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ARTICLE

Effect of Sub-Chronic Administration of *Tetracera potatoria* Roots Extract and Betulinic Acid from the Plant on Haematology and Serum Biochemistry of Wistar Rats

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ABSTRACT

Tetracera potatoria is used as natural remedy for a wide range of diseases in West Africa. Anti-ulcerogenic and anti-inflammatory effects of *T. potatoria* are reported to be induced by Betulinic acid (BA). This study was aimed at assessment of the safety of *T. potatoria* and BA using acute and sub-chronic toxicity methods.

Methanol extract of *T. potatoria* root (100, 500 and 1000 mg/kg) and BA (10, 20 and 40 mg/kg) isolated from the extract were administered to Wistar ratsfor 28 consecutive days in the sub-chronic toxicity study. Each experiment had control groups (n=5) which were administered with distilled water (10ml/kg). Whole blood and serum samples were collected from the rats for hematology and serum biochemistry on day 29.

T. potatoria extract induced significant decreases in PCV from 42.8±1.5% (control) to 32.7±2.2% (1000mg/kg) and RBC from 12.8±0.4 $X10^6$/µl to 11.6±9.1$X10^6$/µl, but increased WBC levels from 7.8±2.3 $X10^3$/µl (control) to 9.1±1.2 $X10^3$/µl (500mg/kg). BA induced decreases in PCV from 42.8±1.2% to 41.8±0.4%, and RBC from 12.7±7.6$X10^6$/µl to 6.1±1.1 $X10^6$/µl. Serum biochemistry showed significant increases in ALT from 48.5±2.2 U/L to 66.75±12.5 in rats administered with BA (40mg/kg), but relatively normal AST. Triglyceride levels were non-significantly reduced in *T. potatoria*-treated rats but were increased from 74.0±3.8 mg/dl (control) to 139.3±4.8 mg/dl in rats administered with BA (40 mg/kg).

In conclusion, sub-acute administration of *T. potatoria* root was relatively non-toxic, but BA showed relatively more toxicity to blood cells and a tendency to cause dyslipidemia.

Keywords: *Tetracera potatoria*, Betulinic acid, haematology, serum biochemistry

INTRODUCTION

Medicinal plants are used worldwide, especially in developing countries and are assuming greater importance in primary health care (Erah *et al*., 2003). More than 80% of populations in these countries use herbal products to treat many diseases because the herbal medicines are believed to have intrinsic activity (Bruno, 2013). However, the markets in these countries are not adequately regulated and many herbal products in circulation are unregistered by national regulatory bodies

(WHO, 1996). Consequently, there is a general indiscriminate use of large numbers of medicinal plants and plant products, with little or no information about their effects on body systems.

There is therefore a need for detailed scientific analysis and documentation of the physiological and pharmacological effects of commonly used medicinal plants (Nevin and Vijayammal, 2005). A method of assessing the safety or toxic potential of a medicinal plant is to evaluate its effect on the haematological and biochemical parameters (Akanabiatu *et al*., 2005; Aboderin and Oyetayo, 2006). Alterations in normal physiological levels of these parameters after administration of an exogenous substance, is an indication of deleterious effects of such substance on living organism (Cheesborough, 1991).

Tetracera potatoria, belonging to the plant family Dilleniaceae, is a climber found in most parts of the world. It is used extensively in ethnomedicinal practice in West Africa for the treatment of jaundice, haemorrhoids, toothache and cough (Betti, 2004). Root decoction of the plant is also used as a remedy for gonorrhoea and other veneral diseases. The antifungal properties of ethanol, cold water and boiled water extracts of the root of *Tetracera potatoria* have also been reported (Adekunle *et al*., 2000). In addition, Adesanwo *et al*. (2003) and Oyebanji *et al.* (2013) reported the anti-ulcerogenic and antinociceptive effects of this plant respectively. Betulinic acid (BA) (3β, hydroxy-lup-20(29)-en-28-oic acid), was isolated and identified as the bioactive compound responsible for these latter effects of *T. potatoria*.

BA is a pentacyclictriterpenoid which has been reported to have a range of therapeutic effects. For instance, BA extracted from the leaves of *Vite xnegundo* demonstrated antibacterial activity against *Bacillus subtilis* (Chandramu *et al*., 2003). BA isolated from *Triphyophyllum peltatum* and *Ancistrocladu sheyneanus* have been reported to exhibit moderate to good *in vitro* antimalarial activity against asexual erythrocytic stages of the human malaria parasite, *Plasmodium falciparum* (Bringmann *et al*., 1997). *T. potatoria* is widely used traditionally mostly in its crude form for the treatment of various diseases, but has not been scientifically evaluated for its safety. The same goes for BA, the much touted bioactive compound responsible for the therapeutic effects of *T. potatoria*. The purpose of this study was to determine the toxic potential of the crude methanol extract of *Tetracera potatoria* and its bioactive constituent, betulinic acid on haematological and biochemical parameters of rats.

MATERIALS and METHODS

Preparation of Plant Extract

The root of *Tetracera potatoria* was collected in April, 2009 from the garden of Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. These were identified and authenticated by Mr. Ademoriyo of the Department of Botany, Obafemi Awolowo University, Ile-Ife. The roots were air-dried at room temperature; pulverized using a hammer mill and 1.0 kg of the powdered plant material was extracted in 3 liters of methanol by cold maceration for 72 h. The extract was filtered and the filtrate was concentrated using a rotary evaporator at 35°C. A residue of 180 g of dry extract (a yield of 18%) was obtained and this was preserved in a desiccator until further reconstitution for administration to test animals or purification.

Fractionation of the crude extract was carried out to isolate betulinic acid. The methanol extracts of the root of *T. potatoria* was subjected to Accelerated Gradient Chromatography (AGC) and the resulting fractions were pooled by Thin Layer Chromatography. The resulting fraction was further purified by Vacuum Liquid Chromatography and the structure of the resulting fraction was verified by Nuclear Magnetic Resonance (NMR) spectroscopy to confirm the active ingredient obtained was betulinic acid. The structure of the compound was compared to existing literature, thus confirming the isolated compound is betulinic

acid.

Acute and Sub-chronic toxicity study

Female Wistar rats weighing between 150-180 g were obtained from the Animal House of the Faculty of Pharmacy, ObafemiAwolowo University, Ile-Ife, Nigeria. The rats were kept in plastic cages placed in well-ventilated house conditions, and were allowed free access to rat pellets and water *ad libitum*. The acute toxicity study was carried out using five doses of the extract (100, 500, 1000, 2000 and 5000 mg/kg). Groups of five rats each were orally administered with the different doses of the extract and observed for 24 hours for signs of toxicity and/or mortality, according to the OECD Test Guidelines 423 recommendation adopted in 2001.

In the sub-chronic toxicity study for *T. potatoria*, twenty rats were randomly and equally divided into four groups. Group 1: control rats were administered with distilled water (10ml/kg) for 28 consecutive days. Groups 2, 3 and 4 rats were administered with methanol extract of *T. potatoria* at doses of 100, 500 and 1000 mg/kg body weight of the reconstituted extract, respectively for 28 days. Twenty rats were also grouped into four which included a control group and three groups administered with graded doses of betulinic acid at 10, 20 and 40 mg/kg for 28 days. These groups of rats were designated as control, BA 10, BA 20 and BA 40 groups.

Haematological and Biochemical evaluation

On day 29, 5 ml of blood was collected by cardiac puncture from diethyl ether-anaesthetized rats into heparinised bottles for haematological studies and blood samples collected in clean non-heparinised bottles were allowed to clot. The serum was separated and centrifuged according to groups into well-labelled bottles for biochemical analysis. Determination of haematological parameters was carried out as described by previous researchers (Jain, 1986; Duncan *et al*., 1994). Packed cell volume, red blood cell count and other red cell indices, total white blood cell (WBC) counts and the differentials were determined. Biochemical parameters determined included total protein, alanine amino transferase, Aspartate amino transferase, creatinine and bilirubin. All parameters were assayed spectrophotometrically using assay kits from Randox Diagnostics, United Kingdom.

Statistical Analysis

All the values were expressed as mean ± standard deviation. Statistical analysis was carried out using the GraphPad PRISM software package (version 5.0). Statistical significance was determined by the one way analysis of variance (ANOVA). The level of significance was determined at $p<0.05$.

RESULTS

Acute Toxicity Test

The acute toxicity test of *T. potatoria* extract and betulinic acid did not show any clinical adverse effect of substance-related toxicity to the rats in the 24 hour observation period. Similarly, there was no mortality or morbidity observed at any tested dose. The lethal dose 50 (LD_{50}) could not be determined at the doses administered in the acute toxicity study. The extract was thus considered safe according to OECD recommendations for acute toxicity test and was progressed to the sub-chronic toxicity test.

Sub-chronic Toxicity Test

Haematological Parameters

The mean packed cell volume (PCV) (42.5±2.1%, 34.0±1.2% and 32.7±2.2%), red blood cell count (RBC) (11.3±9.7X10^6/µl, 11.2±7.6X10^6/µl and 11.6±9.1X10^6/µl), haemoglobin concentration (14.8±0.8g/dl, 10.9±0.4g/dl and 10.8±0.7g/dl) and mean corpuscular haemoglobin concentration (MCHC) (34.0±0.4%, 34.7±0.3% and 33.0±0.1%) of rats administered with the graded doses of the extract of *T. potatoria* showed dose-dependent reductions compared to the control rats (42.8±1.5%, 12.8±0.4X10^6/µl, 15.0±0.4g/dl, 35.0±0.4%).

Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) increased. A significant ($p<0.05$) increase in the population of white blood cells, particularly the lymphocyte count ($6.7\pm1.8X10^3/\mu l$, $5.8\pm1.7X10^3/\mu l$ and $5.6\pm1.6X10^3/\mu l$) was observed in these rats.

Rats administered with betulinic acid at the doses of 10mg/kg (41.8±0.4%) and 20mg/kg (41.8±7.3%) also showed decreases in their PCV, but an increase was observed at 40mg/kg (44.4±8.3%). RBC was significantly decreased in rats administered the three doses of BA ($6.9\pm0.25X10^6/\mu l$, $7.28\pm0.4X10^6/\mu l$ and $6.1\pm1.1X10^6/\mu l$) compared to the control rats. Haemoglobin concentration was decreased but MCV and MCH levels of these test rats were increased compared to the control rats. WBC ($11.4\pm1.3X10^3/\mu l$, $10.5\pm0.7X10^3/\mu l$ and $7.6\pm2.3X10^3/\mu l$) and the lymphocyte count (62.0±3.1%, 66.0±4.2% and 52.0±1.4%) also increased compared to the control rats ($7.8\pm1.2X10^3/\mu l$ and 54.0±1.4%).

Serum liver enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST) were increased in rats administered with *T. potatoria*, while the total protein levels were significantly ($p<0.05$) decreased compared to that observed in control rats. Creatinine ($2.0\pm0.9mg/dl$, $1.5\pm0.4mg/dl$ and 1.2±0.1mg/dl), and biliribin levels increased ($2.1\pm0.2mg/dl$, $1.1\pm0.2mg/dl$ and 2.9±0.3mg/dl) in *T. potatoria*-treated rats compared to the control rats (1.1±0.1mg/dl and 0.6±0.1mg/dl).

The serum biochemistry of rats administered with graded doses of betulinic acid showed minimal increase in ALT levels but AST was significantly increased. Total protein and creatinine levels reduced while bilirubin levels increased compared to the control rats. Triglyceride levels were also increased from 74.0±3.8 mg/dl in the control rats to 103.4±18.4, 117.8±3.4 and 139.3±14.8mg/dl in the test rats.

Serum Biochemical parameters

TABLE I: EFFECT OF SUB-CHRONIC ADMINISTERED OF CRUDE METHANOL EXTRACT OF *TETRACERA POTATORIA* ON RED AND WHITE BLOOD CELL INDICES OF RATS

Parameters	Control	100 mg/kg	500 mg/kg	1000 mg/kg
PCV (%)	42.8±1.5	42.5±2.1	34.0±1.2*	32.7±2.2*
% PCV_c	100.00	99.30	79.44	76.40
$^6/\mu l$)	12.8±0.4	11.3±9.7	11.2±7.6	11.6±9.1
% RBC_c	100	88.28	87.50	90.63
Hb (g/dl)	15.0±0.4	14.8±0.8	10.9±0.4*	10.8±0.7*
	32.1±1.2	32.7±2.2	34.4±4.0	38.8±3.4*
	11.4±0.5	12.5±0.3	12.1±1.2	10.3±1.1
MCHC (%)	35.0±0.4	34.0±0.4	34.7±0.3	33.0±0.1*
WBC ($X10^3/\mu l$)	7.8±2.3	10.5±1.5	9.1±1.2	9.7±1.8
% WBC_c	100	134.62	116.67	124.36
Neutrophil ($X10^3/\mu l$)	3.0±1.2	3.3±1.8	3.0±1.8	4.1±1.1
Lymphocyte ($X10^3/\mu l$)	4.3±0.8	6.7±1.8*	5.8±1.7	5.6±1.6
Eosinophil ($X10^3/\mu l$)	0.2±0.01	0.1±0.08*	0.2±0.01	0.2±0.02

% PCV_c, % RBC_c, % WBC_c - Percentage value of test rats compared to values of control rats. *Significant ($p<0.05$) difference compared to control value

TABLE II: SERUM LIVER ENZYMES, LIPIDS, PROTEINS AND NON-PROTEIN METABOLITE LEVELS OF RATS ADMINISTERED WITH THE CRUDE METHANOL EXTRACT OF *TETRACERAPOTATORIA* FOR 28 DAYS.

Parameter	Control	100 mg/kg	500 mg/kg	1000 mg/kg
AST(U/L)	25.8±1.5	30.0±7.1	30.0±8.7	26.0±1.2
ALT(U/L)	48.5±2.2	43.4±5.0	42.3±6.1	55.2±5.4
Total Protein (g/dl)	8.9±0.3	7.9±0.3	7.3±1.7	6.8±0.4*
Triglycerides (mg/dl)	74.0±3.8	72.2±1.0	72.3±4.2	70.4±5.0*
Creatinine (mg/dl)	1.1±0.1	2.0±0.9	1.5±0.4	1.2±0.1
Total Bilirubin (mg/dl)	0.6±0.1	2.1±0.2*	1.1±0.2*	2.9±0.3*

*Significant ($p<0.05$) difference compared to control value

TABLE III: HAEMATOLOGICAL CHANGES OBSERVED IN RATS ADMINISTERED WITH BETULINIC ACID FOR 28 DAYS

Parameter	Control	BA10	BA20	BA40
PCV (%)	42.8±1.2	41.8±0.4	41.8±7.3	44.4±8.3
% PCV_c	100.00	97.66	97.66	103.74
RBC ($X10^6$/µl)	12.7±7.6	6.9±0.25	7.28±0.4	6.1±1.1
% RBC_c	100	54.33	57.32	48.03
HB (g/dl)	15±0.4	13.7±0.4	13.3±0.7	12.3±0.5
MCV (*f*l)	32.1±3.0	59.3±1.7	57.4±0.5	58.0±0.26
MCH (ρg)	11.4±1.0	19.25±0.5	18.36±0.4	18.0±7.4
MCHC (g/dl)	35±0.02	32.5±1.0	31.8±0.6	30.0±2.9
WBC ($X10^3$/µl)	7.8±1.2	11.4±1.3	10.5±0.7	7.6±2.3
% WBC_c	100	146.15	134.62	97.44
Neutrophils (%)	40.0±1.1	35.0±4.03	33.2±4.3	27.2±4.0
Lymphocytes (%)	54.0±1.4	62±3.1*	66.0±4.2	52.0±1.4
Eosinophils (%)	1.0±0.6	0.3±1.8	1.2±0.6	0.1±0.1

% PCV_c, % RBC_c, % WBC_c - Percentage value of test rats compared to values of control rats.*Significant ($p<0.05$) difference compared to control value

TABLE IV: SERUM LIVER ENZYMES, PROTEINS, LIPID PROFILE AND NON-PROTEIN METABOLITES OF RATS ADMINISTERED WITH BETULINIC ACID (BA) FOR 28 DAYS

Parameter	Control	BA 10	BA 20	BA 40
AST (U/L)	25.75±1.5	26.9±0.8	26.8±1.3	24.0±2.0
ALT (U/L)	48.5±2.2	64.4±1.8*	60.8±5.8*	66.75±2.5*
ALP (U/L)	262.5±35.2	308.2±41.0*	249±49.1	243±30.4
Total protein (g/dl)	8.9±0.3	7.22±0.26	7.16±0.4	6.43±0.76*
Triglyceride (mg/dl)	74.0±3.8	103.4±18.4*	117.8±3.4*	139.3±48*
Creatinine (mg/dl)	1.15±0.04	0.72±0.06	0.86±0.04	0.88±0.03
Bilirubin (mg/dl)	0.62±0.1	1.21±0.6*	1.16±0.06*	1.23±0.7*

Significant ($p<0.05$) difference compared to control value

DISCUSSION

The methanol extract of *T. potatoria* caused a significant ($p<0.05$) decrease in the levels of packed cell volume (PCV) and haemoglobin concentration (Hb) at 500 and 1000 mg/kg, but white blood cell counts increased. This is suggestive of a tendency of the plant to cause anaemia. The anaemia observed was regenerative as shown by the increased mean corpuscular volume (MCV) and decreases in the mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). This is a macrocytic hypochromic anaemia that is characteristic of regenerative responses of the bone marrow in which immature red blood cells (reticulocytes) are released into the circulation before they are fully haemoglobinated (Jelkman, 1992). Reticulocytes are morphologically characterized by increased MCV and decreased MCHC (Adamson and Longo, 2001). Anaemia observed in this study may have also occurred due to an increase in osmotic fragility and progressive destruction of RBCs which may have led to the significant decrease in the level of PCV (Balani *et al*., 2011). The certainty of the latter suggested mechanism is limited by the scope of this study, as osmotic fragility of the red blood cells was not determined.

A staggering number of medicinal plants have been reported to induce anaemia or polycytemia as one of their unwanted effects in animals and humans. For instance, the methanol extract of the plant *Tripterygium wilfordii*, which contain alkaloids and is used as an antifertility agent (Quain, 1987) has been reported to induce anaemia in rats (Pyatt *et al*., 2000). Also, *Azadirachta indica* and *Abrus precatorius* have been reported to cause anaemia in Brown Hisex chicks (Ibrahim *et al*., 1992) and Lohman broiler chicks (Omer *et al*., 1992) respectively. In contrast, the extract of *Telfara occidentalis* is used to treat anaemia and has been shown to significantly increase PCV, RBC, and Hb levels following treatment after experimental induction of anaemia (Dina *et al*., 2000; Alada, 2000).

An increase was observed in population of white blood cells (WBC) which was mainly due to an increase in lymphocyte count. The lymphocytosis observed in this study may be due to the fact that lymphocytes respond to antigenic stimulation by proliferating, thereby expanding the antigen specific lymphocyte clones and producing lymphokines, thus amplifying immune responses (Mackay, 1993). Lymphocytosis is usually associated with increased immunologic activities in the body (Schillaci *et al.*, 1994; Guyton and Hall, 2006a) as lymphocytes are responsible for the immune status of the body. A number of plant extracts have been reported to boost the humoral (Rehman *et al*., 1999) and cell-mediated immunity (Upadhyay *et al*., 1992) against microorganisms such as viruses (Calixto *et al*., 1998), bacteria (Boyanova and Neshev, 1999), fungi (Ali *et al*., 1999) and protozoa (Sharma *et al*., 1998).

Therefore, the stimulatory effect of the *T. potatoria* root extract on lymphocyte count may be advantageous in the management of various disease processes including chronic viral infections, tuberculosis, AIDS, and cancer (Tamez-Guerra and Rodríguez-Padilla, 2008), especially when used to supplement the standard therapeutic drugs for these ailments. Further research may be warranted to explain molecular details of the observed lymphocytosis. Neutrophil levels on the other hand were unchanged in these rats and clinically, changes in neutrophil count are usually related to on-going inflammatory response in the body (Guyton and Hall, 2006b). Neutrophils are usually mobilised in the blood as part of inflammatory response to antigenic stimulation such as from trauma and bacterial endotoxins (Saba *et al.*, 2009).

Haematology of rats administered with betulinic acid (BA) on the other hand showed a similar pattern to that observed in rats administered with *T. potatoria*. In a holistic view, the PCV of rats administered with BA were within range of that observed in the

control, but BA was more toxic to the blood cells compared to *T. potatoria*. RBC levels were as low as 48.03% of the control animals and the WBC were as high as 146.15% of control rats. These derangement in the red and white blood cells parameters are significant ($p<0.05$) in comparison to rats administered with the crude *T. potatoria* extract. This observation is in accordance with findings by previous researchers which showed that a medicinal plant is better used as a whole extract as each plant contains protective substances which may be screening off in purification processes (Iwu, 1996).

Oral administration of the methanol root extract of *T. potatoria* induced a significant decrease in the triglyceride level at the 1000 mg/kg dose. Triglycerides are the main form of fat in the body providing energy, insulation and protection to visceral organs (Sethi and Vidal-Puig, 2007). Unfortunately, high serum triglycerides, is usually implicated in cardiovascular diseases, obesity and diabetes (Saba and Oridupa, 2012). This study has shown that *T. potatoria* does not have an atherogenic tendency. In contrast, BA was observed to increase triglyceride levels in the rats which indicate that BA may have an atherogenic potential and caution must be exercised in its use. The contrasting picture shown in this biochemistry may be explained by the difference in concentration of BA in *T. potatoria* compared with the pure compound administered to the BA-treated rats.

Serum enzymes of rats administered with *T. potatoria* assessed in this study showed non-significant changes in the aminotransferases; AST and ALT. This suggests that the extract is did not induce significant cellular damage to the liver, even when administered in high doses. However, serum protein levels decreased while bilirubin increased. These may indicate an impairment of protein synthesis by the liver and excretion of bilirubin via the bile ducts (Saba *et al.*, 2010). The increase in bilirubin levels supports our findings in the haematology which showed decreases in RBC and Hb. Bilirubin is a metabolite of heme, a part of haemoglobin in red blood cells (Otterbein *et al.*, 1995). Breakdown of red blood cells releases the heme which is recycled for red blood cell production or further metabolized to the excreted form – bilirubin. Increased breakdown increases to amount of heme metabolized to bilirubin which may impair normal hepatic functions, or even lead to clinical conditions presented as jaundice.

BA on the other hand showed increases in AST levels while ALT remained within range of the control values. This may be explained by the significant ($p<0.05$) decrease in RBC as increased destruction of red blood cells may result in increased AST, but normal ALT levels. This supports our finding in the haematology of BA-treated rats above. Serum protein levels were also decreased and bilirubin levels increased. It can thus be inferred from this study that BA does not induce any significant hepatotoxicity.

Creatinine levels in both *T. potatoria*-treated and BA-treated rats were within range of that observed in control rats. Creatinine is excreted from the body in the urine via the kidneys. As a result creatinine measurement is used almost exclusively in the assessment of kidney function. Elevation of plasma creatinine is indicative of under-excretion, suggesting kidney impairment (Bartels *et al.*, 1972; Ranjna, 1999). The insignificant changes in creatinine levels observed in this study, suggests that the plant extract did not alter the functional capacity of the kidneys.

In conclusion, sub-chronic administration of *T. potatoria* root appears to be relatively non-toxic to animals. The alterations observed in the hematological and serum biochemical parameters suggest selective toxicity of the plant when repeatedly consumed on a daily basis at high doses for long period of time. Betulinic acid on the other hand showed relatively more toxicity to blood cells and a tendency to cause dyslipidemia which was not observed with *T. potatoria* extract.

This is probably due to the higher concentration of the BA per unit volume of blood.

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