World Journal of Science and Research. 5 (2): 01-11 (2020)

ISSN: 2455 2208

Available online at http://www.harmanpublications.com

World Journal of Science and Research

Research Article Biochemistry

ACUTE AND SUBACUTE TOXICITY OF ETHANOL EXTRACT OF *Caralluma indica* STEM ON HAEMATOLOGICAL, BIOCHEMICAL AND HISTOLOGY OF THE LIVER IN RATS

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ABSTRACT

Caralluma indica stem is a small erect, succulent plant up to 8 inches (20cm) tall. The leaves are small and reduced to scales. The flower stalks are up to 0.2inch (5mm). The stem is the major part of the plant. Therefore, the present study deals in acute and sub-acute (30-day) toxicity studies of *Caralluma indica* stem extract (CISE) on male and female Wistar rats administered through oral gavage. Acute toxicity study was conducted at a single oral dose of 1000, 1500, and 2000 mg/kg, body weight (b.wt.) for 14 days with a special emphasis on the first four hours after drug administration to find out any mortality and morbidity. In sub-acute toxicity, the extract at the doses of 100, 250 and 500 mg/kg, b.wt., was administered orally for 30 days. Important parameters such as general behaviour, body and organ weight, urinalysis, haematological and biochemical profile, organ macroscopy and microscopy were conducted. Organ sample of liver, kidney, pancreas, heart, lungs, and stomach were taken from both male and female rats, whereas the sample of testis and ovary was taken from male and female rats respectively for organ necropsy and histopathological studies. Neurobehavioral toxicity was conducted by performing a functional observational battery (FOB) and locomotor activity on the initial and final week of the 30-day study period. No mortality or any major signs of morbidity was recorded for acute toxicity except for the limit dose (2000 mg/kg, b.wt.) which produced a slight short-term sedative effect. In sub-acute toxicity, no major alteration was observed in the evaluated parameters. However, few minor changes were recorded for high dose (500 mg/kg, b.wt.) group. The results of the present study showed that oral administration of *Caralluma indica* ethanolic stem extract did not produce any severe toxic effects in both acute and sub-acute studies in Wistar rats. Therefore, usage of an appropriate amount of *Caralluma indica* stem extract preferably at low doses for its traditional use should be considered safe.

Article Info:

Received on 10th March2020
Accepted on 28th June 2020
Online June 2020

Keywords:
Acute and Sub-acute toxicity, Caralluma indica stem, Biochemical, Histology

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INTRODUCTION

A toxicological investigation is considered very essential for the development of new drugs. The US Food and Drug Administration (FDA) have stated that it is important to screen new molecules for toxicity and pharmacological activity in animals (Parasuraman, 2011). Many developing countries are using herbs and herbal products for their health care needs. In fact, few allopathic/western medicines are also derived from plants which are being used for treatment of several diseases in developing as well as developed countries. Ayurvedic medicine is still practiced in India where approximately 85% of the Indian population uses crude plant extract/formulations for the treatment of various diseases. However, the use of plants by ethnic people for curing diseases without knowing its adverse effects may cause health complications in later stages (Cock, 2015).

Worldwide, especially in developing countries there is a general old age myth that herbal drugs are safe and non-toxic but according to Zhang et al. (2015), the traditional uses of plants may cause adverse effects in humans or animals. A number of plants and their constituents traditionally used as medicines are suspected of being carcinogens to rodents and/or humans. Some of the traditionally used medicinal plants such as *Larrea tridentata (DC.), Piper methysticum G. Forst., Atractylis gummifera L., Callilepsis laureola DC.* etc., are known to cause human liver injuries (Moreira et al., 2014). Further, there is a scarcity in scientific evidence on the safety and efficacy of herbal drugs on the increase in a number of its users which has raised concerns regarding toxicity and detrimental effects of these herbal remedies. Thus, there is a need to evaluate the safety and efficacy of these medicinal plants thoroughly to maximize their benefits for mankind (Mohamed et al., 2011). Thus, the present work deals with the evaluation of acute and sub-acute (30-day) toxicity of *Caralluma indica* stem administered on Wistar albino rats through oral gavage.

MATERIALS AND METHOD

Animals

Male albino rats of Wistar strain approximately weighing 180-200 were used in this study. They were healthy animals procured from Sri Venkateswara Enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature 27±2ºC and 12 hrs light / dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (VRK Nutritional, Maharashtra, India) and water ad libitum. They were acclimatized to the environment for 1 week prior to experimental use. The experiment was carried out according to the guidelines of the Committee (Ethical No: PM/265/a/11/2019) for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Plant materials

The fully mature *Caralluma indica* stem was collected from Kathattiipattii, Sengipatti, village in the month of November 2017, Thanjavur District, Tamil Nadu, India. The stem was identified, authenticated (Figure 3.1) by Dr.John Britto, Director, and voucher specimen (RSV01) has been deposited at the Rapinat Herbarium, St. Josephs College, Thiruchirappalli, Tamil Nadu, India.

Preparation of plant extract

The collected stem of Caralluma indica were cut into small pieces and shade dried at room temperature and makes a fine powder using grinder mixture. The powder material of Caralluma indica stem were macerated with 70% ethanol at room temperature for 3 days. After 3 days, the supernatant was transferred into china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at 45oC. A semi solid extract was obtained after complete elimination of alcohol. The obtained residue was kept in the refrigerator for further use. The extract was made up to a known volume in distilled water just before oral administration.

Acute toxicity studies (OECD 2001)

Male Albino rats were randomly assigned into two groups of each six rats. Group 1 is control group, fed daily with only normal laboratory diet and water. Group 2 was treated with a dose of 5,000 mg/kg body weight for 14 days through an oral needle following a period of 10-h fasting. All animals were maintained on standard laboratory diets with water ad libitum. After administration of the extract, animals were monitored continuously for every two hours for a day to detect acute changes in morphological and behavioral responses, spontaneous activity, irritability, corneal refex, tremors, convulsion, salivation, diarrhea, lethargy if any, and also monitored for any mortality during the course of toxicity study.

Sub acute toxicity studies (OECD 2001)

Male Albino rats were randomly assigned into four groups of each six rats.

Group 1 is control group, fed daily with only normal laboratory diet and water. Group 2 to 4 was treated with a doses of 100, 250 and 500mg/kg body weight for 28 days through an oral administration. All animals were maintained on standard laboratory diets with water ad libitum.

Collection of blood and preparation of serum sample

At the end of the experimental period, the animals were anaesthetized using chloroform vapour prior to dissection. The blood, serum and plasma were collected with or without EDTA as anticoagulant. The blood was allowed to clot by standing at room temperature for 30 minutes and then refrigerated for another 30 minute. The resultant clear part was centrifuged at 3000rpm for 10minutes, and then the serum (supernatant) was isolated and stored at refrigerated until required for analysis. Plasma was also separated for the estimation of various biochemical parameters.

Haematological analysis

Haemoglobin was estimated by Cyanmethaemoglobin method (Dacie and Lewis, 1968) (Beacon Diagnostic Kit); RBC and WBC counting of the fish blood samples was carried out by the method of Ochei and Kolhatkar, (2000); Packed cell volume was determined by Ochei and Kolhatkar, (2000).

Biochemical Estimation

Malondialdehyde was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978); Reduced glutathione (GSH) was estimated by method of Moron et al (1979); Superoxide dismutase activity was determined by the procedure of Kakkar et al (1984); The level of ascorbic acid was estimated by the method of Omaye et al (1979); □-tocopherol was estimated by the method of Baker et al (1980); The serum alkaline phosphatase activity was estimated by the method of Kind and King's (1954); The serum GOT was estimated by the method of Reitman and Frankel (1957); The serum GPT was estimated by the method of Reitman and Frankel (1957); Urea was estimated by the method of Natelson (1957); Serum creatinine was carried out by alkaline picrate method of Boneses and Taussy (1954); The serum total bilirubin was estimated by the method of Malloy and Evenlyn (1937); Albumin was estimated by the method of Rodkey (1965); Protein was estimated by the method of Lowry et al. (1951); Globulin = Total protein – Albumin; A/G ratio = Albumin/Globulin.

Histological studies

After sacrifice the organ like liver of animals from each group were subjected for histological examinations. After fixing the tissues in Buins solution the tissues were dehydrated by passing through ethanol gradation and made ready for paraffin block preparation. The tissues were sectioned at the thickness of about 5µ. Routine histological studies were performed by using Haemotoxylin stain carried out by the method of (Ochei and Kolhatka, 2000).

Statistical Analysis

Data were expressed as mean ± standard deviation (n=6). The results were analyzed using one way ANOVA. Post hoc was also conducted to determine the level of significant difference between each treatment and the control group using Tukey-Kramer Multiple Comparisons Test. Test significant was considered at $P < 0.05$. The percentage mortality values are converted to probit values by reading the corresponding probit units from the probit table and plotted against the log10 of dose of extract (Lorke, 1983). From the graph equation of probit against log dose of the ethanolic extract, the log dose that responded to probit 5 (50% deaths) was calculated and its anti-log gave the LD50 value (Kasolo et al., 2011).

RESULTS AND DISCUSSION

There is increasing concern about the safety of use of the medicinal plants. There are general and herb specific concern regarding medicinal plants and their ability to produce toxicity and adverse effects (Saad *et al.*, 2006). Toxicity of medicinal plants may be related to the mixture of active compounds that they contain and stability of active ingredients in tissues. Phytotherapy having its pervasive use is substantiated by the affordability, its medicinal value and the belief of their harmlessness (Springfield *et al.*, 2005).

Lethal dose (LD50)

The animals having received ethanol extract did not exhibit marked behavioral changes but showed weak and less active movement followed by gradual death. The LD50, calculated from equation of % of mortality vs log dose (Fig. 1) of *Caralluma indica* stem ethanolic extract was found to be 2454.70 mg/Kg.

Table.1: LD_{50} Determination of *C. indica* stem extract against albino rats

Dose (mg/kg)	Log $_{10}$ Dose (mg/kg)	Exposed animal	Mortality	% of mortality
100	2.00	6	0	0.00
250	2.39	6	0	0.00
500	2.69	6	0	0.00
1000	3.00	6	1	16.66
3000	3.47	6	3	50.00
5000	3.69	6	5	83.33
LD_{50} Determination				
LD_{50} (50% of mortality) mg/kg			2454.70 mg/kg	

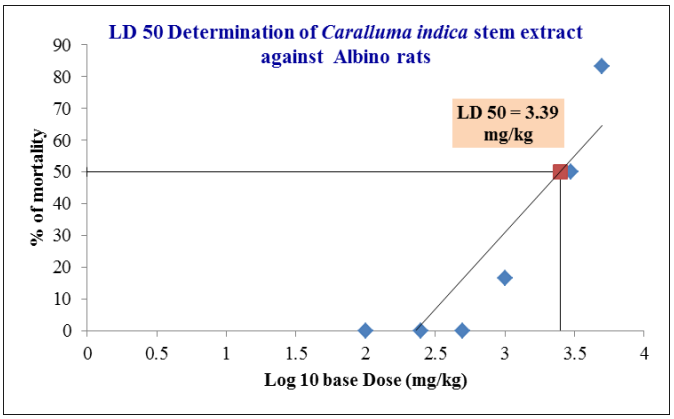


Fig.1: LD_{50} Determination of *C. indica* stem extract against albino rats

Accordingly most of the herbal preparations do not have drug regulatory approval to demonstrate their safety and efficacy (Seth and Sharma, 2004). It is therefore pertinent to establish the safety of medicinal plant preparations through toxicological assessments. Liver, being the primary organ for the detoxification and distribution of drugs, and the kidney, the major excretory organ, could be assessed to establish the safety of a substance (Gupta et al., 1994). The result of the current study showed that the LD50 of the crude aqueous extract of the plant was found to be greater than 2000mg/kg, which may be accepted as safe (OECD 2001). Other workers have reported different LD50 values for different plant extracts. The oral (rat) LD50 of ethanol extract of Vitex leucoxylon leaf (>3000mg/kg), cold water infusion extract of the same plant (1050mg/kg), ethanolic extracts of Ailanthus excelsa (1000mg/kg), Toddalia asiatica (350mg/kg) and Araucaria bidwilli (250 mg/kg) have been reported (Dahanukar et al., 2000). The LD50 of Boerhavia diffusa has been reported to be >2000mg/kg body weight in both mice and rats (Orisakwe et al., 2003). A. chevalieri leaf extract was also reported to be greater than 3000 mg/kg in rats (Saidu et al., 2007b).

ACUTE TOXICITY STUDIES

Effect of oral Caralluma indica stem ethanolic extract on body weights

The body and organ weights of rats organs of both treated and control groups are presented in Table 2 and 3. The acute oral ingestion of Caralluma indica stem ethanolic extract over 14 days caused no significant changes in the weights of the body and organ in the treated as compared to the control rat. The slight differences were due to normal biological growth of rat with time.

Table.2: Effect of extract on animal weight and mortality rate in acute toxicity study

S.No	Animals	Normal	Extract treated
1.	Animal weight		
	Initial (gm)	190±6.32	195±8.61
	Final (gm)	200±7.07	205±9.17
2.	Animal live (Nos.)	6	6
3.	Animal dead (Nos.)	Nil	Nil
4.	% of Mortality	Nil	Nil

Acute Oral Toxicity Effects of extract on rats were investigated. There were no animal deaths in rats receiving 2000 mg/kg of extract. No sign of toxicity was observed in the wellness parameters during the 14-day observation period. Therefore, the approximate acute lethal dose (LD_{50}) of extract in rats was estimated to be higher than 2454.70mg/kg.

Table.2a: Acute toxicity study of extract in wellness parameters of rats

Observations	Response	
	Normal	Extract (2,000 mg/kg body wt)
Consciousness	+	+
Grooming	-	-
Touch response	+	+
Sleeping duration	+	+
Movement	+	+
Gripping strength	+	+
Righting re flex	+	+
Food intake	+	+
Water consumption	+	+
Tremors	-	-
Diarrhea	-	-
Hyper activity	-	-
Pinna reflex	+	+
Corneal reflex	+	+
Salivation	+	+
Skin color	+	+
Lethargy	-	-
Convulsion	-	-
Morbidity	-	-
Sound response	+	+

Note: + indicate normal - indicate absent

Table.3: Effect of extract on liver, kidney and spleen weight in acute toxicity study

S.No	Organ (s)	Normal	Extract treated
1	Liver (g)	5.74±0.55 [a]	6.94±0.88 [a]
2	Kidney (g)	1.23±0.05 [a]	1.57±0.33 [a]
3	Spleen (g)	0.88±0.07 [a]	0.68±0.05 [a]

Values are expressed as Mean ± SD for six rats, Mean values within the row followed by different letters (Superscript) are significant ($P < 0.05$) level different from each other and same letter are non-significant were comparison by student "t" test.

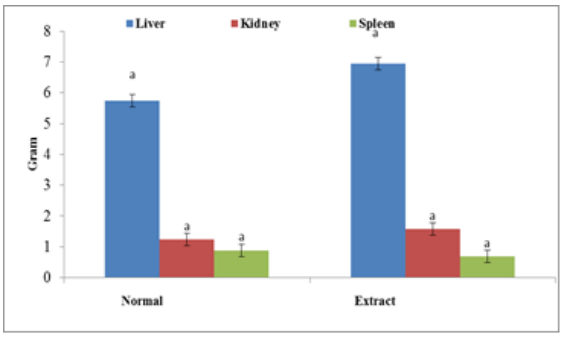
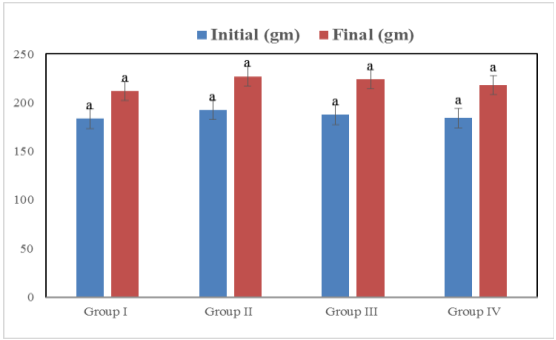


Fig.2: Effect of extract on liver, kidney and spleen weight

SUB ACUTE TOXICITY STUDIES

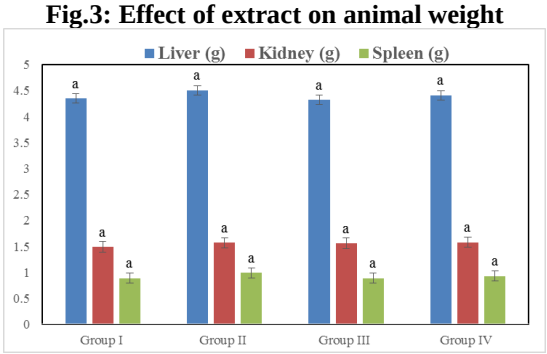
Effect of oral *Caralluma indica* stem ethanolic extract on body weights:

The body weight of rats organs of both treated and control groups are presented in figure 3 and 4. The Sub acute oral ingestion of *Caralluma indica* stem ethanolic extract over 14 days caused no significant changes in the weights of the body in the treated as compared to the control rat. The slight differences were due to normal biological growth of rat with time.



Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.3: Effect of extract on animal weight

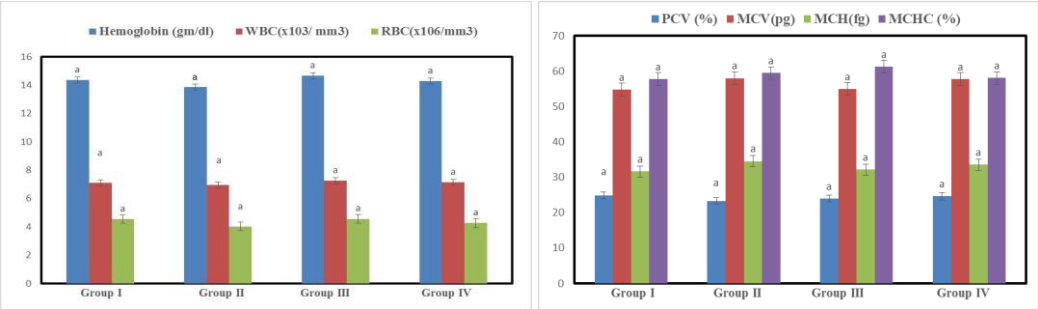


Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.4: Effect of extract on liver, kidney and spleen weight

Effect of oral *Caralluma indica* stem ethanolic extract on the hematological parameters

The haematopoietic system is one of the most responsive targets for toxic compounds and an important manifestation of physiological and pathological status in man and animal (Mukinda and Syce, 2007). The hematological parameters (i.e. RBC, WBC and haemoglobin) showed non-significant changes ($P > 0.05$) in the treated rats at as compared to the control rats (figure 5) was observed. These statistical differences (i.e. increases and decreases) appeared to be biologically relevant and consistent within the group suggesting they were treatment-related.

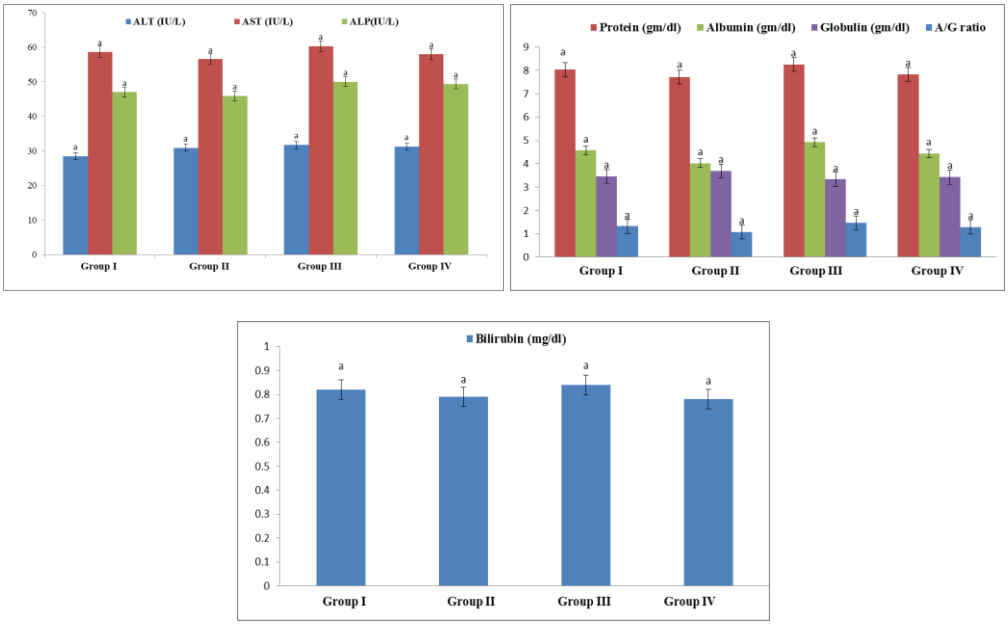


Values are expressed as Mean ± SD for six rats, Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.5: Effect of extract on Hematological profile

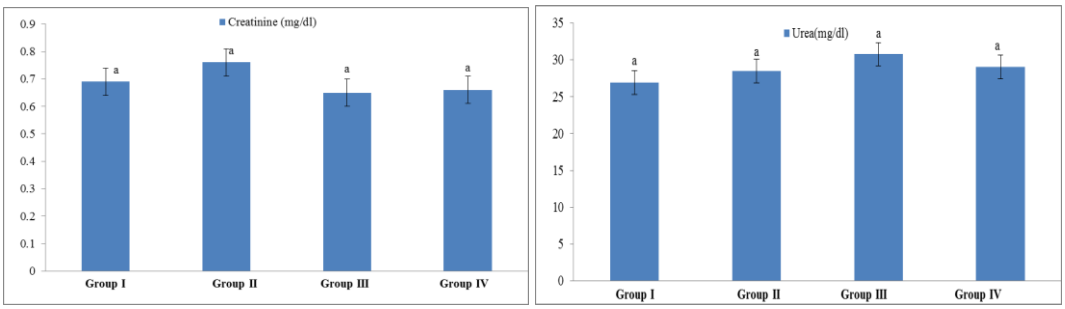
Effect of oral *Caralluma indica* stem ethanolic extract on liver and kidney markers parameters

The liver marker parameters (i.e. SGOT, SGPT, ALP, Albumin, Protein, Globulin, A/G ratio and Bilirubin) and kidney markers (i.e. urea and creatinine) showed non-significant ($P > 0.05$) in the treated rats at doses of 100, 250 and 500 mg/kg as compared to the control rats (Figure 6 and 7). These statistical differences (i.e. increases) appeared to be biologically relevant and consistent within the group suggesting they were treatment-related.



Values are expressed as Mean ± SD for six rats, Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.6: Effect of extract on Liver markers

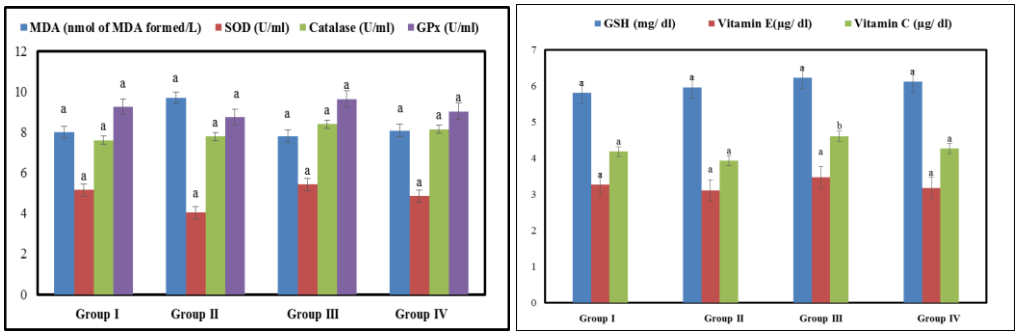


Values are expressed as Mean ± SD for six rats, Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.7: Effect of extract on Kidney markers

Effect of oral *Caralluma indica* stem ethanolic extract on oxidative stress marker parameters

The oxidative stress marker parameters (i.e. MDA) showed non-significant ($P > 0.05$) in the treated rats at doses of 100, 250 and 500 mg/kg as compared to the control rats (Figure 8). These statistical differences (i.e. increases) appeared to be increasing the oxidative stress and consistent within the group suggesting they were treatment-related.



Values are expressed as Mean ± SD for six rats, Mean values within the row followed by different letters (Superscript) are significant ($p<0.05$) level different from each other and same letter are non-significant were comparison by Tukey-Kramer multiple comparison test.

Fig.8: Effect of extract on MDA and antioxidant enzymes

In the current work there was a progressive increase in the body weights of the rats treated with different sub chronic doses of the 100, 250 and 500mg/kg extract of *Caralluma indica* stem. This may be an indication that the drug does not affect the feed utilisation ratio of the animals. The body weights of animals treated with sub-chronic doses of extracts of *Boerhavia diffusa* (Orisakwe *et al.,* 2003) and *A. chevalieri* leaf (Saidu *et al.,* 2007b) were also reported to increase progressively. Increased plasma total protein concentration observed in the current work at high doses may be due to dehydration and/or increased plasma immunoglobulin concentration due to infection.

The enzymes, AST, ALT and ALP showed progressive increase in activities at 100, 250 and 500 mg/kg doses administered, with most significant effect at the 500mg/kg. These findings imply that the extract, may at these doses, affect the liver. Serum ALT, ALT and ASP are useful indices for identifying inflammation and necrosis of the liver (Tilkian *et al.,* 1979). ALT has its highest concentration in the liver with kidney and skeletal muscles having lesser activity of the enzyme. ALT measurements are however more liver specific than the AST and its activity is usually greater than AST activity at early or acute hepatocelluar disease (Whitby *et al.,* 1989). AST on the other hand tend to be released more than the ALT in chronic liver diseases such as cirrhosis (Whitby *et al.,* 1989). Liver, bone placenta and intestine are clinically important sources of the plasma activity of ALP. The activity of this enzyme is increased in many clinical states; the most important being bone and liver diseases.

The extract of the stem of *Caralluma indica* causes a significant increase in serum urea and without change in creatinine level. This may be indicative that the extract may have little adverse effect on the kidney. This assertion may be due to the fact that creatinine is usually a more accurate marker of kidney function than urea. Although elevated levels of creatinine and urea are positive risk of renal impairment. A lower blood level of creatinine does not indicate impairment. The increased in serum urea levels recorded in the present study may be due to factors other than kidney problem (Baker *et al.*, 2001). A measured level of MDA is used as a direct index of oxidative stress and tissue damage associated with lipid peroxidation. It reacts with thiobarbituric acid and produce red-coloured product (Ohkawa *et al.*, 1979). In the present study, significantly increased level of MDA was observed in higher doses, suggested that higher doses of *Caralluma indica* stem increase the stress. Among the various doses, minimal effective dose as 250mg/kg is better for further studies.

Histopathological studies

Histological studies revealed no abnormalities in liver tissue in extract treated rats. After Histopathlogical studies of subacute toxicities in liver of control and treated group of rats were 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 predominant nucleus. Similar type of observation was also seen by Bello *et al*. (2016) in rat liver.

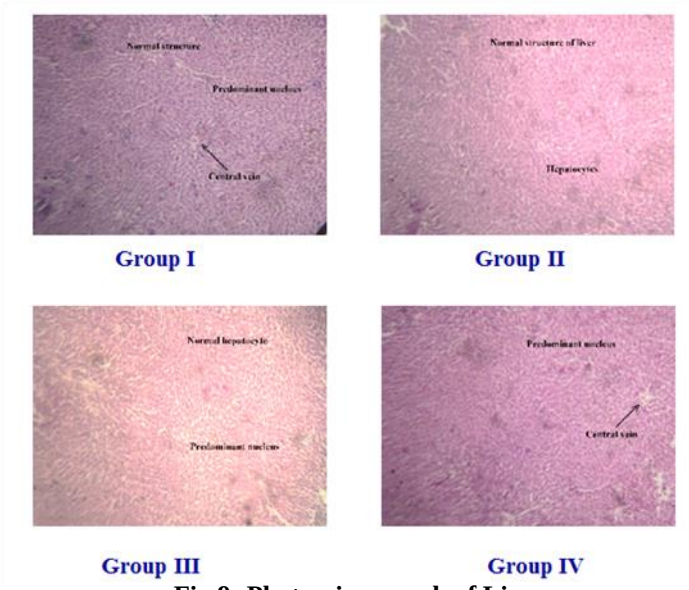


Fig.9: Photomicrograph of Liver

CONCLUSION

Results from this study provides important information and data on toxicological properties of a rare and scientifically unexplored mangrove plant *Caralluma indica* stem, which is traditionally used as a potent pain reliever by the local healers. In the evaluation of acute toxicity, oral administration of *Caralluma indica* stem at a dose up to 2000 mg/kg b.wt., did not produce any major toxicological effects except for mild short-term sedation after administration of the limit test dose of 2000 mg/kg b.wt. In the sub-acute study, no severe treatment-related toxicity was observed during the study after the rats were administered with *Caralluma indica* stem up to a dose of 500 mg/ kg b.wt., for a period of 30 days. Among the different dose, minimal effective dose as 250 mg/ kg b.wt., chosen for further experimental study. Almost all the parameters were normal without any major changes, however minor alterations in few parameters were observed which may or may not be treatment related thus carrying little or no toxicological importance. However, further study is required to investigate and confirm its safety and effectiveness in humans

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Source of support: Nil;
Conflict of interest: None declared