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91-day Oral Toxicity Evaluation of a Herbal Preparation Used in the Management of Breast Tumour in Southwestern Nigeria using Mice

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ABSTRACT

Joloo is a traditional herbal formulation used in the management of tumour of the breast in southwestern Nigeria. The acute and subchronic toxicity studies of Joloo have been reported previously but the chronic toxicity has not been investigated. The study was undertaken to evaluate the long-term toxic effect of Joloo on mice. Ethanolic extract of Joloo was administered to four groups of mice weighing 27.5±1.4 (N = 10/group) Repeated doses (400, 800 and 1600 mg kg^{-1} b.wt.) were administered orally for 91 days. Parameters observed include, body and relative organ weights, haematology, biochemical analysis, antioxidant activities and histologic studies. There were no adverse effects on the general condition, body and relative organ weight, red blood cells and white blood cell. However there were significant increase in leucocytes, GPx, CAT and SOD at 800 and 1600 mg kg^{-1} b.wt. The histoarchitecture of the liver, heart and the spleen revealed slight alteration (mild necrosis) and there was dose-dependent though insignificant increase in some of the biochemical analytes (ALT and AST) at 1600 mg kg^{-1} b.wt. Based on these findings it can be inferred that Joloo is devoid of toxicity at 400 and 800 mg kg^{-1} b.wt. and possess strong antioxidant activities, whereas high dose (1600 mg kg^{-1} b.wt.) may be associated with some toxicity concerns.

Key words: Joloo, chronic toxicity, antioxidants, biochemical analytes, histology

INTRODUCTION

Herbs have been used traditionally since ancient times across the world for the management and cure of myriads of ailments (Tajik *et al*., 2011; Aliyu *et al*., 2007). Joloo is a herbal formulation extracted from seven medicinal plants. It is used in folk medicine practice for the management and treatment of breast cancer and some other health malaise in southwestern Nigeria. Joloo comprise of *Allium ascalonicum* Linn. (Liliaceae: Alliaceae), *Butyrospermum paradoxum* Gaertn (Sapotaceae), *Hoslundia opposita* Vahl (Labiatae), *Olax subscorpioidea* Olive (Olacaceae), *Xylopia aethiopica* Dunal A. Richard (Annonaceae), *Securidaca longepedunculata* Fresen (Polygalaceae) and *Tetrapleura tetraptera* Schum and Thonn (Leguminosae: Mimosoideae) and prepared in specific ratio as reported by Oloyede *et al*. (2008).

It has also been scientifically evaluated to be devoid of acute toxicity (Oloyede *et al*., 2009) where its LD_{50} was realized. Joloo has been scientifically described to possess some anti-inflammatory and analgesic properties (Oloyede *et al*., 2008). Joloo was also devoid of subchronic toxicity as reported by Oloyede *et al*. (2011).

This study was, therefore conducted to assess the chronic toxicity of Joloo for its safety using physical changes, haematology, serum chemistry, histopathological changes and antioxidant potentials as indices of toxicosis.

MATERIALS AND METHODS

Animals: Swiss mice weighing 24-30 g of either sex, bred in the animal house of the Redeemer's university were used for this study. Approval was obtained from College of Natural Sciences on the use of animals for research purposes. The animals were maintained under standard environmental conditions as described by the method of Mbagwu *et al.* (2007). They had access to water and standard feed *ad libitum*.

Plant collection identification and preparation of extract: The research is a continuous study whereby specimens had already been identified and deposited in herbarium as reported by Oloyede *et al.* (2008) and extract was prepared according to Oloyede *et al.* (2008, 2009, 2011).

Study design: Animals divided into four groups of 10 mice each were administered with Joloo for 91 days by oral gavage using a curved blunt tipped stainless steel feeding needle. Group one (control) was administered with distilled water 10 mL kg^{-1} while groups two, three and four received Joloo at doses of 400, 800 and 1600 mg kg^{-1} b.wt., respectively. The weights of the animals were monitored and recorded weekly for seventeen weeks after which animals were sacrificed. The organs and tissues were then collected, weighed and processed on day 91 for relevant assays and histopathologic analysis.

Mortality and clinical signs: During the administration period of 91 days, animals were observed for general appearance, mortality and clinical signs.

Haematology, relative organ weight and necropsy: The mice were fasted for 16-19 h on the autopsy day and anesthetized with ethyl ether. All the animals were then euthanized by exsanguination and blood samples collected from the abdominal aorta into EDTA vials for routine hematological investigation.

The spleen, heart, liver, kidneys, brain and lungs were harvested and weighed to determine the absolute organ weight. The relative organ weight of each animal was then calculated based on body weight measured on the day of sacrifice as follows:

$$\text{Relative organ weight (g)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

The organs were thereafter preserved in 10% buffered formalin for histopathologic examinations. The tissue biopsies were dehydrated and embedded in paraffin, cut into 4-5 μm sections with rotary microtome (LEICA RM 2235 Rotary Microtome) and stained with hematoxylin-eosin for photomicroscopic examination.

Biochemical analysis: The livers were homogenized in 0.1 mol L^{-1} sodium phosphate buffer (pH 7.4) and centrifuged (8000 rpm for 20 min at 4°C). The homogenate derived from each sample

was used for all assays which included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol, creatinine kinase (Cknac), protein, urea and albumin. Spectrophotometer optimal sp 3000 model was used for this study.

Estimation of lipid peroxidative indices: Lipid peroxidation as evidenced by the formation of TBARS was measured by the method modified by Rukkumani *et al.* (2004). In brief, 0.1 mL of tissue homogenate (tris-HCl buffer, pH 7.5) was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in a water bath for 15 min, cooled and centrifuged at room temperature for 10 min at 1,000 rpm. The absorbance of clear supernatant was measured against reference blank at 535 n.

Determination of non-enzymic antioxidant status

Estimation of reduced glutathione: Reduced glutathione (GASH) was determined by the method modified by Rukkumani *et al.* (2004). To the homogenate added 10% TCA, centrifuged. The 1.0 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5'-dithiobis nitrobenzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Determination of superoxide dismutase, catalase and glutathione peroxidase: Superoxide dismutase (SOD) was assayed using the technique of Rukkumani *et al.* (2004). A single unit of enzyme was expressed as 50% inhibition of NBT (nitroblue tetrazolium) reduction/min/mg protein.

Catalase (CAT): Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as μmoles of H_2O_2 consumed/min/mg protein as described modified by Rukkumani *et al.* (2004). The reaction mixture (1.5 mL) contained 1.0 mL of 0.01 M pH 7.0 phosphate buffer, 0.1 mL of tissue homogenate and 0.4 mL of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

Glutathione peroxidase (GPx): Glutathione peroxidase (GPx) activity was measured by the method described by Rukkumani *et al.* (2004). Briefly, reaction mixture contained 0.2 mL of 0.4 M phosphate buffer pH 7.0, 0.1 mL of 10 mM sodium azide, 0.2 mL of tissue homogenate (homogenized in 0.4 M, phosphate buffer pH 7.0), 0.2 mL glutathione and 0.1 mL of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 mL of 10% TCA and centrifuged. Supernatant was assayed for glutathione content by using Ellman's reagent.

The total protein was estimated by total protein and albumin kit RANDOX.

Statistical analysis: Data were expressed as Means±SEM and analysed using GraphPad prism 5. Results were considered significant at $p<0.05$.

RESULTS

Clinical observation: All mice survived until scheduled necropsy and showed normal growth and appeared healthy through the study. Daily general observations, ophthalmoscopy and clinical examinations revealed no treatment-related changes.

The changes of body weights were not significantly different ($p<0.05$) in mice between treatment and control groups (Table 1).

There were no significant changes in the relative weights of the heart, liver, lung and kidneys of all the animals. However for organs like the brain and spleen, there were significant variations ($p<0.05$) between controls and treated group at 800 and 1600 mg kg^{-1} b.wt. (Table 2).

The biochemical analytes from the liver (Table 3) did show that ALT was significantly different from control at 1600 mg kg^{-1} b.wt. while AST was significantly different from control at all doses after Joloo administration for 91 days. Among the biochemical indicators of kidney function tested (Table 4), ALB was significantly different from control at 1600 mg kg^{-1} b.wt. dose and AST was significantly different from control at all the doses.

Joloo exhibited a gradual dose-dependent increase in all hepatic and kidney antioxidant enzymes, CAT, at 1600 mg kg^{-1} b.wt., SOD at 800 and 1600 mg kg^{-1} b.wt. and GPx significantly different ($p<0.05$) from control at both 800 and 1600 mg kg^{-1} b.wt. MDA showed a significant reduction ($p<0.05$) from control (Table 5, 6).

Table 1: Effects of oral administration of Joloo for 13 weeks on body weight

Period	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Control	24.56±1.15	26.63±1.40	26.30±1.00	27.67±1.00	28.85±0.64	29.16±0.62	29.35±0.80
400 mg	29.90±4.51	28.48±2.41	27.31±0.95	26.76±1.35	27.06±1.41	28.23±1.40	28.13±1.62
800 mg	28.33±0.70	28.60±0.78	29.23±0.97	29.23±1.00	29.71±0.84	29.94±0.59	29.33±0.60
1600 mg	25.45±0.90	28.22±0.80	28.79±0.73	28.84±0.70	29.30±0.67	29.57±0.60	29.78±0.70
Period	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	
Control	28.98±0.70	29.86±0.70	30.07±0.63	30.76±0.45	31.24±0.36	31.48±0.26	
400 mg	28.16±1.63	29.28±1.75	29.39±1.80	29.83±1.70	30.07±1.60	29.96±1.55	
800 mg	28.73±0.33	30.78±0.83	30.98±0.77	31.26±0.70	31.66±0.52	31.70±0.53	
1600 mg	30.16±0.50	29.51±0.50	30.15±0.53	30.05±0.40	29.92±0.42	30.68±0.54	

Values are Mean±SEM (N = 10/group), *Significantly different from controls ($p<0.05$)

Table 2: Effects of oral administration of Joloo for 13 weeks on relative organ weight

Dose (mg kg^{-1} b.wt.)	Hearts	Liver	Lungs	Brain	Spleen	Right kidney	Left kidney
Control	0.13±0.03	1.45±0.09	0.54±0.14	0.32±0.02	0.10±0.03	0.17±0.05	0.16±0.04
400	0.11±0.02	1.18±0.08	0.18±0.02	0.34±0.04	0.10±0.02	0.17±0.05	0.16±0.04
800	0.13±0.04	1.20±0.07	0.21±0.05	0.37±0.04*	0.09±0.02	0.18±0.03	0.16±0.04
1600	0.13±0.03	1.34±0.10	0.25±0.04	0.37±0.04*	0.16±0.30**	0.19±0.04	0.17±0.03

Values are Mean±SEM (N = 10/group), *,**Significantly different from controls at $p<0.05$ and $p<0.01$, respectively

Table 3: Biochemistry values from the liver of mice treated with Joloo extract for 13 weeks

Dose (mg kg^{-1} b.wt.)	ALB (g L^{-1})	ALT/GPT (U L^{-1})	AST/GOT (U L^{-1})	CHOL (mmol L^{-1})	UREA (mg dL^{-1})	PROT (g L^{-1})
Control	44.23±0.83	22.67±0.77	47.40±0.64	2.87±0.10	28.44±0.25	36.48±0.18
400	45.32±0.34	21.96±0.53	52.62±1.18*	2.83±0.05	23.95±0.58	33.53±0.41
800	45.81±0.26*	23.09±0.49	61.98±0.84*	2.86±0.07	25.06±0.76	33.85±0.78
1600	46.94±0.56*	26.00±0.69*	62.1±1.44*	2.9±0.11	26.83±0.55	38.18±0.46*

Values are Mean±SEM (N = 10/group), *,**Significantly different from controls at $p<0.05$ and $p<0.01$, respectively

Table 4: Biochemistry values from the kidney of mice treated with Joloo extract for 13 weeks

Dose (mg kg^{-1} b.wt.)	ALB (g L^{-1})	ALT/GPT (U L^{-1})	AST/GOT (U L^{-1})	CHOL (mmol L^{-1})	UREA (mg dL^{-1})	PROT (g L^{-1})
Control	44.96±0.30	28.92±0.30	31.46±1.05	2.84±0.06	28.94±0.34	35.42±0.50
400 mg	47.79±0.46	21.45±0.31	56.29±1.39*	2.77±0.07	25.78±0.39	36.45±0.28
800 mg	48.52±0.62*	22.12±0.75	66.73±1.00*	2.81±0.07	28.61±0.41	37.01±0.46*
1600 mg	54.60±0.84*	24.50±0.60	71.85±1.76**	2.83±0.08	28.83±0.68	37.09±0.38*

Values are mean±SEM (N = 10/group), *Significantly different from controls at $p<0.05$ and $p<0.01$, respectively

Table 5: Antioxidant activity assay values from the liver of mice treated with Joloo extract for 13 weeks

Dose (mg kg^{-1} b.wt.)	CAT (µmol/min/mg/protein)	SOD (SOD/min/mg protein)	MDA (nmol mL^{-1})	GSH (µmol mL^{-1})	GPx (µmol mL^{-1})
Control	2.96±0.07	96.72±0.90	44.36±0.67	1.58±0.44	3.81±0.11
400 mg	2.68±0.16	93.95±0.58	36.97±0.60	1.00±0.09	4.17±0.11
800 mg	2.77±0.13	100.38±0.94*	40.25±0.89*	1.03±0.08	4.38±0.16**
1600 mg	3.02±0.18*	109.53±1.32*	40.99±0.57*	1.91±0.40	4.82±0.05***

Values are Mean±SEM (N = 10/group), *,** and ***Significantly different from controls at $p<0.05$ and $p<0.01$, respectively

Table 6: Antioxidant activity assay values from the kidney of mice treated with Joloo extract for 13 weeks

Dose (mg kg^{-1} b.wt.)	CAT(µmol/min/mg/protein)	SOD(SOD/min/mg protein)	MDA (nmol mL^{-1})	GSH (µmol mL^{-1})	GPx (µmol mL^{-1})
Control	3.3±0.17	96.97±0.69	42.74±0.27	1.09±0.04	4.38±0.11
400 mg	3.45±0.11	95.12±0.93	37.56±0.52	1.09±0.08	4.15±0.12
800 mg	3.59±0.18	100.95±0.82*	38.00±0.51	1.10±0.13	4.65±0.10*
1600 mg	4.03±0.11**	105.71±1.09*	40.97±0.83*	1.17±0.07	4.71±0.12**

Values are Mean±SEM (N = 10/group), *significantly different from controls at $p<0.05$ and $p<0.01$, respectively

Table 7: Haematology values of mice treated with Joloo extract for 13 weeks

Dose (mg kg^{-1} b.wt.)	PCV (%)	WBC (mm^3)	N (%)	L (%)	E	M (%)	B
Control	28.50±0.42	2200±11.89	47.50±3.12	52.50±3.12	-	-	-
400	20.67±1.01	1633.33±6.80	58.67±1.74	40.00±1.77	-	2	-
800	31.50±0.58	1850±4.33	39.00±1.58	61.00±0.51*	-	2	-
1600	23.00±1.01	2233.33±8.70	37.33±0.51	62.67±0.51*	-	-	-

Values are Mean±SEM (N = 10/group), *Significantly different from controls at $p<0.05$ and $p<0.01$, respectively

The result of hematology examination is shown in Table 7. There were no statistically significant difference in the values of PCV, WBC, but leucocytes counts was statistically different from control at 800 and 1600 mg kg^{-1} b.wt.

DISCUSSIONS

Medicinal plants play important role in the socio-cultural, spiritual and medicinal arena of rural people in many parts of the world, however, they are not devoid of toxicity (Abdelgadir *et al*., 2010; Sulaiman *et al*., 2010). The repeated oral administration of Joloo for a period of 91 days in mice showed minor but insignificant variations in the body and some organ weight. The relative organ weight of the heart, liver, lungs and kidneys were comparable to control without difference. However the brain and spleen were significantly different from control at higher doses. It is well established that hematological tests form the very front-line investigations on which diagnosis of various diseases is based. Unaltered hematological parameters in PCV and WBC as well as significant increase in leucocyte in median and higher doses suggest that Joloo did not cause any adverse effects on the general health, but then can boost the animal immunity due to increase in leucocytes in the animals. Joloo did not show any toxic manifestation on body weight; however abnormality in the histoarchitecture of organs has been regarded as reliable indication of toxicity. In this chronic study, beside minor insignificant variations observed in the body weight and some major accessory organs, the histoarchitecture of the liver, heart and spleen at the highest dose were characterized with mild necrosis which probably is an indication of early toxicity. The necrosis observed in the histoarchitecture of the spleen may be an indication of early immunotoxicity. The presence of pathological necrosis in the liver and spleen is not unconnected with the fact that the liver is the main organ of biotransformation in the body (Maphosa *et al*., 2008), while the spleen

filters foreign matters from the blood and involved in many metabolic disturbances (Kumar *et al.*, 2007), hence, the organs may be exposed to the toxic/active agents, especially during prolonged usage and at high dose of 1600 mg. Enzymes and non-enzyme indices in tissues and body fluids are very essential in aiding disease investigation and diagnosis, enzymes are released into circulation from affected organ or tissue due to damage (Hassan *et al.*, 2011). Liver injury is often evaluated by monitoring of biochemical parameters such as ALT, AST, ALP and GGT which are among the most sensitive markers of hepatocellular damage (Natanzi *et al.*, 2010; Ghazi-Khansari *et al.*, 2005). The histologic evidence juxtaposed with the biochemical analytes, which showed a significantly elevated ALT and AST in the liver and AST alone in the kidney against control. ALT concentration is highest in the liver and therefore is a more sensitive test for hepatocellular damage than AST (Kuete *et al.*, 2010). However, the detected levels of ALT and AST in this study are within the range of reference value (Levine, 1995), more so, the significant increase in albumen and total protein in both liver and kidney cells is an indication that no serious damage has occurred in the organs. But then, this is an indication that the liver and kidney might be target candidates for toxicity when exposed to prolong administration of Joloo at high dose of 1600 mg.

Oxidations of foods help in energy production for living organisms; however Reactive Oxygen Species (ROS) capable of damaging cellular components are formed as by-products (Koneru *et al.*, 2011). Antioxidant protects aerobic organisms from the harmful effects of reactive oxygen metabolites. Proteins, carbohydrates, lipids and nucleic acids are targets for reactive free radical molecules, therefore structural proteins, DNA, RNA, simple and complex sugars, cellular proteins and membranes are susceptible to oxidative damage (Balouchzadeh *et al.*, 2011). Joloo exhibited significant ($p<0.05$) dose-dependent increase in antioxidant enzymes and reduced lipid peroxidation during the chronic toxicity studies due to significant reduction of MDA. A significant increase in GPx was observed in the chronic studies, which implies that Joloo can protect cells against oxidative injury of any type since GPx activity is not limited to H_2O_2, but covers a wide range of substrates from H_2O_2 to organic hydroperoxides (Rukkumani *et al.*, 2004). The significant increase in SOD and CAT is an indication that Joloo can protect cells against oxidative stress, as these enzymes act as preventative antioxidant which plays important role in protection against deleterious effects of lipid peroxidation (Rukkumani *et al.*, 2004). It can also protect and enhance the activity of the heart, since increased oxidative stress with decreased antioxidant enzyme activities has always been associated with coronary heart disease (Ima-Nirwana *et al.*, 2011). The antioxidant mechanism of Joloo may be attributed to one or more of the following; inhibition of oxidative enzymes like cytochrome P450, chelating and disarming oxidative properties of metal ions, scavenging or neutralizing of free radicals, interacting with oxidative cascade and preventing its expression and oxygen quenching and reducing its availability for oxidative reaction (Rukkumani *et al.*, 2004).

CONCLUSION

This chronic study has indicated that lower doses 400 and 800 mg of Joloo are devoid of toxicity, whereas high dose (1600 mg kg^{-1} b.wt.) may be associated with some toxic potential especially under prolonged usage. This study has given the scientific basis for the non toxicity of Joloo with prescribed doses of 800 and 1600 mg under the study period.

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