ORIGINAL ARTICLE

Acute, Subacute and Subchronic Toxicological Studies of *Carissa Carandas* Leaves (Ethanol Extract): A Plant Active against Cardiovascular Diseases

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ABSTRACT

Objective: The Purpose of this research study was to examine the toxicological effects of aqueous: ethanol (1:1) extract of *Carissa carandas* leaves extracts in rats.
Methodolgy: Acute toxicity studies were conducted to check the LD_{50} values in experimental animals. Autopsy after acute toxicity revealed that no gross changes were observed in organs like liver, spleen, heart and kidney among the animals of group N (control) and S (treated). The appearance of organs of Group S animals was comparable with that of Group N animals.
Results: No signs of toxicity and mortality were observed in treated group after sub acute toxicity as compared to the control group. The histopathological studies after subchronic toxicity in doses of 1750mg/kg (p.o.) and 5000mg/kg (p.o) showed no toxic effects on organs like liver, heart, kidney and spleen. While chronic toxicity in dose 5000mg/kg (p.o.) showed some histological changes.

Key words: Carissa carandas, acute toxicity, subacute toxicity, chronic toxicity

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INTRODUCTION

Herbal medicines have been used for thousands of years. The practice continues today because of its biomedical benefits and place in cultural believes in many parts of the world. The economic reality of the inaccessibility of modern medications for many societies has also played a major role in the broad use of herbal medicines. The World Health Organization has recognized the contribution and value of the herbal medicines used by a large segment of world's population. A growing interest in usage has created the need for greater precision in preparation and evaluation and has stimulated research into herbal medicines' various uses and applications[1]. With the advances in knowledge, revitalization of the old system of medicine seems to be difficult. But, there are a large number of secrets of nature that are still needed to be discovered. The nature has gifted us with a rich flora, because of varied climatic conditions, soil and a vast area of cultivation there is a wide scope of research on natural source of medicines. In addition to the efficacy of medicinal plants it is observed they have fewer side effects as compared to known synthetic sources[2].

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Carissa carandas (Auct) belongs to family apocynaceae which consists of 300 genera and 1000 species. It is a large shrub with simple thorn and commonly cultivated throughout Pakistan for hedges and is called "Kakronda". The different parts of this plant have been used for various systems of medicine. Cardiotonic activity was found in root of this plant. This plant has been mentioned in the old chemical literature as purgative, antihelmintics and antidote for snake-bite. The physical characteristics of oil from the fruits of *Carissa carandas* were determined by using standard methods[3].

The roots of plant contain salicylic acid and cardiac glycosides causing a slight decrease in blood pressure. Also reported are carissone; the D-glycoside of B-sitosterol; glucosides of odoroside H; carindone, α terpenoid; lupeol; ursolic acid and its methyl ester; also carinol, a phenolic lignan. Bark, leaves and fruit contain an unnamed alkaloid[3].

MATERIALS & METHODS

Acute Oral Toxicity Studies On Mice:

Acute oral toxicity was carried out in vivo. All solutions were prepared using 2 ml of 0.9% Saline solution and administered per os using gastric tube[4]. The Acute oral toxicity study was conducted using the limit dose test of up and down procedure according to EPA Health Effects Test Guidelines on Acute Oral Toxicty OPPTS

870.1100 developed by United States Environmental Protection Agency (EPA12-C-02- 190 December 2002), at a limit dose of 5000mg/kg, starting with 1750mg/kg keeping n=10. Preliminary estimates of the LD50 helped in detecting a dose progression factor and a starting dose for testing[5]. Each animal was observed for body weight, signs of toxicity and mortality were observed after the administration at the first, second, fourth and sixth hour and then once daily for 14 days. On the day 15, all rats were fasted for 16-18 h, and then sacrificed for microscopic examination[6].

Subacute Oral Toxicity On Mice:

According to the procedure adopted by Welt et al., 2007, mice were divided into 2 groups of 6 mice in each group weighing between 25-30 gm. One group served as control group (N) and another group as treated group (S). Animals were fed for 14 days with the 5000 mg/kg/day of C.C.L.E. leaves extract[7]. The same pattern of procedure was adapted for the treatment with the Herbal Product (MUYM) 5000 mg/kg/day[6].

Histopathological Studies of Subacute Toxicity:

The Liver, heart, kidney and spleen of control and treated rats were taken out. These organs were weighed and examined for the evidence of gross lesions. Similar samples were fixed in 10% buffered formaline for 24 hours before being trimmed and then further processed by tissue processing method[8] i.e., dehydrated in graded (80-100%) alcohol, cleared in xylene, and placed and embedded in paraffin wax and in the form of small cubes of wax these organs were preserved. To perform histology of tissues, 6-8 µm sections were prepared with the help of Microtome (Leica, RM 2145). These sections then deparaffinated in xylene, passed through 80% to 100% alcohol, and stained with hematoxylin and eosin (H & E) for the assessment of liver, heart, kidney and spleen tissues histology of control and treated rats[9]. The slides prepared by this process were observed under microscope for the study of their cells[10]. (Curran 1990). These slides were photographed through Nikon Advanced Research Microscope OPTIPHOT Model X2T-21E equipped with Nikon Microphotography system; Model UFX-DX-35 and phase contrast N plan[11].

Chronic Toxicity on Rats:

Rats were divided in two groups of eight rats in each group. One group was control (N) and the other were test (S) with C.C leaves extract (C.C.L.E.) for eight weeks[12,13].

Biochemical Studies of ChronicToxicity:

At the end of eighth week, the blood samples from rats, approximately 6 to 7ml from each rat, were drawn by cardiac puncture with sterile disposable syringe, before dissecting the animals. Serum was separated by centrifugation at 2000 rpm for 15min by using BHG Hermle Z230 Centrifuge machine. The Chemical Kits were used (Diagnostica Merck, Germany) for biochemical analysis[7]. The serum level of Cholesterol, Triglyceride, HDL, LDL, SGPT, SGOT, Bilirubin (Total and direct), Gamma-G.T, Alkaline Phosphatase, Creatinine kinase, Glucose, Albumin, Creatinine, Urea, and Uric acid were determined by spectrophotometry using Hitachi U-2000 spectrophotometer on the same day[14].

Histopathological Studies of ChronicToxicity:

The Liver, heart, kidney and spleen of control and treated rats (C.C.L.E. Extract 5000mg/kg for 8 weeks) were taken immediately after the collection of blood; these organs were weighed and examined for the evidence of gross lesions. Similar samples were fixed in 10% buffered formaline for 24 hours before being trimmed and then further processed by tissue processing method[15]. i.e., dehydrated in graded (80-100%) alcohol, cleared in xylene, and placed and embedded in paraffin wax and in the form of small cubes of wax these organs were preserved.

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Statistical Analyses:

Data obtained during various tests were statistically analyzed using Student's t-test, one-way analysis of variance (ANOVA) and two-way ANOVA. All differences are considered significant at 5% level, therefore P-values less than 0.05 ($P<0.05$) were considered statistically significant. Our results are expressed as mean ± S.E.M[18,19].

RESULTS

Acute Toxicity in Mice:

Acute toxicity studies were conducted to check the LD50 values in experimental animals.

Behaviour

None of these animals showed any sign of toxicity for LD 50.

Table 1: Change in weight of control and C.C.L.E. (1750mg/kg) single dose (LD_{50}) treated mice after 14 days observation.

C.C.L.E. (1750mg/kg)	1st Day body wt.(g)	14^{th} Day body wt.(g)	% change in wt.	Mortality
Control (N)	27.4±1.026	27.8±1.772	-1.11±2.66	No expiry
Treated (S)	24.56±0.72	24.4±0.515	0.53±1.70	No expiry

n=10, Mean ± SEM

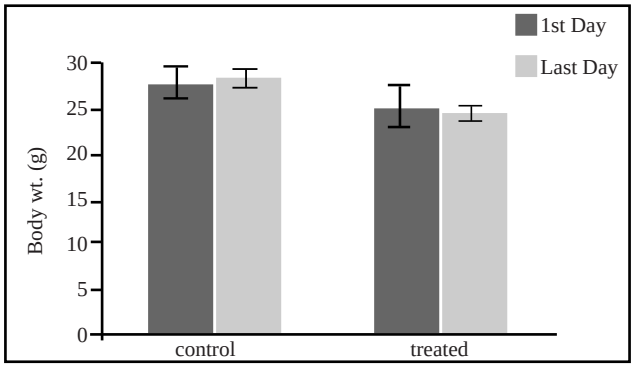

Fig. 1: Change in weight of control and C.C.L.E. treated mice (1750mg/kg) (LD_{50})

Table 2: Change in weight of control and C.C.L.E. treated mice (5000mg/kg) single dose (LD_{50}) after 14 days observation.

C.C.L.E. (5000mg/kg)	1st Day body wt.(g)	14^{th} Day body wt.(g)	% increase in wt.	Mortality
Control (N)	27.4±1.02	27.93±2.03	1.48±3.55	No expiry
Treated (S)	26.13±0.76	28.1±0.61	7.84±1.61	No expiry

n=10, Mean ± SEM

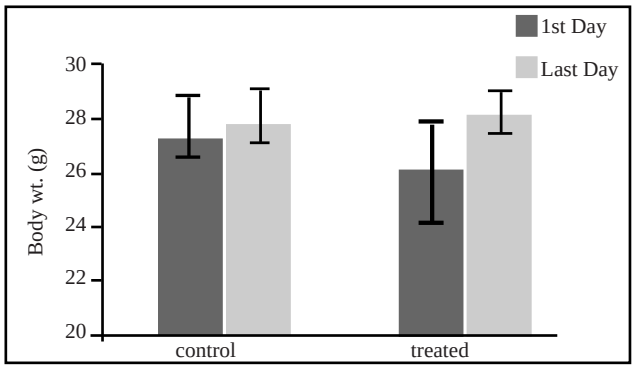

Fig-2 Change in weight of control and C.C.L.E. (5000mg/kg) treated mice for LD_{50}. * = $P<0.05$

SUBACUTE TOXICITY IN MICE

Body Weight (g)

Table 3: Change in weight of control and C.C.L.E. treated mice (5000mg/kg) after 14 days treatment.

(5000mg/kg)	1st Day body wt.(g)	14^{th} Day body wt.(g)	% increase	Mortality
Control (N)	23.575±2.13	23.856±2.12	1.24±1.55	No expiry
Treated (S)	30.65±1.76	31.833±1.36	3.24±2.49	No expiry

n=6, Mean ± SEM

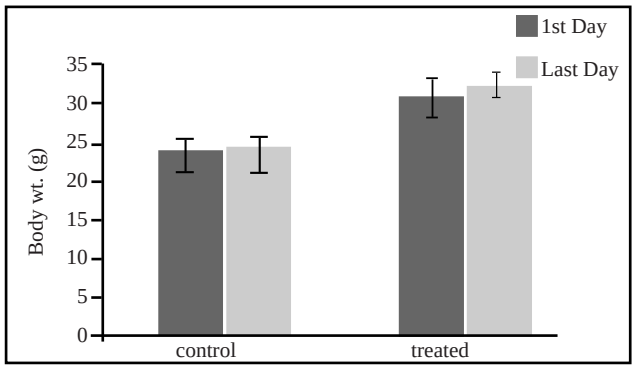

Fig. 3: Change in weight of control and C.C.L.E. treated mice (5000mg/kg) after 14 days treatment.

HISTOPATHOLOGICAL STUDIES OF SUBACUTE TOXICITY IN MICE:

CONTROL GROUP:(N)

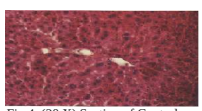

Fig 4. (20 X) Section of Control mice Liver for Subacute Toxicity

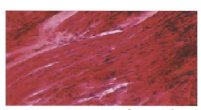

Fig 5 (20 X) Section of Control mice Heart for Subacute Toxicity.

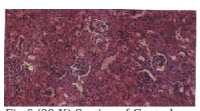

Fig 6 (20 X) Section of Control mice Kidney for Subacute Toxicity.

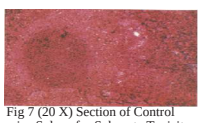

Fig 7 (20 X) Section of Control mice Spleen for Subacute Toxicity.

C.C.L.E. LEAVES TREATED GROUP: (S)

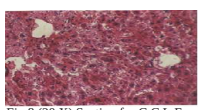

Fig 8 (20 X) Section for C.C.L.E. Leaves Extract effcects on Liver of mice showing Subacute Toxicity

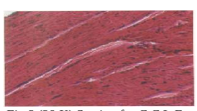

Fig 9 (20 X) Section for C.C.L.E. Leaves Extract effects on Heart of mice after Subacute Treatment

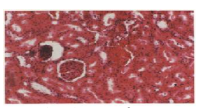

Fig 10 (20 X) Section for C.C.L.E. Leaves Extract effects on Kidney of mice showing Subacute Toxicity

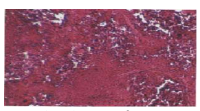

Fig 11 (20 X) Section for C.C.L.E. Leaves Extract effects on Spleen of mice showing Subacute Toxicity

DISCUSSION

For Acute toxicity studies, male and female mice treated with C.C leaves after single dose administration i.e.,1750 mg/kg (p.o) and 5000 mg/kg (p.o) respectively, did not show any mortality during the 14 days period of observation.

A non significant increase in body weight of control animals, and non significant decrease in treated animals' weight was observed at the end of 14th day of observation of single high dose oral treatment i.e., 1750 mg/kg. (Table-1, Fig- 1).

A non significant increase in body weight of control animals, and significant increase in treated animals' weight ($P<0.05$) was observed at the end of 14th day of observation of single high dose oral treatment i.e., 5000 mg/kg. (Table-2, Fig-2).

Autopsy revealed that no gross changes were observed in organs like liver, spleen, heart and kidney among the animals of group N (control) and S (treated). The appearance of organs of Group S animals was comparable with Group N animals.

For the 14 consecutive days the 5000 mg/kg/day dose was administered to mice to check the subacute. No signs of toxicity and mortality were observed in treated group as compared to the control group. Table 3 shows the effects of C.C.L.E. on the body weight. A non significant increase in body weight was detected on 14th day (Fig 3).

After Histopathlogical studies of subacute toxicities in control group of mice (N) it was observed that the liver cells are arranged into lobules in both control and treated slides. Liver cells hepatocytes are flat and arranged. A discontinuous layer of cells lines the sinusoids. Central vein is lined by epithelial cells and filled with biconcave Red Blood cells. Hepatic sinusoids appear to radiate from central vein. Fig 4 (20 X)

Cardiac muscles showed cross-striations and each muscle fiber showing a central nucleus. A distinguishing and characteristic feature of the intercalated disks. The myofibrils within each cell are well displayed. Histological pictures of cardiac muscle, demonstrating that the fiber dividing, then recombining and then spreading again. Each muscle cell possesses centrally located oval nuclei.Occasionally muscle cell posses two nuclei. Intercalated disc indicating intracellular junction between two cardiac muscle cells. The intercellular areas are richly supplied by capillaries[20]. Fig.5 (20X)

Kidney cortex components were observed. The Renal corpuscles in the center display a slight shrinkage artifact and thus clearly demonstrate Bowman's space. The renal corpuscles are surrounded by cross sections of proximal convulated tubules, distal convulated tubule and macula densa[20,21]. Fig 6 (20 X).

Spleen is subdivided into red pulp and white pulp. White pulp is arranged as a cylindrical sheath of lymphocytes i.e., periarterial lymphatic sheath (PALS) it surrounds a blood vessel known as central artery as shown in both control and treated spleen. Red pulp consists of sinusoids. While treated spleen contains an area of germinal center. Fig 7(20 X)

Histopathlogical studies of subacute toxicities in treated group (S) with *Carissa carandas* leaves extract. The liver showed spotty necrosis and mild inflammation[4]. Fig 8 (20 X).

Heart tissues are comparable to that of control animals Fig 9 (20 X).

The kidney slide show that, the cells lining the tubule are necrotic. The capillaries of the boundary zones are greatly distended and the stroma is edematous. Fig 10 (20 X).

Spleem slide depicted that venous sinuses are dilated and Endothelial cells lining them are prominent. The walls of the sinusoids are thicker and more fibrous than normal Fig 11 (20 X).

CONCLUSION

It is concluded by the toxicological studies of the extract on treated animals that the Extract C.C.L.E. is very safe in acute treatment even with a very high dose i.e., 5000mg/kg which did not show any mortality. But, the same dose has produced some deleterious effects on different tissues in mice, when treated sub acutely. These effects were further cross checked by treatment of the same high dose for a longer period on different group of animals now having rats as treated animals i.e., Chronic toxicity testing. Results have shown that some histopathological changes are occurring only at the renal levels which are confirmed by the Biochemical testing showing a high level of creatinine, albumin and glucose in serum. Other organs like Heart, Liver and Spleen are not affected as the biochemical data also confirms the safe use of this Extract.

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