

ACUTE & SUB-ACUTE TOXICITY STUDIES OF PHARMACOLOGICALLY ACTIVE SEABUCKTHORN LEAF EXTRACT

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ABSTRACT

Background: Seabuckthorn (SBT, *Hippophae rhamnoides* L.) is an important medicinal plant of cold desert areas. Therapeutic significance of various parts of this plant has been mentioned in many traditional medicinal systems, especially Chinese and Tibetan systems. There are many pharmacological reports suggesting therapeutic usefulness of SBT leaf extracts, however, relatively very few toxicity studies are available on these extracts.

Objective: The present study was undertaken to evaluate preclinical acute and subacute toxicity of a 75% ethanolic extract of SBT leaves in Sprague-Dawley rats.

Methods: Firstly, the SBT Leaf extract was characterized by total phenol content estimation and HPLC fingerprinting analysis. Further, toxicity was evaluated in Sprague-Dawley rats after single dose oral administration of the extract in the dose range of 0.625, 1.25, 2.50 and 5 g/kg body weight (acute model) and 28 days administration in the dose range of 0.625 and 1.25 g/kg body weight (Subacute model).

Results: The results of the acute toxicity study showed the LD₅₀ of the extract to be higher than 5 g/kg body weight via oral route. Subacute toxicity study did not show a significant change in any of the hematological and biochemical parameters. The animals which received the dose of 1.25 g/kg body weight per day were subjected to histopathological examination. No significant changes were however observed as compared to the control group.

Conclusion: On the basis of observations of the present study, it can be concluded that 75% ethanolic extract of SBT leaves is practically non toxic; however, further studies are needed to confirm long term toxicities.

Keywords: Seabuckthorn, Acute model, Subacute model, Histopathological examination.

INTRODUCTION

Hippophae rhamnoides L., commonly known as Seabuckthorn (SBT; Family: Elaeagnaceae) is a branched, thorny nitrogen fixing deciduous shrub, found in cold desert areas. Reports suggest that SBT is a rich source of various bioactive phytochemicals [1] and has a long history of use in Europe and Asia for food and pharmaceutical purposes [2, 3]. Conventionally, SBT is evident to be used for its varied therapeutic potential [4]. Moreover, the plant has also got a prominent position in Tibetan, Chinese and Ayurvedic literature [5]. SBT is one of the most fascinating but underworked plant in the world and can be termed as natural biofactory capable of synthesizing more than 200 biomolecules having immense medicinal, nutraceutical and cosmeceutical values. The SBT leaves are reportedly found to be rich in tannins, phenolic acids and volatile compounds [6-8]. SBT extracts have also been reported to exhibit many bioactivities like antioxidant [9], antibacterial [10] and immunomodulatory activities [11]. Literature reports also revealed the usefulness of SBT extracts in various diseases like gastric ulcers [12-13] skin disorders [14] cardiac disorders [15-16] and radiation induced oxidative damage [17] etc.

All these studies, which prove the tremendous nutritional and health properties of SBT, make its toxicity studies very important. Despite all the reports regarding pharmacological efficiency of SBT leaf extracts, very few reports focus on the toxicological evaluation of SBT leaf extracts. In this background, it is suggestive to conduct toxicity studies on SBT leaf extract for actual assessment of their risk-benefit profiles. As per Saggi et al (2007), rich antioxidant content of SBT extracts is mainly responsible for the precipitation of various therapeutic properties. In our previous work on bioactivity guided extraction of SBT leaves, 75% ethanolic extract has been observed to show higher antioxidant activity than other solvent extracts [18]. With reference to above mentioned observations, in the present study an effort has been

made to evaluate the acute and subacute toxicity of 75% ethanolic SBT leaf extract through the assessment of its effect on various behavioral, hematological, biochemical and histopathological parameters.

MATERIALS AND METHODS

Plant material

SBT leaves were collected from Leh (Ladakh), India. These were further authenticated by National Institute of Science Communication and Information Resources, New Delhi, India. Leaves were cleaned, dried, powdered and stored at ambient temperature till extraction.

Preparation of extract

Cold percolation method was used for preparation of 75% ethanolic extract of dried SBT leaves [1]. Powdered leaves were extracted with 75% ethanol for 24 hours and filtered with 80 mesh nylon cloth. The raw material to solvent ratio used was 1:8. The extraction process was repeated 5 times. To avoid contamination, clean and sterile conditions were maintained during the extraction process. The filtrates obtained after each extraction were combined and stored at ambient temperature. The combined filtrates were again filtered with 250 mesh nylon cloth to get the liquid extract. This extract was then concentrated under reduced pressure till a solid mass was obtained.

Characterization of the extract

The 75% ethanolic SBT leaf extract was characterized by estimation of total phenol content and HPLC finger printing at 368 nm.

Total Phenol Content

Folin - Ciocalteu Reagent (FCR) based assay was used for estimation of total phenol content in above prepared SBT extracts [18]. To the

aliquot (50 μ l) taken from stock solution (1mg/ml) of the extract, 3.5 ml distilled water and 250 μ l of FCR was added, the mixture was kept at room temperature for 1 – 8 min and 750 μ l of 20% sodium carbonate solution was added. Mixture was kept at room temperature for 2 hrs and absorbance of the color developed was recorded at 765nm with the help of a UV-Visible spectrophotometer against blank. Total phenols (mg/g) in the SBT leaf extracts were expressed as Gallic Acid Equivalent (GAE), using standard curve ($R^2 = 0.986$) prepared from gallic acid (0.1 mg/ml) solution.

HPLC fingerprinting

HPLC analysis of the 75% ethanolic extract of SBT leaves was carried out using a system equipped with HIQ SIL C18V reversed-phase column and diode array detector at 368 nm [18]. The mobile phase used, was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. Flow rate and injection volume were 1.0 ml/min and 10 μ l, respectively. All chromatographic operations were carried out at ambient temperature.

Experimental animals

A total of 42 Sprague-Dawley rats (Male), weighing 170 \pm 20 g, were used for the study. The animals were maintained under controlled environment at 25 \pm 1°C. The animals were fed standard animal food pellets and water *ad libitum*. The experiments were performed after clearance from the Institutional Animal Ethical Committee.

Acute toxicity

Acute toxicity studies were performed on 24 animals using 4 different oral doses (0.625, 1.25, 2.50 and 5 g/kg body wt.). These animals were divided in to 4 groups, each containing 6 animals. The animals were fasted overnight and the above selected doses of the extract were administered orally to different groups. After drug administration the animals were provided with food and water immediately and closely observed in their cages for any mortality/ any adverse signs of severe toxic effects like hypo-activity, anorexia, salivation, diarrhea, syncope, muscle cramping, convulsions, if any. The general behavior of animals were continuously monitored for 1 h after dosing, periodically during the first 24 h, with special attention given during the first 4 h and then daily thereafter, for a total of 14 days [19].

Subacute toxicity

Subacute toxicity was carried out as described by OECD guidelines 407 with some modifications [20]. Total 18 animals were used for this study. The animals were divided in to three groups viz. i) First experimental group getting 0.625 g/kg body weight of the extract, ii) Second experimental group getting 1.25 g/kg body weight of the extract and iii) the Control group getting normal saline. Food and water were freely available to the animals during the experiment.

Hematological and biochemical analysis

After 28 days of treatment, animals were fasted overnight but allowed access to water *ad libitum*. They were then anesthetized

with ether and blood samples were obtained from orbital sinus [21] using capillary tubes for hematological and biochemical analysis, with and without anticoagulant, ethylenediaminetetraacetic acid (EDTA), respectively. Blood with the anticoagulant was used immediately for the determination of hematological parameters, while blood without the anticoagulant was centrifuged at 4000 rpm for 10 min at 4°C and the serum obtained was stored at -20°C until analyzed for biochemical parameters.

Histopathological study

After blood collection, animals were sacrificed by decapitation under light ether anesthesia and vital organs (liver, spleen, heart, kidneys, lungs and brain) were removed and weighed. Ratio of each organ to body weight was determined. Kidney and liver were preserved in 10% buffered formalin solution to observe the histopathological changes, if any.

Statistical analysis

Results were expressed as means \pm SE. Statistical comparisons between the data for the control and treatment groups were performed using the paired t-test. Values of $p < 0.05$ were considered as significantly different. The Statistical Package SPSS 12.0 for Windows was used to analyze the data.

RESULTS

Characterization of the extract

Total phenol content in the 75% ethanolic extract of SBT leaves was found to be 402.19 \pm 2.20 mg/gm in terms of Gallic Acid Equivalents. The HPLC profile of the SBT extract has been given in figure 1.

Acute toxicity study

Results of acute toxicity study (Table 1) were observed to have zero mortality and no adverse effects. So, in context with LD₅₀, 50% of the rats did not die within 24 h of treatment. Moreover, there was no adverse effect or mortality observed even after 14 days of oral administration of extract to the rats.

Subacute toxicity study

In case of subacute toxicity study, all the animals which were given subacute doses i.e. 0.625 and 1.25 g/kg body weight, orally for 28 days, remained active and healthy throughout the period of study. No symptoms of adverse effects were recorded during the course of study.

Hematological and biochemical parameters

Similarly, in case of hematological parameters also, no significant changes were observed in RBC count, WBC count, platelet count and hemoglobin content of extract treated groups when compared to control. However, a slight increase in WBC, platelet count and hemoglobin contents were observed in extract treated groups. In addition to this, the hematocrit of extract treated groups was found to be higher than that of control group but again these were non significant (Table 2).

Table 1: Effect of 75% ethanolic SBT leaf extract on animal behavior

Dose (g/kg body weight)	Toxic symptoms	Mortality
0.625	None	None
1.25	None	None
2.50	None	None
5	None	None

Table 2: Effect of 75% ethanolic SBT leaf extract on hematological parameters

Parameters	Control	Extract (0.625 g/kg/day)	Extract (1.25 g/kg/day)
WBC ($\times 10^3/\mu$ l)	6.7 \pm 0.6	7.1 \pm 0.7	7.7 \pm 0.5
RBC ($\times 10^6/\mu$ l)	7.3 \pm 0.2	7.1 \pm 0.2	7.4 \pm 0.2
Hemoglobin (g %)	14.4 \pm 0.3	14.6 \pm 0.5	15.5 \pm 0.4
Hematocrit (%)	42.6 \pm 0.7	43.0 \pm 1.0	44.1 \pm 0.6
MCV (fQ)	59.8 \pm 0.9	60.8 \pm 0.7	58.6 \pm 1.5
Platelets ($\times 10^3/\mu$ l)	853.8 \pm 61.1	863.5 \pm 71.8	872.7 \pm 49.4

WBC: White Blood Cells; RBC: Red Blood Cells; MCV: Mean Corpuscular Volume

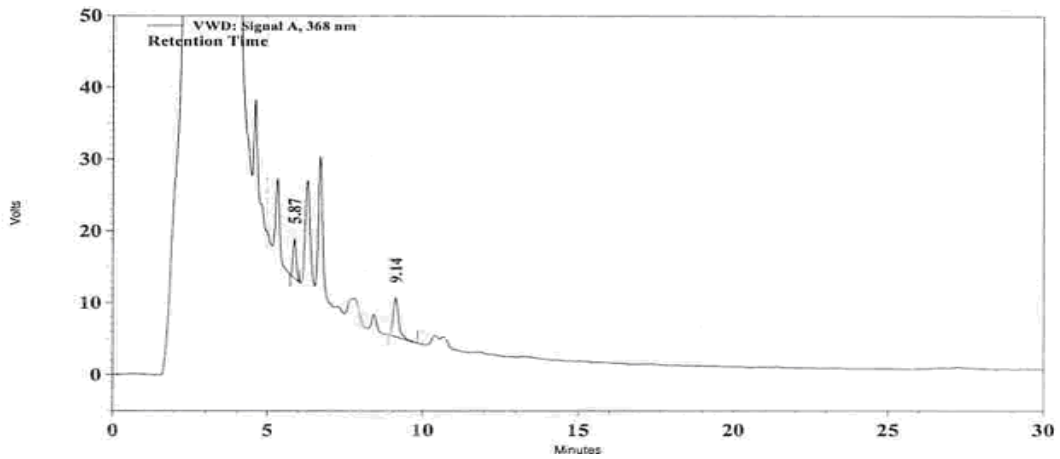


Fig. 1: HPLC fingerprinting profile of 75% ethanolic SBT leaf extract at 368 nm

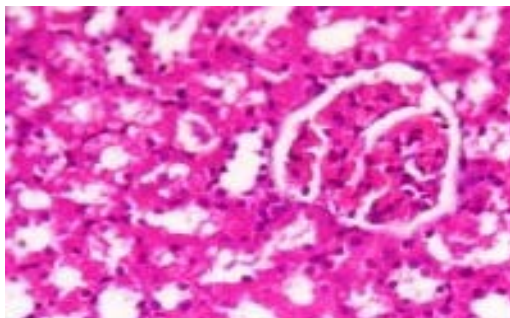
Table 3: Effect of 75% ethanolic SBT leaf extract on biochemical parameters

Parameters	Control	Extract (0.625 g/kg/day)	Extract (1.25 g/kg/day)
Cholesterol (mg/dl)	95.27 ± 0.6	94.73 ± 1.0	94.87 ± 0.35
Creatinine (mg/dl)	0.34 ± 0.01	0.32 ± 0.05	0.33 ± 0.01
Bilirubin (mg/dl)	0.31 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
SGPT (IU)	6.6 ± 0.02	6.6 ± 0.04	6.6 ± 0.03
Protein (g/dl)	6.31 ± 0.02	6.31 ± 0.03	6.30 ± 0.03

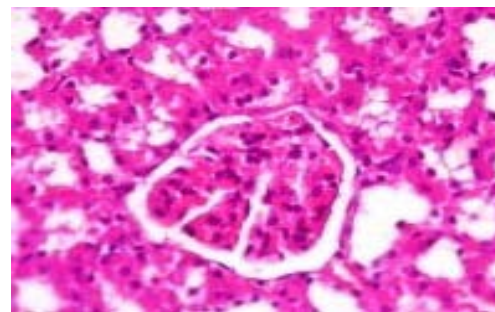
SGPT: Serum Glutamic Pyruvic Transaminase

Table 4: Effect of 75% ethanolic SBT leaf extract on organ weight/body weight ratio

Organ	Control	Extract (0.625 g/kg/day)	Extract (1.25 g/kg/day)
Liver x 10 ⁻³	31.25 ± 0.05	31.28 ± 0.05	31.18 ± 0.04
Heart x 10 ⁻³	3.61 ± 0.04	3.60 ± 0.03	3.67 ± 0.05
Kidney x 10 ⁻³	3.21 ± 0.04	3.17 ± 0.04	3.21 ± 0.08
Spleen x 10 ⁻³	2.06 ± 0.05	2.05 ± 0.04	2.13 ± 0.06
Lungs x 10 ⁻³	4.88 ± 0.03	4.93 ± 0.04	4.87 ± 0.05
Brain x 10 ⁻³	6.24 ± 0.09	6.24 ± 0.06	6.10 ± 0.08

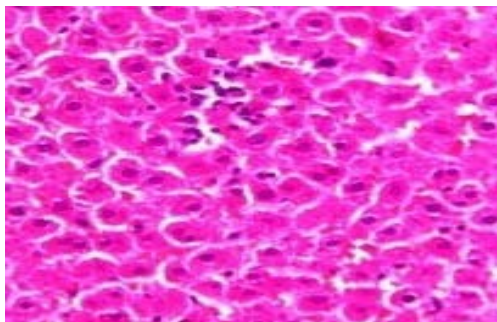


(A) Normal rat kidney

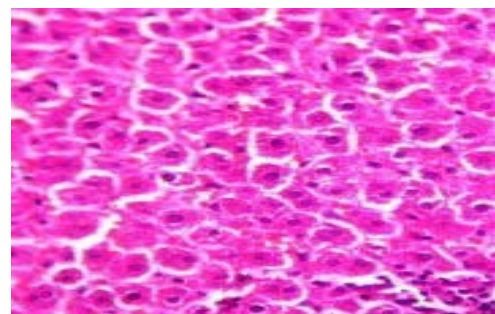


(B) Extract treated rat kidney

Fig. 2: Effect of 75% ethanolic SBT leaf extract on histopathology of rat kidney



(A) Normal rat liver



(B) Extract treated rat liver

Fig. 3: Effect of 75% ethanolic SBT leaf extract on histopathology of rat liver

None of the biochemical parameters of both the extract treated groups showed any significant changes in comparison to control group. Although there were very slight increase in bilirubin content in extract treated groups, no clinically significant changes were observed (Table 3).

Histopathological study

All vital organs (kidney, spleen, liver, heart, lung and brain) showed no significant changes in the organ weight/body weight ratios in 0.625 and 1.25 g/kg/day treated groups in comparison to controls (Table 4).

The microscopic examination of internal organs like kidney (Figure 2) and liver (Figure 3) of the animals administered with the extract at the dose level of 1.25 g/kg/day did not reveal any significant changes in color or texture when compared with control animals. Moreover, they did not indicate any histological abnormalities in the organs examined.

DISCUSSION

Medicinal plants, due to their natural origin, were used to be considered safe for human use. But various reports suggest the potential risks involved with such plants [22]. SBT is also a medicinal herb with huge nutritional and therapeutic potential. In spite of tremendous therapeutic properties and great history of use, there are very few studies in the literature which involved the safety evaluation of SBT leaf extracts. One such study conducted by Saggu et al. (2007) was based on safety assessment of the aqueous extract of SBT leaves using acute (with 4 different doses i.e. 1, 2, 5 and 10 g/kg body weight) and subacute rat models (with 2 different doses i.e. 1 and 2 g/kg body weight) for 14 days. In the present study, acute and subacute toxicity studies of 75% ethanolic extract of SBT leaves has been done in rats with single time oral dosing of 0.625, 1.25, 2.50 and 5 g/kg body weight (acute study) and after 28 days oral administration of 0.625 and 1.25 g/kg/day (subacute study).

The results of the acute toxicity studies indicated no mortality even after 14 days of extract administration, suggesting that LD₅₀ of 75% ethanolic SBT leaf extract is possibly higher than 5 gm/kg body weight in case of oral administration in rats. There are various studies on LD₅₀ determination of plant extract which report that substances with LD₅₀ higher than 5 g/kg via oral route may be considered practically non toxic [23-24], therefore, the observations from acute toxicity studies suggest that 75% ethanolic SBT leaf extract is practically non toxic up to a dose level of 5 g/kg body weight per day via oral route.

Similar observations i.e. no mortality and no symptoms of adverse health were recorded in case of subacute toxicity studies during 28 days. None of the hematological parameters in extract treated groups showed any significant changes when compared to control group. On the basis of these observations, it can be said that oral administration of SBT extract did not affect hematopoiesis, indicating the absence of anemia in extract treated animals. The changes in the hematological parameters in animals have been considered to be a significant indicator of human toxicity [25]. Thus, the hematological observations of the present investigation (Table 2) also suggest that 75% ethanolic extract of SBT leaves can be used for further toxicity studies in human subjects. However, a slight and non significant increase in most of the hematological parameters like WBC, platelet count, hemoglobin and hematocrit of the extract treated animals direct towards the need of further detailed investigation of the effect of this extract on various hematological parameters.

Examination of the biochemical parameters of the blood serum indicated no significant changes in extract treated groups (Table 3). Non significant difference in SGPT content of control and extract treated groups, indicates the absence of any harmful effects on liver. The plasma cholesterol level in extract treated animals remained unchanged which indirectly indicates normal liver functioning. Similarly, non significant difference in other biochemical parameters like creatinine indicate that there is no adverse effect on kidney functions after 28 days oral administration of 75% ethanolic SBT leaf extract in the dose range of 0.625 and 1.25 g/kg body weight [24, 26]. Determination of plasma protein may act as an indicator of synthetic

capacity of liver [27]. Unchanged plasma protein therefore suggests absence of any abnormality in the synthetic capacity of liver.

In addition to the hematological and biochemical observations, the results of the organ weight/ body weight ratio demonstrate that 75% ethanolic SBT leaf extract did not produce organ swelling, atrophy or hypertrophy. Further, absence of significant changes in organ weight/body weight ratio of kidney supports the results of biochemical evaluation (Table 4). Similarly, organ weight/body weight ratio of liver supports absence of any abnormality in liver of extract treated groups (Table 4). However, Saggu et al (2007) observed an increased liver organ weight/body weight ratio after oral dosing of 1 g/kg/day and decreased kidney organ weight/body weight ratio after oral dosing of 2 g/kg/day of aqueous SBT leaf extract in rats. It may be because these authors used aqueous extract of SBT leaves. Moreover, histopathological examinations of the rat treated with the extract at the dose level of 1.25 g/kg/day also evident the absence of any toxic effects on liver and kidney in extract treated groups when compared with control group (Figure 2 & 3).

CONCLUSION

On the basis of findings of the present study, it can be concluded that 75% ethanolic extract of dried SBT leaves was practically non toxic in rats after oral administration. It did not produce any behavioral, hematological, biochemical or histopathological symptoms of toxicity after 28 days of continuous oral administration (up to a dose of 1.25 g/kg/day), which supports the use of SBT leaves in traditional systems of medicine. However, further studies need to be performed in order to evaluate long term toxicities.

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CONFLICT OF INTEREST

No conflict of interest

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