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ABSENCE OF ORGAN SPECIFIC TOXICITY IN RATS TREATED WITH TONICA, AN AQUEOUS HERBAL HAEMATINIC PREPARATION

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Abstract

The sub-chronic toxicity of Tonica, an aqueous herbal haematinic prepared from the stem barks of *Khaya senegalensis*, *Mitragyna stipulosa* and *Kigelia africana*, was investigated in male Sprague-Dawley rats at 28, 280 and 560 mg kg^{-1} day^{-1}, representing the normal human dose, 10x and 20x that dose, respectively for 6 weeks. The growth rate of animals over the period of treatment and certain serum biochemical and haematological indices as well as urinalysis and weight of selected organs at termination, were determined. Results show that the extract did not affect the weight gain of the animals with time or the mean wet weights of selected organs. Although there were slight but insignificant ($p>0.05$) elevations in WBC (16-27%) and PLT (8-11%) counts in Tonica-treated animals compared to controls at 10x and 20x the normal dose, most serum biochemical, haematological and urinalysis data indicated no significant differences ($p>0.05$) between tests and control rats. There were also no changes in the morphology of liver, kidney, lung and heart tissues as a result of Tonica treatment. These findings suggest that Tonica is safe at the dosage regimens administered to the animals in this study, and there appears to be no overt organ specific toxicity associated with it.

Key words*: Khaya senegalensis; Mitragyna stipulosa; Kigelia africana;* sub-chronic; toxicity

Introduction

There is growing concern worldwide about the efficacy, safety, quality, availability, preservation and further development of plant medicines for health care delivery. Although many traditional medicines have promising potentials, many are untested and their use not monitored. As a result, knowledge of their potential side effects is limited making the identification of the safe and most effective therapies and the promotion of their rational use very difficult (WHO, 2002).

Tonica is an aqueous herbal haematinic prepared from the stem barks of *Khaya senegalensis*, *Mitragyna stipulosa and Kigelia africana*. *K. senegalensis* belongs to the Mahogany family (Meliaceae). Studies have shown that aqueous and ethanolic extracts of the stem bark of *K. senegalensis* have anti-helminthic, anti-proliferative, gastrointestinal motility and anti-inflammatory activities (Ademola et al., 2004; Egesie et al., 2004; Androulakis et al., 2006). However, the ethanolic extract of *K. senegalensis* administered to rats at 2 mg kg^{-1} body weight for 6 and 18 days showed signs of renal toxicity (Adebayo et al., 2003).

M. stipulosa belongs to the Madder family (Rubiaceae). It is widely used for the treatment of inflammation, hypertension, headache, rheumatism, gonorrhea and broncho-pulmonary disease. The methanolic extract of *M. stipulosa* has been demonstrated to possess anti-inflammatory activity, and that alkaloids and kaemferol derivative, may be responsible for its anti-inflammatory properties (Dongmo et al., 2003).

K. africana belongs to the bat flower family (Bignoniaceae). Generally, the stem bark of Kigelia is noted to contain iridoid and its derivatives, napthaquinones, monoterpenoids, isocourmarins, lignans, sterols and flavonoids (http://www.healthbells.co.za//kegelia Tech.htm.). The aqueous extract and two major iridoids

showed significant antimicrobial activity, validating the anecdotal use of the plant in traditional medicine as a natural antibacterial and antifungal agent (Akunyili et al., 1991). *In vitro* studies demonstrated that four naphthoquinoids from Kigelia root bark showed anti-malarial activity against chloroquine-sensitive (T9-96) and resistant (KI) *Plasmodium falciparum* strains (Weiss et al., 2000).

Despite the extensive use of these plants there is no evidence of their use either singly or in combinations for the treatment of anaemia, making Tonica a novel natural product for the treatment of anaemia. Recent clinical evaluation of Tonica at the Centre for Scientific Research into Plant Medicine (CSRPM) indicated that it is an effective and safe haematinic for use in humans (Adusi-Poku et al., 2008).

The pre-clinical safety evaluation of new medicinal products, involving the determination of their effects on certain haematological, blood chemistry and histopathological parameters as indices of organ specific toxicity, is a pre-requisite for their clinical studies and subsequent registration by drug-regulatory agencies like the Food and Drugs Board (FDB) of Ghana, Committee on Safety of Medicines (CSM) of the UK and the Food and Drugs Administration (FDA) of the USA. Hence the pre-clinical safety evaluation of Tonica in Sprague-Dawley rats in our laboratory prior to its evaluation in humans is presented in this paper.

Materials and Methods

Reagents and Chemicals

Test kits: aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamylaminotransferase (GGT), bilirubin (direct and total), albumin, creatinine, urea, creatinine kinase (CK-MB) were purchased from Cypress Diagnostics (Belgium). Urine test strips (UroColor^{TM} 10) were supplied by Standard Diagnostics Inc. (Kyonggi-do, Korea). Pentobarbital was obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were purchased in the purest form available from British Drug Houses (BDH) Ltd. (Poole, UK).

Preparation of Tonica Extract

Tonica was prepared from the stem barks of three different plant species: *Khaya senegalensis, Mitragyna stipulosa* and *Kigelia africana* **(**CSRPM voucher specimen numbers 175, 233 and 306, respectively**)** in kilogram quantities (8:4:2) in 110 litres of sterile distilled water. This was boiled for one hour, cooled to room temperature, and re-boiled for another hour. The extract was sieved and freeze-dried (Heto Power Dry LL 3000, Denmark) to give a dry extract yield of 72000 mg kg^{-1} plant raw material mixture (7.2% w/w) and stored in a cool dry place. The extract was reconstituted in sterilized distilled water before administration to animals.

Animals

Male Sprague-Dawley rats (200-250 g) were obtained from the Animal Unit of the CSRPM, Mampong-Akuapem, in the Eastern Region of Ghana. The animals were fed *ad libitum* on powdered feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were allowed free access to sterile distilled water. Studies were conducted in accordance with internationally accepted principles for laboratory animal use and care.

Treatment of Animals

Four groups of six rats each were kept in four separate cages. Group one was kept as control and received sterilized distilled water for six weeks. Groups two, three and four were treated with 28, 280 and 560 mg kg $^{-1}$ body weight, respectively of Tonica daily by oral gavage for six weeks. The animals in each group were weighed on day zero (baseline) and weekly thereafter.

Histology

At the termination of treatment, four rats each from control and Tonica-treated groups were euthanized by cervical dislocation and the heart, lungs, liver, kidney and spleen were excised and weighed by a Mettler balance (Mettler Toledo, Switzerland). With the exception of the spleen, the other four organs were fixed in 10% formaldehyde and dehydrated with a progressively increasing concentration of alcohol. The tissues were cleared with chloroform and impregnated with paraffin wax. Sections were cut, stained with haematoxylin and eosin and mounted on slides for light microscopic examinations. Tissue sections of the four organs of four other rats at baseline were also prepared (Baker and Silverstone, 1985).

Urinalysis

Urine samples of the control and Tonica-treated rats produced as a result of involuntary discharges were collected on clean ceramic tiles at baseline and at termination of treatment and analysis of urine for glucose, bilirubin, ketones, specific gravity, pH, proteins urobilinogen, nitrate, blood and leukocytes was done using urine reagent strips (UroColor^{TM} 10, Standard Diagnostic Inc., Korea).

Blood Sampling

Blood samples of control and Tonica-treated rats were obtained by tail bleeding, at baseline and at termination of treatment, into Eppendorf tubes without anticoagulant, centrifuged at 4000 x g for 5 min (Denley BS 400, England) and serum stored at -40ºC for biochemical analyses. Other blood samples were collected into separate tubes pre-coated with tri-sodium citrate (Westergreen E.S.R, UK) for haematological analysis within 24 hrs.

Serum Biochemical Analysis

Serum ALT, AST, GGT, total and direct bilirubin, albumin, CK-MB, creatinine and urea levels of samples of control and Tonica-treated rats were determined using protocols from Cypress diagnostic kits (Belgium) with a semi-automated blood chemistry analyzer; photometer 4040 (Robert Riele G & Cole-2000, Germany).

Haematological Analysis

Red blood cell (RBC) counts, mean cell volume (MCV), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell (WBC) counts, haemoglobin (Hb), platelet counts (PLT) mean platelet volume (MPV), Platelet distribution width (PDW) and lymphocytes (LYMPH) of control and Tonica-treated animal blood samples were determined with Haema-screen 13 (Hospitex Diagnostics, Italy) in accordance with established protocol. The equipment is computerized to automatically determine and display the above hematological profile for each blood sample.

Statistical Analysis

One-way analysis of variance (ANOVA) and independent sample t-Test was conducted between control and test to determine statistical significance. The 5% level of probability was used as criterion of significance in all instances. All statistical tests were performed with SPSS statistical software version 11.0

Results

Changes in Rat Body and Organ Weights

Figure 1 shows a graphical representation of the percentage change in body weight following treatment of rats with Tonica extract. There were significant increases ($p< 0.05$) in body weight over the experimental period for all treatment groups. The slight reductions in growth between test and control groups were insignificant ($p>0.05$) and not dose-dependent. Table 1 shows the effect of Tonica on mean organ wet weights expressed as percentage of body weight at termination of treatment. There were insignificant differences ($p>0.05$) in the relative weights of the heart, lungs, liver, kidney and spleen between control and Tonica-treated animals.

Histology

The effect of Tonica extract on the morphology of the rat heart, kidney, liver and lungs at termination are represented in Figures 2-5 (see legends of figures). Results showed no significant morphological changes in cardiac tissue (Figures 2 a-d), glomerular and renal tubular areas of the kidney (Figures 3 a-d), and the liver (Figures 5 a-d) between control and Tonica-treated animals. The lungs also did not show any significant changes in Clara and alveolar cells as well as the bronchiolar epithelial lining between Tonica-treated and control animals (Figures 4 a-d).

Table 1: Mean Organs Wet Weights at Termination of Treatment

	Organ weight /body weight ratio (%)			
	Treatment group			
		Tonica		
Organ	Control	28 (mg kg $^{-1}$)	280 (mg kg $^{-1}$)	560 (mg kg $^{-1}$)
Heart	0.30 ± 0 .02	0.33 ± 0.01	0.30 ± 0.03	0.32 ± 0.01
Lungs	0.51 ± 0.05	0.65 ± 0.13	0.51 ± 0.01	0.48 ± 0.01
Liver	3.09 ± 0.06	3.11 ± 0.42	3.38 ± 0.02	3.42 ± 0.10
Kidney	0.53 ± 0.05	0.57 ± 0.04	0.59 ± 0.02	0.65 ± 0.02
Spleen	0.22 ± 0.01	0.19 ± 0.02	0.21 ± 0.01	0.19 ± 0.01

Results are means ± SEM of n = 6

Table 2: Urine Parameters at Termination of Treatment [a]

	Treatment group			
		Tonica		
Parameter	Control	28 (mg kg $^{-1}$)	280 (mg kg $^{-1}$)	560 (mg kg $^{-1}$)
Urobilinogen (mg dL^{-1})	N	N	N	N
Glucose (mg dL^{-1})	–	–	–	–
Bilirubin (mg dL $^{-1}$)	–	–	–	–
Ketones (mg dL $^{-1}$)	±	±	±	±
Density (g mL $^{-1}$)	1.03	1.03	1.03	1.03
Blood (RBC µL $^{-1}$)	–	–	–	–
pH	6.83	6.33	7.00	6.67
Protein (g L $^{-1}$)	+	+	+	+
Nitrite	–	–	–	–
Leukocytes (WBC µL $^{-1}$)	–	–	–	–

Figures represent means of 6 determinations. (–): Absent; (N): Normal; (±): Trace; (+): Positive. [a] Baseline values of treatment groups were not different from controls at termination of treatment

Table 3: Serum Biochemical Data at Termination of Treatment [a]

	Treatment group			
		Tonica		
Parameter	Control	28 mg kg $^{-1}$	280 mg kg $^{-1}$	560 mg kg $^{-1}$
AST (U L $^{-1}$)	46.0 ± 1.41	47.0 ± 5.37	44.3 ± 4.68	48.6 ± 5.66
ALT (U L $^{-1}$)	26.2 ± 1.59	24.2 ± 1.16	26.2 ± 4.67	28.3 ± 2.29
GGT (IU L $^{-1}$)	1.54 ± 0.19	1.51 ± 0.20	1.39 ± 0.31	1.42 ± 0.34
Total bilirubin (µmol L $^{-1}$)	2.12 ± 0.47	2.50 ± 0.62	1.94 ± 0.33	2.64 ± 0.89
Direct bilirubin (µmol L $^{-1}$)	0.72 ± 0.12	0.64 ± 0.27	0.70 ± 0.20	0.86 ± 0.23
Urea (mmol L $^{-1}$)	7.99 ± 0.40	8.04 ± 0.36	7.79 ± 0.46	6.26 ± 0.20
Creatinine (µmol L $^{-1}$)	54.8 ± 2.75	53.2 ± 2.46	70.6 ± 4.02	65.6 ± 2.48
CK-MB (U L $^{-1}$)	416 ± 129	331 ± 40.5	390 ± 42.6	362 ± 16.9
Albumin (g L $^{-1}$)	57.2 ± 5.47	57.4 ± 4.46	55.6 ± 6.15	53.8± 5.47

Results are means ± SEM of n = 6. [a] Baseline values of treatment groups were not different from controls at termination of treatment.

Table 4: Haematological Parameters at Termination of Treatment [a]

	Treatment group			
		Tonica		
Parameter	Control	28 (mg kg $^{-1}$)	280 (mg kg $^{-1}$)	560 (mg kg $^{-1}$)
RBC (x10^{6} µL $^{-1}$)	8.48 ± 1.00	8.38 ± 0.90	8.14 ± 1.04	9.02 ± 1.15
MCV (µ^{3})	64.4 ± 0.81	65.4± 0.93	61.2 ± 2.35	64.8 ± 2.56
HCT (%)	58.9 ± 3.06	61.1 ± 1.08	59.8 ± 2.54	58.2 ± 7.40
MCH (pg)	20.5 ± 2.05	20.8 ± 1.44	18.9 ± 0.76	18.1 ± 1.48
MCHC (g dL $^{-1}$)	35.7 ± 6.75	32.0 ± 2.82	30.9 ± 1.17	28.2 ± 2.45
RDW (%)	29.3 ± 2.20	28.9 ± 0.93	28.1 ± 2.04	30.7 ± 1.87
WBC (x10^{3} µL $^{-1}$)	11.9 ± 1.57	11.1 ± 1.56	13.8 ± 1.68	15.1 ± 1.22
Hb (g/l)	16.7 ± 0.87	16.9 ± 1.11	15.1 ± 1.56	15.7 ± 0.84
PLT (x10^{3} µL $^{-1}$)	762 ± 200	761 ± 52.6	846 ± 36.1	814 ± 29.3
MPV (µ^{3})	11.0 ± 0.59	10.3 ± 0.43	10.7 ± 0.56	10.6 ± 0.20
PCT (%)	1.21 ± 0.29	0.79 ± 0.09	1.45 ± 0.44	0.91 ± 0.13
PDW (%)	26.2 ± 2.33	27.9 ± 4.71	25.1 ± 1.39	23.6 ± 0.37
LYMPH (%)	96.4 ± 1.71	96.9 ± 1.60	98.0 ± 1.39	99.0 ± 0.44

Results are means ± SEM of n = 6. [a] Baseline values of treatment groups were not different from controls at termination of treatment

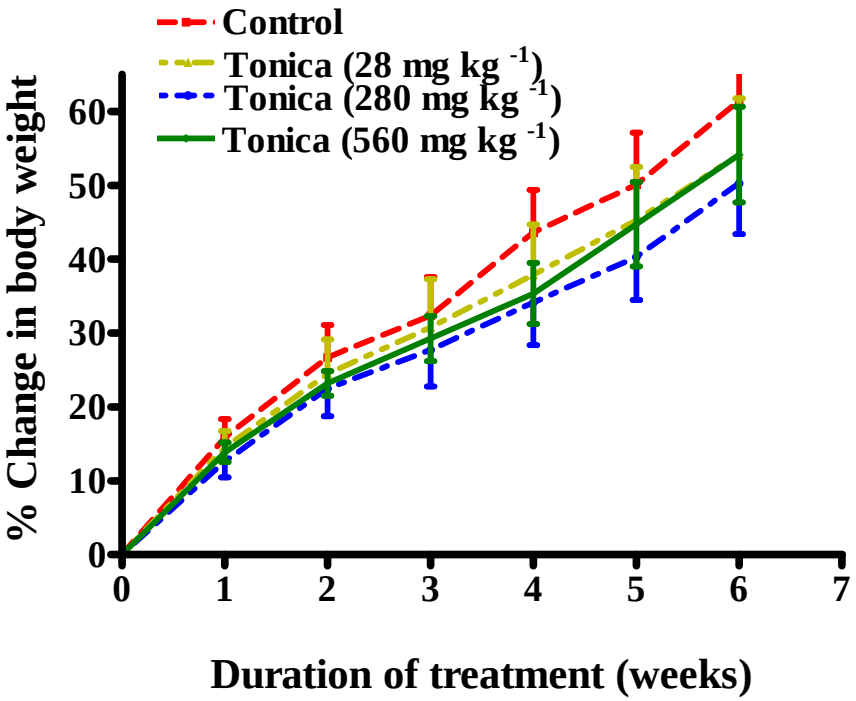

Figure 1: Percentage changes in mean body weight of rats with duration of Tonica treatment. Each point represent mean ± SEM (n = 6). For details of treatment regimen, see "Materials and Methods"

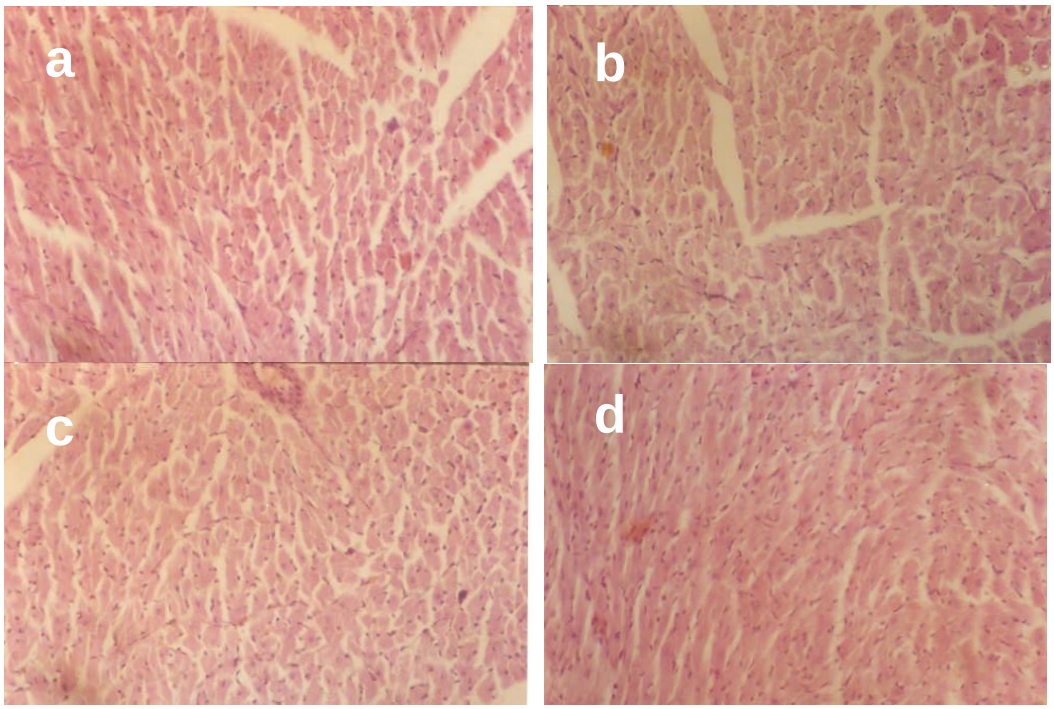

Figure 2: Histological appearance of the heart of control animals (a) and animals treated with normal dose of Tonica extract: 28 mg kg $^{-1}$ (b), 10x the normal dose: 280 mg kg $^{-1}$ (c) and 20x the normal dose: 560 mg kg $^{-1}$ (d) at termination showing no differences in morphology of cardiac tissue. Magnification: x66.

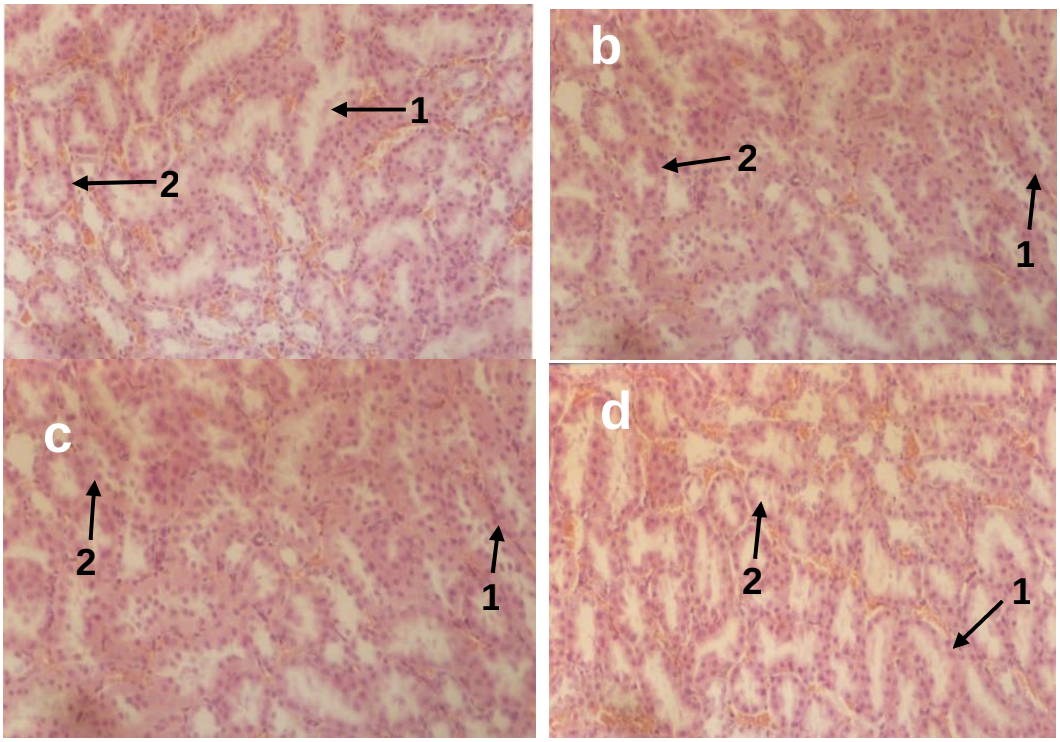

Figure 3: Histological appearance of the kidney of control animals (a) and animals treated with normal dose of Tonica extract: 28 mg kg $^{-1}$(b), 10x the normal dose: 280 mg kg $^{-1}$(c) and 20x the normal dose: 560 mg kg $^{-1}$(d) at termination showing no differences in tubular (1) and glomerular (2) areas compared to control. Magnification: x66.

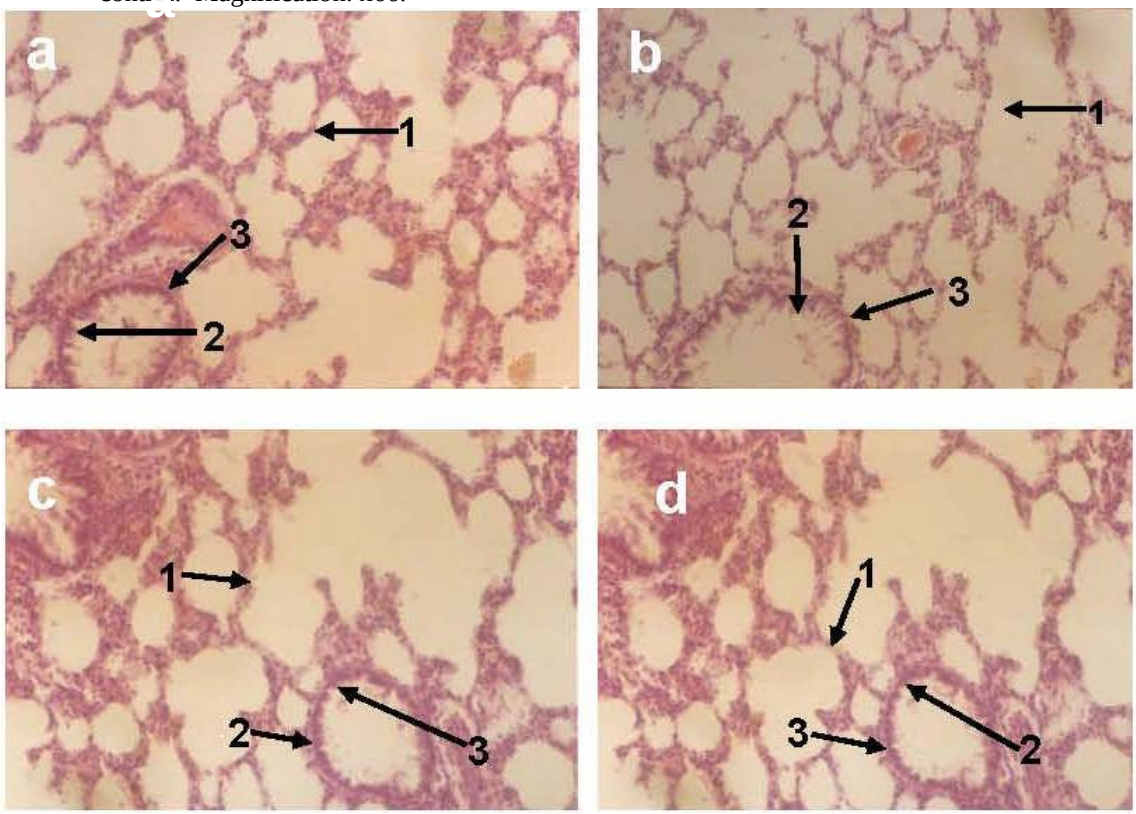

Figure 4: Histological appearance of the lung of control animals (a) and animals treated with normal dose of Tonica extract: 28 mg kg $^{-1}$(b), 10x the normal dose: 280 mg kg $^{-1}$(c) and 20x the normal dose: 560 mg kg $^{-1}$(d) at termination showing normal alveolar areas (1) and Clara cells (2) lining a normal bronchiolar epithelial wall (3). Magnification: x66.

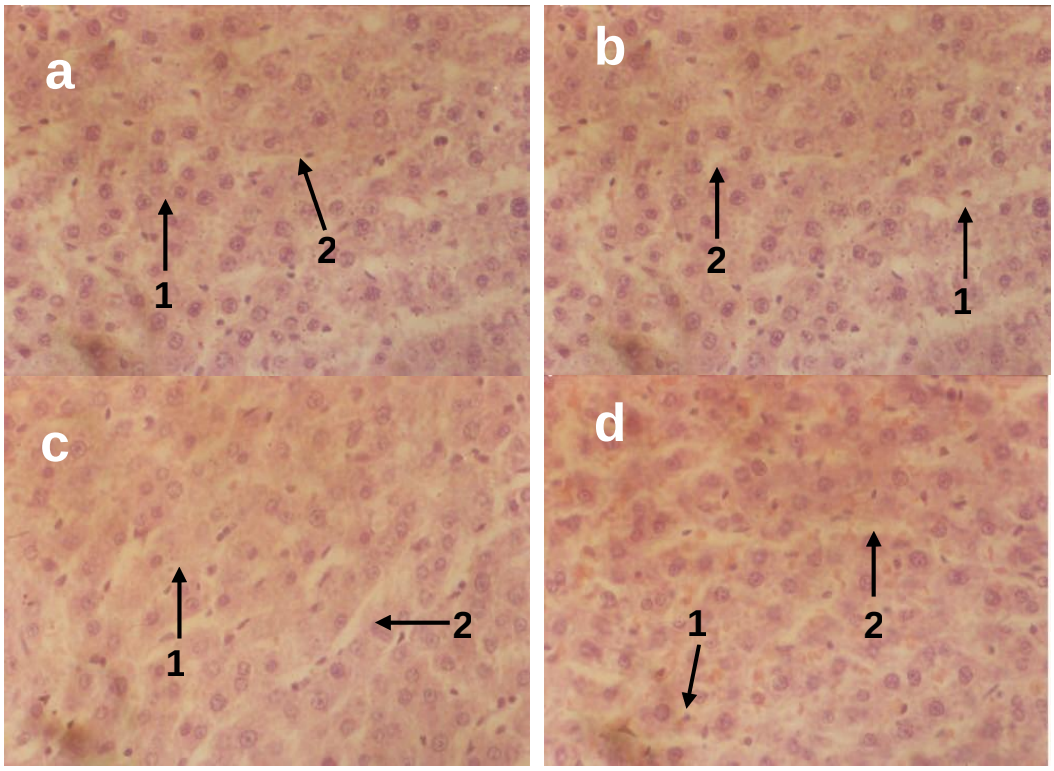

Figure 5: Histological appearance of the liver of control animals (a) and animals treated with normal dose of Tonica extract: 28 mg kg $^{-1}$(b), 10x the normal dose: 280 mg kg $^{-1}$(c) and 20x the normal dose: 560 mg kg $^{-1}$(d) at termination showing no differences in appearance of hepatocytes (1) and interstitial spaces (2) compared to control. Magnification: x132.

Urinalysis Data

Dipstick urinalysis data of control and Tonica-treated animals at termination of treatment is shown in the Table 2. Results indicated that there were no significant differences in urine blood, bilirubin, urobilinogen, proteins, ketones, glucose, nitrite, leucocytes, pH and specific gravity between Tonica-treated and control animals. Baseline values for each treatment group (results not shown) were similar to control values at termination.

Serum Biochemical and Haematological Data

The effect of Tonica treatment of rats on some serum biochemical and haematological parameters at termination of treatment are presented in Tables 3 and 4. No significant differences ($p>0.05$) were observed in serum levels of ALT, GGT, albumin, AST, total and direct bilirubin, urea, creatinine and CK-MB and the haematological indices measured between Tonica-treated animals and controls. Baseline values for each treatment group (results not shown) were similar to control values at termination.

Discussion

Anaemia is the principal nutritional problem in the world and affects mostly children and pregnant women in developing countries. According to the WHO, nutritional anaemia is a state in which the haemoglobin concentration of the blood is lower than the levels considered normal for the age, gender and physiological state and altitude as a consequence of the shortage of essential nutrients, independent of the cause of this deficiency (De Mayer et al., 1989; Coutinho et al., 2005).

Tonica is a novel aqueous herbal haematinic that has been used by the CSRPM for over 20 years at its clinic for the treatment of anaemia in pregnant women and anaemia due to other causes. Recent studies have shown that Tonica administration to patients over a 14-day period caused the elevation of blood Hb levels at a rate of 1.66 g dL^{-1} per week which is 66% faster than most known allopathic haematinics (Hope et al., 1999; Adusi-Poku et al., 2008).

Haematological studies in the animals indicated that the Toncia extract did not cause any significant changes in haematological indices (Table 4). The WBC and LYMP counts were slightly but insignificantly increased an indication that it may enhance, to some degree, the defences of the body to fight infections or cellular injury and may be of benefit to HIV/AIDS patients whose defences are normally compromised. The absence of any change in Hb levels on Tonica treatment in animals is at variance with the 1.66 g dL^{-1} increase per week found in humans (Adusi-Poku et al., 2008). This suggests species differences in the effect of Tonica on Hb levels, and that the rat may not be a good animal model to study the efficacy of Tonica as a haematinic. Tonica does not appear to directly damage blood cells or indirectly cause damage to the bone marrow.

The absence of any morphological changes in selected organs like the liver, kidney and heart (Figures 2, 3 and 5), which are corroborated by urinalysis and serum creatinine and urea data for renal function, serum AST, ALT, GGT, bilirubin and albumin for liver function and serum CK-MM for the cardiac function (Table 3), are indicative of the fact that Tonica did not adversely affect any of these three organs. Despite the observation that *K. senegalensis*, at a dose of 2 mg kg^{-1} for 6-18 days, caused impaired renal function in animals (Adebayo et al., 2003), Tonica which contains *K. senegalensis* did not show such an effect, suggesting possible modulating effect of the other chemical components provided by the other plants contained in Tonica. Furthermore, morphological studies of the lung did not show any signs of Clara cell and/or alveolar cell damage as a result of Tonica treatment as shown by other pneumotoxicants like butylated hydroxytoluene, 4-ipomeanol and 1,1-dichloroethylene (Krijgsheld et al., 1983; Okine et al., 1986; Forkert et al., 1990). These observations are further supported by the lack of effect of Tonica treatment on the mean wet weight of these organs. Reduction or increase in size and weight of organs may be a reflection of loss or absorption of water as a result of changes in osmoregulation due to either tissue damage or changes in osmolality of the ECF (Curtis et al., 1999).

Tonica has been shown to increase the appetite of patients (Adusi-Poku et al., 2008) and this has been attributed to the presence of *Khaya senegalensis* in the Tonica extract, which is known to stimulate appetite (Mshana et al., 2000). This enhanced appetite can lead to increased rate of growth, especially in children. However, our study showed that Tonica did not significantly affect the growth of the animals. There was also no evidence of increased intake of food to suggest the enhancement of the appetite of the animals as a result of Tonica treatment.

Conclusion

These findings show that Tonica is highly tolerated even at 20x the normal dose and that there is no overt organ specific toxicity associated with it on sub-chronic administration to the rats.

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