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Antidiabetic Activity of Methanolic Extract of *Hygrohila auriculata* in Adult Male Wistar Rats.

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Abstract

Objective:
The objective of this study was to determine the acute toxicity and antidiabetic activity of the methanolic extract of *Hygrophila auriculata* in adult male Wistar rats.

Methods:
Methanolic extract was prepared by optimized maceration procedures. Then, they were administered to groups of six rats in doses of 0, 100, 500, 750, 1000 and 3000 mg/kg body weight. The rats were then observed for signs of acute toxicity and these were duly recorded. Other parameters tested were Chloride and Calcium levels, Alkaline Phosphatase Levels and α-amylase levels.

Results:
The methanolic extract was found to show no oral toxicity even at the highest dose of 3000 mg/kg body weight. Moreover, it was an effective antidiabetic agent at concentrations as low as 10mg/kg body weight.

Conclusion:
The present study shows that the methanolic extract of *H. auriculata* is non-toxic when administered orally even at higher concentrations.

Keywords:
Hygrophila auriculata, Anti-diabetic, Phytochemicals, Methanolic Extract, Acute Toxicity, Wistar Rats, Amylase, Alkaline Phosphatase, Glucose Metabolism

INTRODUCTION

Until very recently, India was titled "the diabetic capital of the world". In 2007, 41 million Indians were estimated to have this lifestyle disease, thereby amounting to 19% of the total population. [1] This means that approximately one in every five diabetics is an Indian. [2] It is estimated that this number will rise to around 66-70 million by 2025. [3, 4] This is expected to reach 79.4 million by 2030. [5] However, the growth is faster than expected as a research study undertaken in 2011 found that India has 61.3 million diabetic patients second only to China with 90 million diabetic patients. [6]

The problem of diabetes in India is further complicated owing to its comparatively conservative nature. Patients prefer natural medicines and traditional remedies as opposed to conventional allopathic medicine. This makes finding efficient herbal remedies for diabetes a top priority. Before these remedies can be released, it is essential that their toxicity and antidiabetic activity be studied in detail. The aim of this study is to identify the acute toxicity effects and determine the *in vivo* efficacy of the methanolic extract of *Hygrophila auriculata* in adult male Wistar rats before it can be used as a treatment for diabetes.

MATERIALS AND METHODS

Plant Material and Extract Preparation

H. auriculata seeds were obtained from the local market in Vellore, Tamil Nadu, India. The methanolic extract was prepared based on the previously optimized procedure. [7]

Experimental Animals

Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 423 11. [8] The institutional ethical committee of VIT University, Tamil Nadu, India approved the protocol for these experiments under number VIT/IAEC/VII/25. Experiments were performed using healthy young adult male Wistar rats, weighing 250-300 g.

Assignment, Housing and Diet of animals

The animals were randomly divided into six groups each containing six rats. The animals were housed in polypropylene cages (55 x 32.7 x 19 cm), with sawdust litter in a temperature controlled environment (23 ± 2ºC). Lighting was controlled to supply 12 h of light and 12 h of dark for each 24-h period. Each cage was identified by a card stating the cage number, number and weight of the animals it contained and dose level. The animals were fed with standard laboratory animal food pellets with water ad libitum.

Acute Toxicity Study

The test substance was administered in a single dose by gavage using specially designed rat's oral needle. Animals were fasted 3 h prior to dosing (only food withheld). Following the period of fasting, animals were weighed and test substance was administered orally at a dose of 0 (control), 100, 500, 750, 1000 and 3000 mg/kg body weight. The administration volume was 2 ml/kg body weight of the animal. Based on the body weight of the animal on the day of treatment, the quantity of the test substance was calculated. Animals were observed for a total of 14 days. All the rats were observed at least twice

daily with the purpose of recording any symptoms of ill-health or behavioral changes. Direct observation parameters included tremors, convulsions, salivation, diarrhea and sleep. Eyes and mucous membrane, circulatory, skin and fur, respiratory, autonomic and central nervous systems were other parameters observed. The time of death, if any, was recorded.

Induction of Diabetes Mellitus

The Diabetes Mellitus (DM) was induced in the rats, after 12 h fasting, by intravenous injection of streptozotocin (STZ) diluted in 0.05M citrate buffer (50 mg/kg body weight). The control group received the citrate buffer injection without STZ. The fasting blood glucose levels of the rats were tested after two days. All the control group rats presented fasting glycaemia of 60-100 mg/dl and the animals chosen as diabetic were those which presented fasting glycaemia above 250 mg/dl. Fasting glycaemia was measured by the glucose oxidase method. [9]

Administration of Extract and Animal Sacrifice

After induction of diabetes, the animals were divided into 6 groups of 6 rats each. The first group served as the normal control (NC). This group consisted of healthy rats that were not diabetic and were administered distilled water. The second group served as the diabetic control (DC). This group consisted of diabetic rats that were administered distilled water. The third group consisted of diabetic rats that were administered Metformin at a concentration of 10 mg/kg body weight ($D_{Metformin}$). The fourth group consisted of diabetic rats that were administered the plant extract at a concentration of 100 mg/kg body weight ($D_{100mg/kg\ bw}$). The fifth group consisted of diabetic rats that were administered the plant extract at a concentration of 50 mg/kg body weight ($D_{50mg/kg\ bw}$). The sixth group consisted of diabetic rats that were administered the plant extract at a concentration of 10 mg/kg body weight ($D_{10mg/kg\ bw}$). All the animals were administered for 4 weeks. At the end of the experiments, the animals were weighed, sacrificed and then they had their blood, livers, hearts, pancreases and kidneys collected.

Chloride Levels

Chloride levels in the sera of the animals were determined by the method outlined in Clinical Chemistry. [10]

Calcium Levels

Calcium levels in the sera of the animals were determined by the method outlined in Clinical Chemistry. [10]

Alkaline Phosphatase Levels

Alkaline Phosphatase levels in the sera, livers, hearts, pancreases and kidneys of the animals were determined by the method outlined in Clinical Chemistry. [11]

α-Amylase Levels

α-Amylase levels in the sera and pancreases of the animals were determined by the method outlined in Clinical Chemistry. [11]

RESULTS AND DISCUSSION

Acute Toxicity Study

No mortality was observed throughout the study period of 14 days, even at the highest dosage of 3000 mg/kg body weight. The oral LD_{50} was indeterminable being in excess of 3000 mg/kg body weight. Testing the extracts at a higher dose maybe unnecessary and, the extracts are practically non-toxic. As illustrated in Table 1, the rats showed absolutely no outward signs of acute toxicity. Even the animals that were administered the highest dosage of 3000 mg/kg body weight showed no difference in behavior or parameters from the control group. This clearly indicates that the methanolic extract of *H. auriculata* is not toxic up till very high concentrations when administered orally. In the past, we analyzed the phytochemical constituents, anti-oxidant activity and *in vitro* anti-diabetic activity of aqueous, methanolic, ethanolic and chloroformic extracts of *H. auriculata* and found the methanolic extract to be rich in many phytochemicals including alkaloids flavonoids, tannins, saponins, terpenoids, etc. It was also the extract with the greatest anti-oxidant activity and *in vitro* anti-diabetic activity. [7] Hence we chose it for acute toxicity studies in rats. *H. auriculata* is commonly used in *Ayurvedic* treatment of diabetes, but few scientific studies have been carried out on it. [12] The large majority phytochemicals found in the extract, like alkaloids, flavonoids, tannins and saponins, are common constituents of cosmetic products and drugs. [13, 14] Hence, they are bound to be safe. The findings of this study are congruent with this.

Chloride and Calcium Levels

The chloride and calcium levels are tabulated in Table 2 as well as shown in Figures 1 and 2 respectively. Both chloride and calcium ions showed a similar trend. While chloride levels remained virtually identical in the NC and DC groups, the calcium levels were drastically raised in the DC group. This spike was best regulated by Metformin. The rats being administered 10 mg/kg body weight of the extract showed near perfect regulation of chloride levels. The extract however, did not seem to have a significant effect on the calcium levels. It was important to study the chloride and calcium levels since they, especially chloride ions, play an important role in glucose transport. [15, 16]

Table 1: Effect of *H. auriculata* extract on rats.

Response	0mg/kg	100mg/kg	500mg/kg	750mg/kg	1000mg/kg	3000mg/kg
Alertness	Normal	Normal	Normal	Normal	Normal	Normal
Lacrimation & Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Pain & Touch Response	Absent	Absent	Absent	Absent	Absent	Absent
Skin Color & Urination	Normal	Normal	Normal	Normal	Normal	Normal
Tremors & Convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent
Pinna Reflux	Normal	Normal	Normal	Normal	Normal	Normal
Grooming	Absent	Absent	Absent	Absent	Absent	Absent

Alkaline Phosphatase Levels

The alkaline phosphatase levels in sera and organs are shown in Figures 3 and 4 respectively. Alkaline Phosphatase is an enzyme found in all types of tissues in the body. It serves a variety of functions in the body, including deactivation of AMP-activated protein kinase α (AMPK). [17, 18] AMPK is a very important enzyme for diabetics as it stimulates insulin secretion, increases glucose uptake, decreases cholesterol synthesis and regulates adipocytes. [19, 20] Raised alkaline phosphatase levels directly imply a lowered activity of AMPK, commonly seen in diabetes patients. The DC group of rats also showed a similar result, with raised alkaline phosphatase levels as compared to the NC group. A well-known mechanism of the action of Metformin is to raise the activity of AMPK, thereby improving the metabolism of fat and glucose in the body. [21] However, this is not brought about by affecting the ALP levels, as can be seen in Figures 3 and 4. The methanolic extract of *H. auriculata* does change the ALP levels. The extract at a concentration of 10mg/kg body weight was most effective in normalizing the concentrations of ALP. As shown in Figures 3 and 4, the ALP levels are most similar in the NC and $D_{10mg/kg\ bw}$ groups.

α-Amylase Levels

The α-amylase activity in sera and pancreases are tabulated in Table 3 and visualized in Figure 5. α-Amylase is the enzyme responsible for the breakdown of starch into glucose. Hence, its activity directly affects the amount of glucose in the body, especially post-prandial glucose levels. [22] Thus, it is extremely important to study the effects of the extract on α-amylase activity. As can be concluded from Figure 5, the diabetic rats have elevated α-amylase activity. Metformin does not decrease the activity, but the methanolic extract of *H. auriculata* does. While the activity is not reduced to normal levels, the extract administered at a concentration of 100mg/kg body weight is most effective at bringing the α-amylase activity to a normal level.

Table 2: Chloride and calcium levels in rats. The values represent Mean±SEM for a set of 6 values.

Group	Chloride(mmol/l)	Calcium(mg/dl)
NC	0693.00±34.65	3.01±0.15
DC	0696.72±34.83	8.35±0.41
$D_{Metformin}$	0824.59±41.23	3.14±0.16
$D_{100mg/kg\ bw}$	1131.15±56.55	8.51±0.42
$D_{50mg/kg\ bw}$	1272.13±63.60	7.52±0.36
$D_{10mg/kg\ bw}$	0673.77±33.68	7.27±0.28

Table 3: α-amylase levels in sera and pancreases of rats. The values represent Mean±SEM for a set of 6 values.

Group	Serum(IU/l)	Pancreas(IU/l)
NC	031.63±1.58	0122.56±06.12
DC	106.70±5.33	0213.49±10.67
$D_{Metformin}$	019.76±0.98	1043.72±52.18
$D_{100mg/kg\ bw}$	003.95±0.18	0123.72±06.18
$D_{50mg/kg\ bw}$	023.72±1.18	0620.70±31.03
$D_{10mg/kg\ bw}$	027.67±1.38	0498.14±24.90

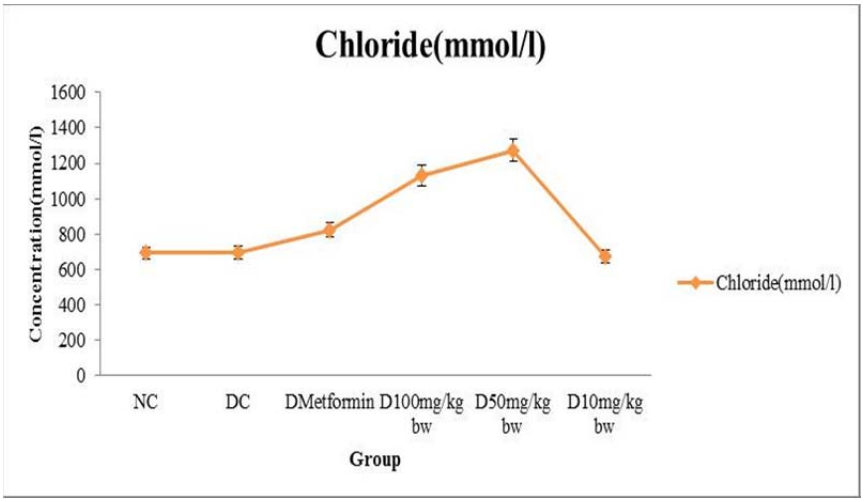

Figure 1: Chloride levels in rats. The values represent the mean for a set of 6 values, with the black bars representing the standard error mean.

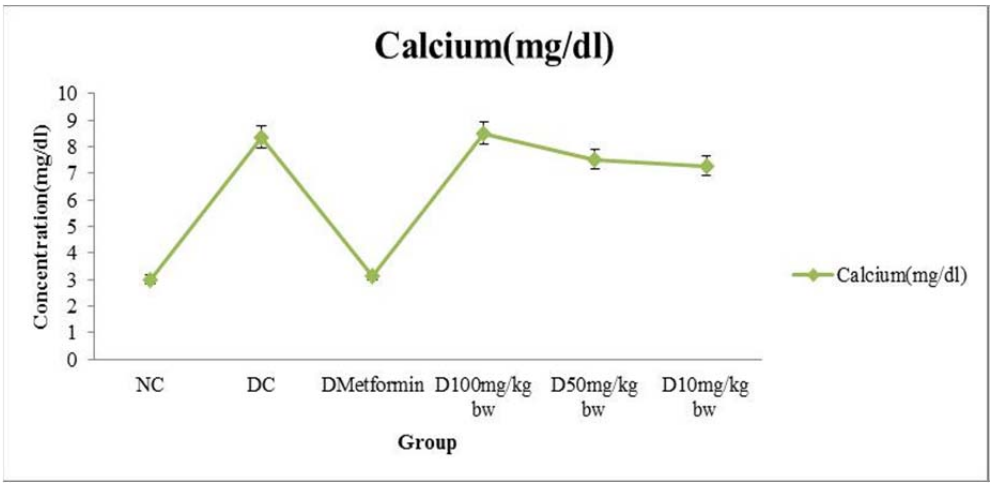

Figure 2: Calcium levels in rats. The values represent the mean for a set of 6 values, with the black bars representing the standard error mean.

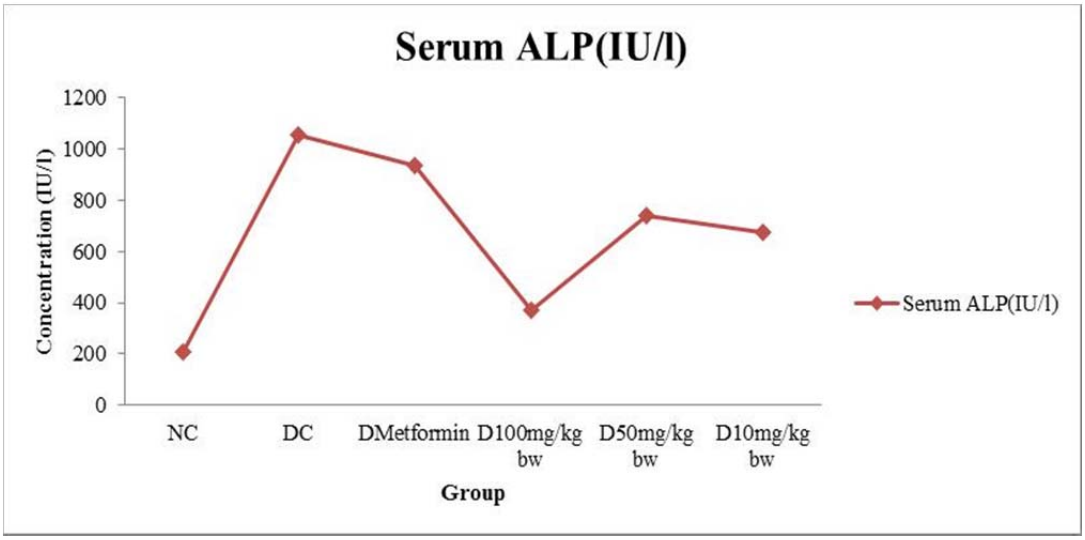

Figure 3: Serum Alkaline Phosphatase levels in rats. The values represent the mean for a set of 6 values, with the black bars representing the standard error mean.

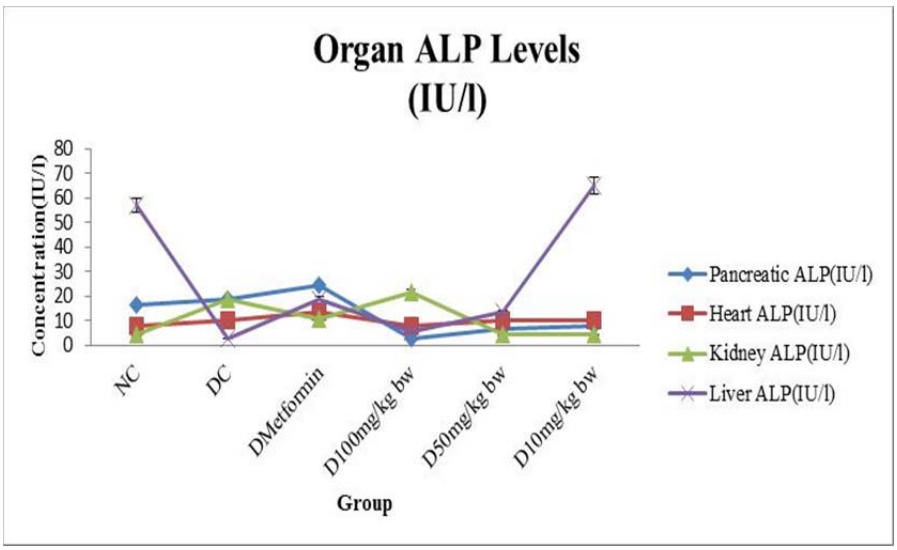

Figure 4: Organ Alkaline Phosphatase levels in rats. The values represent the mean for a set of 6 values, with the black bars representing the standard error mean.

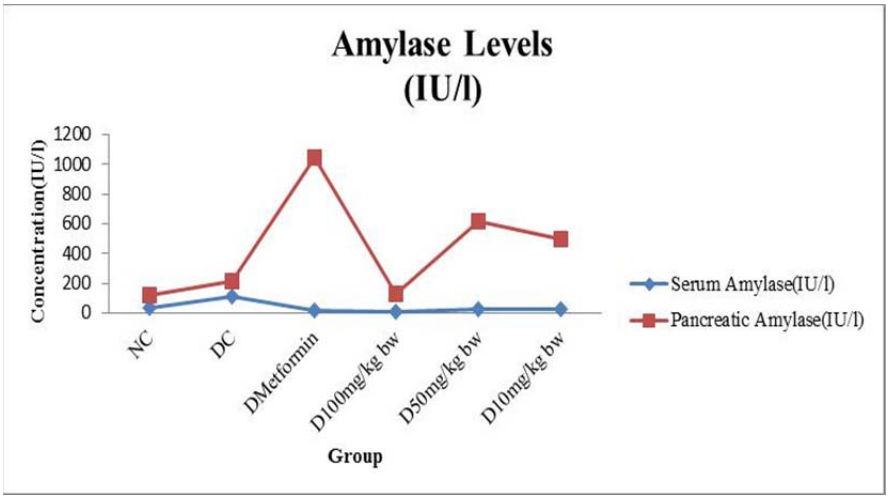

Figure 5: Amylase levels in rats. The values represent the mean for a set of 6 values, with the black bars representing the standard error mean.

CONCLUSION

This study shows that the methanolic extract of *H. auriculata* is non-toxic up till concentrations of 3000 mg/kg body weight. This study indicates that the extract maybe safe when administered orally. Moreover, the extract is very effective at controlling chloride levels which play an important role in glucose uptake. Further the study found that the extract is effective at controlling the activity of ALP, and thereby increasing the activity of AMPK proving very beneficial for diabetics. The extract also lowers the activity of α-amylase which can prevent post-prandial glucose spikes in diabetic patients. The extract is most effective at lower concentrations, and this is probably due to some solvation mechanism. Further studies to elucidate the exact mechanism of action of the extract are planned. The extract is a safe and effective anti-diabetic treatment.

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CONFLICT OF INTEREST

The authors wish to declare that they have no conflict of interest.

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