < 0.01$ for ANOVA and $P < 0.05$ for t-test.

3. RESULTS AND DISCUSSION

3.1. Preliminary Phytochemical Analysis

The results of qualitative phytochemical analysis of the crude powder and the methanol extract of Desmodium gangeticum leaves is shown in Table.1.

Table 1: Preliminary qualitative phytochemical analysis of Desmodium gangeticum leaves

Phytochemical	Test	Methanolic extract
Alkaloids	Dragandroffs test	+
	Mayers test	+
	Wagners test	+
Flavonoids	Shinoda test	+
	Alkaline reagent test	+
Cardiac glycosides	Keller-kilianni test	-
Phlobotannins	HCl test	+
Saponins	Frothing test	+
Steroids	Libbermann-Burchard test	-
Tannins	$FeCl_3$ test	+
Triterpenes	H_2SO_4 test	+

(-): absent, (+): present.

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3.2. Hepatoprotective Studies

Diclofenac induced hepatotoxicity

The results of serum biochemical parameters in pre-treatment of DG with respect to induction of hepatotoxicity using diclofenac are shown in Figure 1. The level of total protein and albumin depleted in the group treated with diclofenac (toxin control) and were significantly decreased ($P < 0.001$) when compared with the normal control group. The BUN and ALP levels increased significantly ($P < 0.01$, $P < 0.001$ respectively) in the group treated with diclofenac. The administration of diclofenac markedly increased serum AST and ALT levels which were significant as compared to normal control group ($P < 0.05$, $P < 0.01$ respectively). The groups that received the pre-treatment of DGL at dose levels of 400 and 600 mg/kg body weight significantly controlled the change in the biochemical parameters. The extract at dose levels of 400 and 600 mg/kg exhibited significant increases ($P < 0.05$) in the serum total protein level as compared to toxin control group. The albumin level in lower as well as in higher dose group increased significantly ($P < 0.01$, $P < 0.001$ respectively) as compared to toxin control group and the effect was comparable with the standard group ($P < 0.01$) treated with silymarin. The BUN level decreased in both the dose groups significantly ($P < 0.05$) as compared to toxin control group. The ALP level also significantly decreased in DGL-400 ($P < 0.05$) as well as in DGL- 600 group ($P < 0.001$). In DGL-600 group, the level of ALT and AST significantly decreased ($P < 0.05$), the result was comparable to that of standard group.

The results of relative liver weight, liver total protein, GSH and antioxidant enzymes in diclofenac induced hepatotoxicity are given in Figure 2. The relative liver weight in toxin control group increased significantly ($P < 0.001$) as compared to normal control group. The total protein and GSH levels from the liver homogenate decreased significantly ($P < 0.001$, $P < 0.01$ respectively) in toxin control group. The catalase (CAT) and GPx activity in the toxin control group was also significantly ($P < 0.001$, $P < 0.05$ respectively) depleted as compared to the normal control group. The mean relative liver weight decreased significantly in DGL-400 ($P < 0.001$) and DGL-600

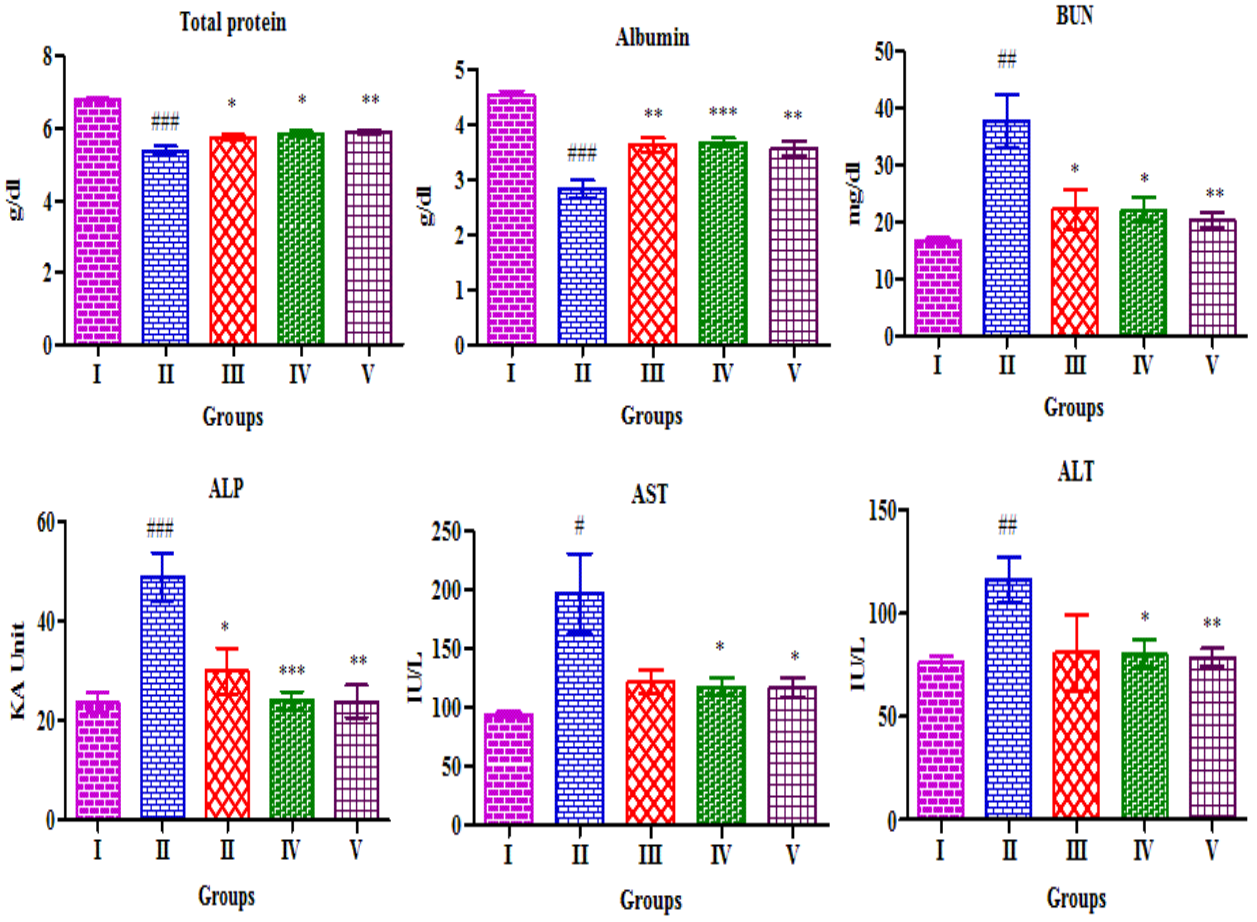

Figure 1: Effect of methanol extract of Desmodium gangeticum Leaves on different serum biochemical parameters in diclofenac (50 mg/kg) induced hepatic damage in rats. Group I: Normal control, Group II: Toxin control diclofenac, Group III: DGL-400 mg/kg + diclofenac, Group IV: DGL-600 mg/kg + diclofenac, Group V: Silymarin-100 mg/kg + diclofenac. Results are expressed as mean ± SEM, (n = 6). #P < 0.05, ##p < 0.01, ###p < 0.001 as compared with normal control group; *p < 0.05, **p < 0.01, ***p < 0.01 as compared with toxin control group.

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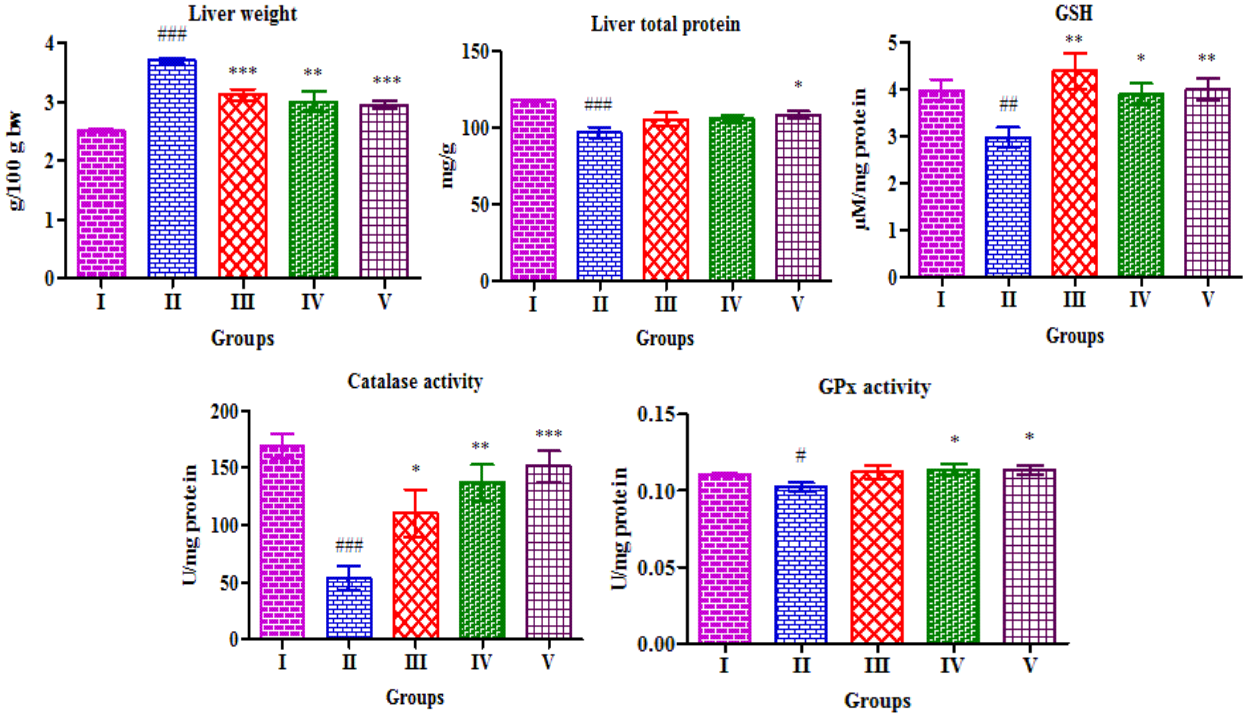

Figure 2: Effect of methanol extract of Desmodium gangeticum Leaves on relative liver weight, liver total protein and different liver antioxidants in diclofenac (50 mg/kg) induced hepatic damage in rats. Group I: Normal control, Group II: Toxin control diclofenac, Group III: DGL-400 mg/kg + diclofenac, Group IV: DGL-600 mg/kg + diclofenac, Group V: Silymarin-100 mg/kg + diclofenac. Results are expressed as mean ± SEM, (n = 6). #p < 0.05, ##p < 0.01, ###p < 0.001 as compared with normal control group; *p < 0.05, **p < 0.01, ***p <0.01as compared with toxin control group. (P < 0.01) treated group as compared to the toxin control group. The result of the higher dose group was comparable to the standard drug treated group (P < 0.001).

The total protein and GSH levels from liver homogenate in DGL treated groups elevated, but total protein level was not significant. However, pretreatment with DGL significantly recovered the diclofenac induced GSH depletion in lower and higher dose group (P < 0.01, P < 0.05 respectively). The catalase and GPx activity increased at both the dose levels; at higher dose DGL exhibited good activity (P < 0.01, P < 0.05 respectively). GPx activity of DGL-600 group was similar to that of standard drug treated group.

Acute Toxicity Study

In acute toxicity study, no adverse reactions or mortality were observed after administration of DGL (450, 1800, and 3600 mg/kg bw) and no behavioral changes were observed during the entire period of experimentation. Some alteration was noticed in daily feed and water intake in both male and female rats treated with single dose of extract as well as in control animals. As compared to the control group, drug treated groups had several consecutive days of reduced/increased feed and water consumption at different times in the study. These periods of reduced/increased feed and water intake were not significant to the overall feed and water consumption rates. Individual body weights were recorded daily during the experimental period. Mean body weight gains were calculated for each group. In control and DGL treated groups, body weight of animals slightly increased during experimental period, but the increase was not significant.

4. CONCLUSION

In physicochemical analysis, crude powder and methanol extract of Desmodium gangeticum leaves were free from heavy metals. In qualitative phytochemical analysis tannins and alkaloids were present in higher amount, while cardiac glycosides and steroids were totally absent. In quantitative analysis of phytoconstituents, total phenol content was higher than flavonoid content. Hence, the determination of pharmacognostical and phyto-physicochemical profile of Desmodium gangeticum leaves may be useful to supplement information in respect to its identification, authentication and standardization of herbal drugs. In other words, the pharmacognostic features examined in the present study may serve as tool for identification of the plant for validation of the raw material and for standardization of its formulations at herbal industrial level in the coming days.

In hepatoprotective studies, the induced diclofenac toxicity elevated levels of serum marker enzymes ALT, AST, ALP

and the level of BUN along with the decrease in total protein and albumin levels. It also increased the relative liver weight and decreased the level of liver total protein and GSH. The activity of catalase and GPx significantly decreased in diclofenac intoxicated animals. The pre-treatment of methanol extract of Desmodium gangeticum leaves at dose levels of 400 and 600 mg/kg had restored the ALT, AST, ALP and BUN levels towards normalization and the effects were comparable with standard drug (Silymarin 100 mg/kg). The total protein, albumin, GSH levels and catalase, GPx activity increased significantly in the animals received pre-treatment of the DGL.

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