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Studies on Acute and Sub-Chronic Toxicities of N-Hexane Seed Extract of Ricom-1013-J in Wistar Rats

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Abstract

The study investigated the acute and sub-chronic effects of the n-hexane seed extract of RICOM-1013-J in biochemical and haematological parameters of mature adult female Wistar rats with an average weight of 110 g. The objective of the study was to determine the immediate, short-term and prolonged toxicological profile of the extract. Thirty-four (34) adult female rats were randomly divided into three groups (n=8) of rats. Two animals (group I) and coded A received 0.2 ml/100 g rat of vegetable oil, and served as control. Two other animals (group II) coded B received 5 mg/kg, subcut. of the extract, a third set of animals (group III) code C was administered 20 mg/kg, subcut. The last two animals (group IV) coded D were injected with 30 mg/kg, subcut. of the extract. The same dosage regimen was administered to a different set of animals of equal number in groups II and III in pairs, and coded accordingly. SGOT, SGPT, urea, total protein, alkaline phosphatase, bilirubin and haematological assays were carried out at weeks I, IV and VIII in groups I, II and III respectively. Findings from the experiment showed no significant (P>0.05) differences in the mean values of the biochemical parameters and haematological indices test groups relative to control. The study concluded that the n-hexane seed extract of RICOM-1013-J is relatively safe in rats when administered subcutaneously.

Keywords: Biochemical parameters, Coded, Haematological indices, Optical density, Ricinus communis, RICOM-1013-J, Toxicological profile.

INTRODUCTION

*Ricinus Communis Linn. (*RICOM-1013-J) *(*Castor bean or castor oil plant) belongs to the species of perennial flowering plant in the spurge family (Euphorbiaceae), and the only species in the family. It is a robust, hairless annual or perennial herb, shrub or small tree. Ricinus communis is indigenous to north-eastern tropical Africa. It is found across the continent of African, from the Atlantic coast to the Red sea and from Tunisia to South Africa, as well as the Indian Ocean islands. The castor bean contains 46-53 % fixed oils which are mainly glycosides of ricinoleic, isoricinoleic, stearic, dihydroxystearic acids (Trease and Evans, 1997). The cake left after expressing the oil contains very poisonous toxins known as ricins, and thereby makes it unfit as cattle feed. Ricin possesses anti-tumour activity. The seed also contains lipases and crystalline alkaloid (ricinine), which is not very toxic. Ricinine is structurally similar to nicotinamide (Trease and Evans, 1997). Ricinus communis is used in ethnomedicine as a purgative due to its powerful trigger effect on intestinal peristalsis, and also has abortifacient property. Okwuasaba *et al.,* (1996) showed that oral administration of 1-5 seeds of RICOM -1013-J protected women against pregnancy for about 9-12 months. Other pharmacological effects demonstrated by RICOM-1013-J are possession of oestrogenic activity; lack of progestational effect; reduction in basal gastric acid secretion and contraceptive activity (Okwuasaba *et al.,* 1997a,b). Isichei *et al.*, (2000) demonstrated the clinical efficacy of RICOM-1013-J in protecting women against pregnancy for 1-year in fifty (50) volunteers. The study investigated the acute and sub-

chronic toxicities of RICOM-1013-J on mature adult female Wistar albino rats.

MATERIALS AND METHODS

Plant Collection

The seeds were supplied by Dr. O. Azija, a traditional medical practitioner and consultant herbalist attached to the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Jos. The plant was identified and authenticated by field botanists/taxonomists in the Departments of Botany at the University of Lagos and Ahmadu Bellow University, Zaria, as well as the Forestry Research Institute, Jos. A voucher specimen of the plant was deposited at the herbarium of the Department of Pharmacognosy, University of Jos, Nigeria.

Extraction and Preparation of Extract

The testae of RICOM-1013-J seeds were removed and the decoated seeds were physically and finely pulverised using motar and pestle. The generated powdery material weighing 134.4 g was subjected to exhaustive soxhlet extraction using 250 ml of n-hexane for 72 h at a temperature of 67-69 °C. The resulting viscous oily extract was allowed to cool and then weighed. The oily residue was stored in the refrigerator at 4 °C until required for experiment. The extraction process yielded 66.5 g or 49.5 % of the total powdered material. In all the experimental session, appropriate extract weight was dissolved in measured volume of olive oil (solvent) to generate the right concentration in order to make up for test solution of desired concentrations (mg/ml), expressed in terms of extract dry weight.

Animal Preparation

Adult female albino rats (Wistar strain) weighing 100-120 g were used for the study. They were housed at the animal facility of the University of Jos. These animals were bred in well ventilated rooms (temperature: 22.0±2.5 °C, relative humidity: 65.0±5.0 %, illumination: 12 h light/ 12 h dark cycle), and were maintained on standard pellet diet (Vital Feeds, Jos, Plateau State, Nigeria). The animals had access to safe drinkable water *ad libitum*.

Drugs, Chemicals and Equipment

Micro-haematocrit centrifuge (Hawskey, England), micro-haematocrit reader (Hawskey, England), microscope (Olympus Tokyo, Japan), mettler balance (Gallen Kamp, United Kingdom), n-hexane (Sigma-Aldrich Inc., USA), stop clock, petri dishes, feeding tubes, dissecting kit, tape measure, weighing balance, colorimeter, mortar and pestle, heparinised tubes, water bath, methylated spirit, needles and syringes, and scissors.

Ethical Consideration

The Institutional Research Ethics Committee of the University of Jos through the Postgraduate School approved the proposal for the work.

Acute Toxicity

The Lorke's method (Lorke, 1983) was used for the determination of the LD_{50}. Adult female rats weighing 100-120 g were divided into five groups (n=5) and were administered graded doses of 1 g/kg, 2 g/kg, 4 g/kg, 8 g/kg, 16 g/kg of n-hexane seed extract subcutaneously. The animals were allowed access to drinkable water and food *ad libitum*. They were observed for any signs toxicity (e.g., change in water and food intake, behavioural change and the number of deaths) occurring within the first 24 h.

Sub-Chronic Toxicity

Adult female rats weighing 100-120 g were divided into three groups (n=8). Two animals in group one (1) coded a received 0.2 ml/100 g rat of vegetable oil, and served as control. Two other animals coded B received 5 mg/kg of n-hexane extract, while third group coded C was administered 20 mg/kg of extract. The last two animals coded D were injected 30 mg/kg of the extract. In another set of experiment, the same dose regimen was injected in groups two and three in pairs, and coded accordingly. Analysis of AST (Aspartate Transaminase) or SGOT (Serum Glutamic Oxaloacetic Transaminase), ALT (Alanine Transaminase) or SGPT (Serum Glutamate Pyruvate Transaminase), urea, total protein, alkaline phosphatase, bilirubin and haematological parameters were undertaken after week-1 (Day 7), week-4 (Day 28) and week-8 (Day 60) in groups 1, 2 and 3 respectively.

Effect of Extract on Biochemical and Haematological Parameters

Blood sample for biochemical analysis was collected and centrifuged (12,000 rpm) with the resulting clear sera subjected to examination using different assay protocols: Transaminases (SGPT and SGOT) (Tietz, 1970); Conjugated bilirubin (Billing *et al.,* 1971); urea (Marsh *et al.,* 1965) and total protein (Biuret method).

Effect of Extract on Red Cell Count

The method of Baker and Silverton (1976) was adopted in the study. The rat was placed on a restrainer and the tail held firmly *via* a hole to the outside. The tip of the tail was then sterilised with 70 % ethanol. Phlebotomy was carried out on the animal and the initial few drops were collected for red cell count determination.

Effect of Extract on the Packed Cell Volume

The procedure described by Baker and Silverton (1976) was adopted. Blood samples were collected from the cut tail of the rat with heparinised capillary tubes up to the three-quarter mark. The free

end of the tubes was sealed off with plasticine. The tubes were then placed in micro-haematocrit centrifuge, and centrifugation performed at 12,000 rpm for 5 min. The packed cell volume was then read using a micro-haematocrit reader.

Effect of Extract on Haemoglobin Concentration

Haemoglobin concentration was determined using the oxy-haemoglobin method. Haemoglobin is converted to oxy-haemoglobin by dilution with ammoniated water. Blood was drawn to the 0.02 ml mark of the haemoglobin pipette and was added to previously measured 2 ml of 0.04 % ammoniated water in a test tube. This was then thoroughly mixed and allowed to stand for 5 min. Another 2 ml of ammoniated water was again added to the test tube making a total volume of 4 ml. The optical density of the mixture (ammoniated water plus blood was then read in a colorimeter at 520 wavelength). The concentration of haemoglobin (g/100 ml of blood) was calculated according to Baker and Silverton, 1976.

$$\text{Hb (g/dl)} = \frac{\text{OD Test} \times [\text{Hb}]}{\text{OD Standard}}$$

OD Test = Optical density of test substance
OD Standard = Optical density of test standard haemoglobin
[Hb] = Concentration of haemoglobin per 100 ml of blood

Effect of Extract on White Cell Count

The method previously described by Baker and Silverton (1976) was used to determine the white cell count. The rat was placed on a restrainer and the tail held firmly *via* a hole to the outside. The tip of the tail was then sterilised with 70 % ethanol. Phlebotomy was carried out on the animal and the initial few drops were collected for white cell count determination.

Effect of Extract on Platelet Count

The protocol described by Baker and Silverton (1976) was employed in the work. The rat was placed on a restrainer and the tail held firmly *via* a hole to the outside. The tip of the tail was then sterilised with 70 % ethanol. Phlebotomy was carried out on the animal and the initial few drops were collected for platelet count determination.

Effect of Extract on Bleeding Time (Duke's method)

Sterilisation of tail of the rats was performed using 70 % ethanol. This was followed by puncturing with a disposable lancet while a stop-watch was started simultaneously. The blood from the puncture site was gently blotted every 15 sec using filter paper until the bleeding stopped. The interval between the time when the skin was punctured to the time the wound stopped bleeding was recorded as the bleeding time.

Effect of Extract on Clotting Time

The protocol for the determination of clothing time was analogous to the Duke's method for bleeding time. In the study, the first 1-2 drops of blood from the puncture site on the rat's tail were collected on a clean glass slide. The tip of a sterilised pin was used to stroke through the blood continuously until a streak of blood was first observed with the pin, an indication of clot formation. This was then recorded as the clotting time.

Statistical Analysis

Results were expressed as the mean±SEM and the level of significance determined by Student's t-test. A probability level of equal to or less than 5 % ($P \leq 0.05$) was accepted as statistically significant.

RESULTS

Acute Toxicity

The LD_{50} of RICOM-1013-J was 63.2±16.0 mg/kg, subcut. RICOM-1013-J did not demonstrate any signs of toxicity (e.g., reduced motor activity, prostration, loss of appetite, rapid respiratory rate, restlessness, weight loss, loss of righting reflex, salivation, convulsion, tremor, gasping, or death) within the acute period of the study. The acute toxicity test revealed that administration of n-hexane seed extract of RICOM-1013-J at a mega dose (16,000 g/kg, subcut.) was safe in experimental animals.

Sub-Chronic Toxicity

Pre-Treatment with n-Hexane Seed Extract of RICOM-1013-J on Biochemical Parameters

Table 1: Day 7 Post Treatment

Serum Parameter	Control	5 mg/kg	20 mg/kg	30 mg/kg
ALP (IU/L)	245.00±33.00	220.00±28.00	250.00±41.00	236.00±39.00
SGOT (IU/L)	46.50±4.60	43.20±9.80	48.60±5.10	44.10±5.80
SGPT (IU/L)	28.40±3.90	30.60±9.80	27.00±4.50	31.50±4.20
Total Protein (g/100 ml)	18.11±1.20	21.33±2.40	18.66±2.00	58.11±2.00
Urea (mg/100 ml)	57.14±3.10	51.14±3.10	56.24±2.40	-
Bilirubin (µmol/L)	-	-	-	-

Table 2: Day 28

Serum Parameter	Control	5 mg/kg	20 mg/kg	30 mg/kg
ALP (IU/L)	202.00±29.00	242.00±32.00	259.00±41.00	234.00±38.00
SGOT (IU/L)	46.50±5.60	43.00±11.0	42.20±10.20	45.60±6.00
SGPT (IU/L)	29.40±4.50	29.6±9.80	32.60±4.20	30.80±9.00
Total Protein (g/100 ml)	24.89±2.40	21.44±1.80	25.11±2.10	24.66±2.10
Urea (mg/100 ml)	57.44±2.00	56.14±2.20	57.43±3.00	57.14±1.80
Bilirubin (µmol/L)	-	-	-	-

Table 3: Day 60

Serum Parameter	Control	5 mg/kg	20 mg/kg	30 mg/kg
ALP (IU/L)	241.00±42.00	259.00±41.00	245.00±38.00	258.00±45.00
SGOT (IU/L)	45.30±5.80	49.40±6.10	44.00±5.80	49.60±5.00
SGPT (IU/L)	29.20±4.50	35.60±3.80	30.10±4.60	31.60±4.30
Total Protein (g/100 ml)	23.90±1.80	24.10±2.30	25.40±2.00	25.40±2.00
Urea (mg/100 ml)	57.10±3.00	54.60±3.20	57.60±2.80	57.10±2.80
Bilirubin (µmol/L)	-	-	-	-

Data was expressed as mean±SEM. No significant (P>0.05) differences in the biochemical parameters between control and the study group (n=6).

Table 4: Effect of n-Hexane Seed Extract of RICOM-1013-J on Haematological Indices Day 7 of the Experiment

Dose (mg/kg)	RBC 10^6/mm^3	WBC X10^3/mm^3	Platelets X10^3/mm^3	BT min	CT min	PCV %	Hb mg/ml
CTR	8.8.40±0.74	29.75±4.55	120.00±7.00	1.52±0.15	2.06±0.46	49.50±0.70	15.70±0.40
5	5.85±0.96	17.30±6.90*	44.50±2.50	2.47±0.56	2.22±0.15	51.00±4.24	16.45±0.95
20	6.38±1.25	18.10±3.50*	36.00±6.00	2.50±0.15	2.21±0.46	47.50±1.50	14.90±0.80
30	7.05±0.03	17.70±2.20*	41.50±2.00	3.34±0.44	1.37±0.10	48.20±2.00	15.10±1.25
	P>0.05	P<0.05 on day-7 for all doses	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
Day 28							
CTR	6.13±0.45	23.05±2.60	80.00±3.00	3.07±0.12	2.12±0.30	47.00±1.00	13.25±0.85
5	6.89±0.41	23.30±2.30	70.00±1.50	3.20±0.20	2.21±0.11	48.60±2.90	12.50±0.38
20	6.40±0.56	23.03±1.80	66.70±1.30	2.47±0.60	2.21±0.40	48.00±1.70	15.70±0.26
30	6.34±0.18	22.67±1.90	119.7±1.70	3.35±0.18	2.25±0.11	51.60±1.80	14.00±0.26
Day 60							
CTR	6.36±0.76	20.00±3.00	115.30±3.10	2.50±0.05	2.00±0.23	54.00±2.50	17.00±0.80
5	6.50±0.45	19.35±1.40	116.50±2.50	3.07±0.08	2.05±0.15	55.50±2.50	16.60±0.40
20	7.10±0.59	22.80±0.59	119.00±1.00	3.00±0.04	1.24±0.18	56.00±2.10	18.20±0.80
30	7.60±0.26	29.16±2.30	116.00±1.70	2.41±0.16	1.19±0.08	53.00±2.50	16.80±0.54

Data was expressed as mean±SEM. No significant (P>0.05) differences between the treated animals relative to control on day-28 and day-60 of the experiment for all the haematological indices. However, there was a significant (P<0.05) difference in all the test doses for WBC count on day-7.

Effect of the Extract on Biochemical Parameters

There was no significant (P>0.05) effects of n-hexane seed extract of RICOM 1013-J on the liver and kidney functions on day-7, day-28 and day-60 in relation to control.

Effect of Extract on Red Blood Cell Count

The n-hexane seed extract of RICOM 1013-J did not demonstrate any significant (P>0.05) effects on the red blood cells.

Effect of Extract on White Blood Cell Count

The effect of the extract on the white blood cells was significant (P<0.05) on day 7 of the study. However, there was no significant (P>0.05) differences between test and control groups on subsequent days post treatment with the extract.

Effect of Extract on Platelet Count

RICOM-1013-J did not demonstrate any biological effects on the platelet count between the test and control groups of the animals.

Effect of Extract on Bleeding and Clotting Time

No significant (P>0.05) differences was observed between test and control experiments.

Effect of Extract on Packed Cell Volume and Haemoglobin Concentration

The two haematological indices did not indicate any significant (P>0.05) differences between the treated animals and control.

DISCUSSION

The study investigated the acute and sub-chronic effects of the n-hexane seed extract of RICOM-1013-J in biochemical and haematological parameters of mature adult female Wistar albino rats. LD_{50} of the n-hexane seed extract of RICOM 1013-J was determined using Lorke's method (Lorke, 1983). Toxicity testing is used to determine the hazardous nature of drugs, chemical substances, edible and medicinal plants, and cosmetics, which are absorbed into our body through various routes (e.g., enteral, parenteral and topical/local). The toxicity of a substance is inversely proportional to the LD_{50} value (i.e., substances with low LD_{50} will be more toxic than those with high LD_{50}). The converse is also true. RICOM-1013-J n-hexane seed extract at 20 mg/kg, subcut. did not produce any signs of toxicity in experimental animals. There was no signs of neurotoxicity *viz.* excitability, changes in motor activity, convulsion, postural or gross behavioural abnormalities. There was also no autonomic dysfunction such as defecation, urination, salivation or lacrimation. Animal testing is used to demonstrate the presence of acute toxicity (short-term toxicity), and is usually characterised by lethality and exposure frequency used in experimental designs. Two major classes of acute toxicity have been reported (Arya and Bist, 2022), and they include, the acute toxicity and STOT-SE (Specific Target Organ Toxicity - Single Exposure). The classification is hinged on the evidence of death, which is commonly reported as median lethal dose or median lethal concentration (LD_{50}/LC_{50}) value. STOT–SE is usually considered where there is a clear and unambiguous pathological evidence of toxicity to a specific organ (e.g., gut, liver, kidneys, lungs, heart, brain etc.), especially when there is absence of death in the experimental animals. In the study, neither acute toxicity nor STOT-SE was experimentally demonstrated in specific organ study with the liver, kidney or blood indices (e.g., tables 1– 4 above). At doses of 200 mg/kg, subcut. and 2000 mg/kg, subcut., no signs of toxicity was observed. This indicates that the extract is relatively non-toxic and safe, and in concordance with the wide range of safety previously reported for LD_{50} (63.2±16 mg/kg, subcut.) (Okwuasaba *et al.*, 1997a, 1997b; Das *et al.*, 2000).

RICOM-1013-J had no significant adverse effects on both the biochemical and haematological parameters in rats, which is in concordance with earlier findings (Das *et al.,* 2000; Dafur *et al.,* 2002). In the study, no significant differences in the mean values of all liver enzymes was observed relative to control. Many liver function tests (LFTs) are based on a wide variety of chemical reaction that may be used to measure different aspects of hepatic function. Since there is no single test which effectively measures total liver function, there is the need to perform several of them such as enzyme assays, total proteins and bilirubin estimations. In the absence of pregnancy and bone diseases, alkaline phosphatase levels generally reflect impaired hepatic excretory functions (Herfindal *et al.,* 1992; Finlayson and Bouchier, 1995). No such disorders was demonstrated in the study, as evidenced by normal enzyme assays at all the three dose (5, 20 and 30 mg/kg, subcut.) levels used in the study. The transaminases have demonstrated to be the most practical indices or measures of hepatocellular injury, and both alanine and aspartate transaminases invariable increase in all forms of liver dysfunction. The effect of RICOM-1013-J at graded doses used in the study did not demonstrate toxicity to the liver, as there was no traces of bilirubin in the serum of the Wistar rats tested. Elevated serum levels of bilirubin, especially conjugated bilirubin is a pointer to hepatic disorder, when there is excretory impairment of conjugated bilirubin as seen in prolonged biliary obstruction or intra-hepatic cholestasis. The values of the total serum protein in the test and control groups did not demonstrate any significant differences. In a similar vein, the n-hexane seed extract of *Ricinus communis* did not produce any toxic effects on the kidneys, as the blood urea profile was not significantly different between test and control groups in the experimental animals. Total serum protein estimation measures the amount of protein in the blood as proteins are critical for healthy growth of the cells and tissues of the body. It is employed in the assessment of the state of the liver and kidneys. In the study, there were no significant differences between the test and control groups in the values of the total serum protein concentrations, an indication that RICOM-1013-J had no adverse effects on hepato-renal function of the test animals.

The haematological indices examined in the work also, did not implicate the n-hexane seed extract of RICOM- 1013-J as having any short-term or long-term potential for haematotoxicity. The result of the blood parameters assay corresponds with that of the biochemical estimation of being relatively non-toxic and safe in animals. In all, the findings from the toxicological screening of the n-hexane seed extract of RICOM-1013-J are in tandem with previous research by Das *et al.,* (2000) and Dafur *et al.,* (2003).

CONCLUSION

The study demonstrated that the n-hexane seed extract of RICOM-1013-J is relatively toxic and safe when administered subcutaneously to experimental animal subjects. These findings suggest their extensive ethnomedical application.

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Contribution of Authors

JUI carried out the laboratory work. FKO designed the study and provided guide for the work. CNN reviewed the experimental data analysis and wrote the manuscript, while PCN and POA proof read the work and made useful suggestions.

CONFLICTS OF INTEREST

The authors had no conflicts of interest to declare.

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