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Safety Evaluation of bioactive Sub-Fraction of *Lawsonia inermis* Linn. leaves in Male Wistar Rats

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ABSTRACT

This study evaluated the safety of bioactive labelled sub-fraction of *Lawsonia inermis* in male Wistar rats. Twenty male rats were used for this study of five rats per group. Three groups were administered methanol bioactive sub-fraction of *Lawsonia inermis* at 2.5, 5 and 10 mg/kg for fourteen days while control received distilled water orally. Physiological weight increased in all treatment groups. The group fed with 10 mg/kg had the highest weight gain compared to other treatment groups and the control. Relative organ weight of the heart, liver, kidneys, testes and pancreas did not increase ($p>0.05$) in all the treatment groups except the pancreas in the group fed with 5 mg/kg that increased significantly ($p<0.05$). The sperm count in rats fed at dose rate of 10 mg/kg increased significantly ($p<0.01$) compared to other treatment groups. There were no significant differences in sperm motility, volume and live/dead ratio in the treatment groups compared to control. Haematological parameters such as, PCV, RBC, WBC and haemoglobin concentration were not significantly different compared to control. The AST increased significantly ($p<0.01$) while urea and creatinine decreased significantly ($p<0.001$) in group fed with 2.5 mg/kg. 5 mg/kg showed significant reduction ($p<0.05$) in total protein, urea and ALP while ALT increased significantly. Serum chloride ion at the dose rate of 2.5 mg/kg decreased significantly ($p<0.05$) when compared with other treatment groups. Sodium ion (Na^+) decreased significantly ($p<0.01$), while Bicarbonates (HCO_3^-) increased significantly in the 5 mg/kg group. It was concluded that the bioactive sub-fraction of *Lawsonia. inermis* was safe at the administered dosages.

Keywords: Andrological parameters; Haematology; *Lawsonia inermis;* Serum chemistry; Sub-fraction; Rats

INTRODUCTION

Medicinal plants have been being explored globally especially in developing countries where traditional medicine play an important part in primary health care delivery (Barliana *et al.,* 2014). Reports have shown that 85% of the populations in most developing countries rely on medicinal plants drugs for their primary health care needs (Schmincke, 2003). These plants contain abundant bioactive phytochemicals which serve as potential drug sources used in managing various disease of both human and animal (Barliana *et al.,* 2014).

These plants are frequently used for treatment of particular diseases, on large scale are reported to be having serious side effects (Susana *et al.,* 2019). Reports have shown that most synthetic drugs were derived from bioactive phytochemicals (Ciddi, 2012). Toxicities shown by crude extracts cannot be mimicked when using pure compound isolated from purified constituents of the plant (Philomena *et al.,* 2009). Toxicity of various plant used for medicinal purpose are usually related to viewpoint of perception because edible foods that are considered relatively safe may possess constituents that could trigger serious allergic reaction (Ernst, 2007).

Lawsonia inermis is a very useful medicinal plants in many parts of the world especially Asia and Africa continent (Aremu and Oridupa, 2022). Leaves of *L. inermis* are used in the treatment of many diseases such as diabetes, poliomyelitis, measles among the Yoruba tribe of South Western Nigeria while the seeds have been reported for its deodorant action used in reproductive disorders (Nawagish *et al.,* 2007; Oladunmoye and Kehinde, 2011). Decoction from the leaves is also used for aseptic cleaning of wounds and healing (Zumrutdal and Ozaslan, 2012). Reports have shown that crude extracts of *Lawsonia inermis* possess significant deleterious effects on haematology, sperm morphology and immunology (Aremu *et al.,* 2022). This study is aimed at exploring the safety potential of bioactive sub-fraction of *Lawsonia inermis* using physiological weight, organ weight, andrological parameters, haematology, serum chemistry and electrolytes in male Wistar rats.

Materials and Methods

Plant Harvesting, Identification and Preparation

Leaves of *Lawsonia inermis* Linn was harvested from a farm land in Oke-oyi in Ilorin East area council of Kwara state, North Central, Nigeria. Taxonomically, it was both identified and authenticated at University of Ibadan Herbarium and a specimen was deposited and assigned a voucher number UIH-22460. Leaves of *Lawsonia inermis* were dried at room temperature (25^0C) under shade in a room for four weeks. The leaves were macerated to powdery form. The powdery leaves of *L. inermis* Linn was used for crude extracts.

Extraction and Separation of bioactive sub-fraction of *Lawsonia inermis* leaves

Two kilograms of powdery leaves of the *L. inermis* was soaked in 5 liter of methanol for 72 hours. Mixture was gently decanted and filtered using filtered paper. The filtrate was immediately evaporated at temperature (40^0C) using a rotary evaporator. The concentrate (wet residue) was dried and stored at 4^0C.

Column chromatographic separation of methanol sub-fraction

The crude methanol extract was subjected to Vacuum Liquid Chromatography (VLC), Column Chromatography (CC) and Thin Layer Chromatography (TLC). Fractions obtained were pooled together using Thin Layer Chromatography (TLC).

Vacuum Liquid Chromatography (VLC)

Lawsonia inermis Leaves (samples) were pre-adsorbed on VLC silica gel to make slurry and loaded to the column in a dry state with adsorbent silica gel 60; Merck 40 - 63 microns (230 - 400 mesh). It was then eluted with different combinations of increasing polarity. Graded combinations of analytical grade of methanol, *n*-hexane and ethyl acetate were used for the separation. Samples were collected into labelled test tubes with particular attention to the solvent used for elution. The progress of elution was monitored with Thin Layer Chromatography (TLC).

Thin Layer Chromatography (TLC)

Pre-coated TLC plates (aluminum foil) were used in this study. TLC Fingerprinting of methanol Fraction of *L. inermis* leaves was done following standard method. Briefly, 15 cm long TLC plate was cut and marked cautiously with a marker, 20 µL of the extract was dotted onto marked plate using a capillary tube. *n*-Hexane: Ethyl acetate: Acetone (1 ml: 9 ml: 2 ml) was used as mobile phase. The plate was kept in a chromatographic compartment containing the individual solvent system and was covered with glass plate to prevent solvent evaporation. The plate was left in the chamber until the solvent travelled a distance of about 10 cm. The plate was then viewed with UV-florescence analysis cabinet at both short and long wavelengths.

Visualization of the Thin Layer Chromatography Plate

The TLC finger printing plate was derivatized with anisaldehyde sulphuric acid reagent followed by heating at 100^0C till colored bands of various secondary metabolites appeared. The observations were taken before and after derivatization in visible as well as ultraviolet light.

Rf values were calculated as follows:

$$Rf = \frac{\text{Distance travelled substance}}{\text{Distance travelled by solvent}}$$

Column Chromatography

The pooled samples were dried and tested on TLC plates to ascertain the various compound(s) in them. Fractions with one or two compounds were selected for further purification using Column Chromatography (CC) method.

Briefly, 10 g of dried and purified methanol fraction of *L. inermis* leaf was dissolved in the mobile phase *n*-Hexane: Ethyl acetate: Acetone (1 ml: 9 ml: 2 ml). This solvent system was exposed to column chromatography (CC). Glass-52 Column filled with 650 g of silica gel was employed for this procedure. Following different color band development within the column, different fractions collected. Fractions gotten were dried using rotary evaporator reduced pressure **(**Abbot and Andrews, 1979).

Ethical Consideration

This study was ethically approved by ACUREC who is the regulatory body in charge of animal use in University of Ibadan. ACUREC issue a full approval with assigned number: **UI-ACUREC/18/0063.** All stress factors such as handling, feeding, housing, environmental conditions etc were adequately provided and the animals were humanly handled.

Experimental Animals and grouping

Wistar rats weighing 120-140 g, (20 rats) were used for this study. Normal temperature and humidity were maintained during the course of this study. The rats were fed with standard animal feed and water which were provided *ad libitum*. Experimental rats were grouped into four with five rats per group. Three groups were administered methanol bioactive sub fraction of *Lawsonia inermis* at 2.5 mg/kg, 5 mg/kg and 10 mg/kg for fourteen days. The last group was the control and were administered distilled water.

Sample collection

On fifteenth day, rats were anaesthetized using ether and haematological samples was collected from the median canthus of experimental animal for haematological and biochemical assays. The experimental animals were sacrificed and various organs were harvested. Sperm was extracted from the testes of all the rats and were analyzed for morphology (abnormal sperm cell) and sperm characterization (volume, concentration, motility and live/dead ratio) using standard method described by Wyrobek (1980).

Results

VLC and TLC

The result of VLC and TLC following solvent combination of chloroform and methanol at increasing ratio showed thirteen different sub-fractions (f1-f10). The TLC result with similar spot and retention factor (Rf) were combined as shown in Figure 1.

Weight Changes

The percentage weight gain increased in all treatment groups but 10 mg/kg have highest weight gain (18.2%) compared to other treatment groups; 2.5 mg/kg (13.8%), 5 mg/kg (13.9%) and untreated control (13.4%) (Table 1).

Relative organ-weight

All the measured organs; heart, liver, kidneys, testes and pancreas increased non-significantly in all the treatment groups except the pancreas of group dosage 5 mg/kg increased (0.29±0.02g) significantly ($p<0.05$) when compared to all other treatment groups and untreated control. There was no significant change in the weight of all organs measured when compared to untreated control (Table 2).

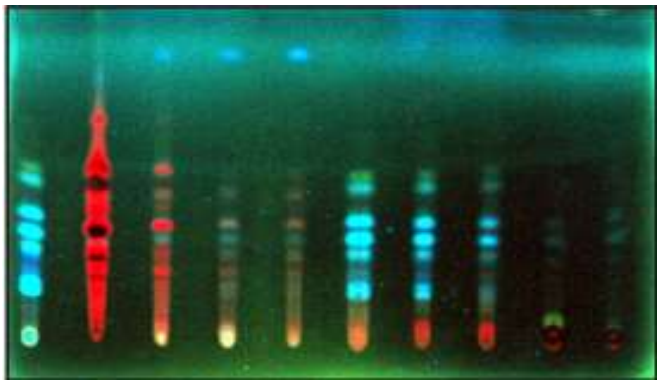

Figure 1: TLC print of bioactive sub-fraction C

Fraction C (4 spot) = f7, f8, f9 and f10 (EA and Acetone) (2:3, 1:4, and 0.5:4.5)

The fractions pulled together conclusively showed 10 major spot and the fraction (C) was cleansed through running through the column silica gel. The total weight of the dried sample recovered was: Fraction-C = 2.2 g

Andrological parameters of Wistar rats administered bioactive sub-fraction(c) of *Lawsonia inermis* Linn leaves

Sperm Characteristics

The 10 mg/kg (136.00±5.29 sperm cell/µL) dose significantly increased ($p<0.01$) sperm count compared to other treatment groups and untreated control. There was no significant alteration in sperm motility, volume and live/dead ratio in other treatment groups when compared to untreated control (Table 3).

Sperm Morphology

Total sperm abnormality decreased ($p<0.05$) significantly in groups dosed with 10 mg/kg (12.07±0.61) when compared to all other treatment groups and untreated control (14.34±1.04 sperm cell/µL) [Table 4]. The bent tail sperm abnormality of 10 mg/kg presented significant ($p<0.01$) reduction (1.97±0.23 sperm cell/µL) when compared to untreated control (3.04±0.34 sperm cell/µL). There was no significant alteration in sperm morphology across the treatment groups (Table 4).

Table 1: Changes in weight (g) and percentage change for Wistar rats administered bioactive sub-fraction of *Lawsonia inermis* leaves.

Weight (G)	Control	2.5 mg/kg	5 mg/kg	10 mg/kg
Day 0	121±7.02	128±5.9	121±8.39	134±10.69
Day7	155±5.51	157±9.54	155±11.02	157±20.88
(% change)	(21.9%)	(18.5%)	(21.9%)	(14.6%)
Day14	179±10.79	178±14.01 (13.8%)	180±12.34 (13.9%)	192±10.12
(% change)	(13.4%)			(18.2%)

Data rep. as Mean ±SD: n=5

Table 2: Relative organ weight of Wistar rats following sub-chronic administration of bioactive sub-fraction of *Lawsonia inermis* leaves

Grp/organ	Heart	Liver	Kidney	Testes	Pancreas	Brain
Control	0.37± 0.11	2.94±0.25	0.70±0.07	1.07±0.06	0.21±0.04	0.87±0.07
2.5 mg/kg	0.39± 0.05	3.41±0.37	0.75±0.05	1.96±0.30	0.23±0.03	0.91±0.07
5 mg/kg	0.41±0.02	3.59±0.32	0.74±0.03	1.10±0.19	0.29±0.02[a]	0.97±0.11
10 mg/kg	0.41±0.03	3.72±0.73	0.75±0.03	1.32±0.19	0.25±0.01	0.93±0.04

Results are shown as Mean ±SD: n=5

Table 3: Effect of bioactive sub fraction C of *Lawsonia inermis* Linn leaves on sperm characterization of Wistar rats following sub-chronic administration.

Group Treatment	Sperm motility	Sperm count	Sperm volume	Live/dead ratio
Control	77.67±5.77	101.00±3.61	5.17±0.06	96.00±1.73
2.5 mg/kg	73.33± 5.77	110.70±6.66	5.17±0.06	97.00±1.73
5 mg/kg	66.67±5.77	108.30±12.34	5.20±0.00	92.67±6.81
10mg/kg	88.33±2.89	136.00±5.29 [a]	5.17±0.06	97.00±1.73

Data rep. as Mean ±SD: n=5; [a] Significant [a]$p\leq0.05$

Haematology of Wistar rats administered bioactive sub-fraction of *Lawsonia inermis* leaves

The haematology following treatment with fraction of *Lawsonia inermis* Linn. showed that PVC, RBC, haemoglobin (Hb) had no significant effect compared to untreated control. WBC and its differential count such as Neutrophils, Lymphocytes and Monocytes also showed similar observation without significant alteration across all the treatment groups when compared to untreated control. However, the platelet of 10 mg/kg ($4.33\pm0.43X10^5$/µl) increased significantly ($p<0.001$) when compared with other treatment groups and untreated control. (Table 5)

Serum chemistry of Wistar rats administered bioactive sub-fraction of *Lawsonia inermis* leaves

Serum chemistry following treatment with fraction(c) of *Lawsonia inermis* Linn. showed that AST increased significantly ($p<0.01$) while urea and creatinine decreased significantly ($p<0.001$) in group dosed with 2.5 mg/kg when compared to untreated control. 5 mg/kg presented a significant reduction ($p<0.05$) in total protein, urea and ALP while ALT increased significantly ($p<0.05$) when compared with untreated control. Highest dosed 10 mg/kg showed a significant ($p<0.001$) decreased urea and creatinine when compared to untreated control (Table 5).

Serum electrolytes following bioactive sub-fraction treatment using *Lawsonia inermis* leaves in Wistar rats

Serum chloride ion of group dosed with 2.5 mg/kg decreased significantly ($p<0.05$) when compared with other treatment groups and untreated control. Sodium ion (Na^+) presented a significant decrease ($p<0.01$) across all the treatment groups when compared with untreated control. Bicarbonate (HCO_3^-) increased significantly in 5 mg/kg when compared to other treatment groups and untreated control. Potassium (K^{2+}) and calcium (Ca^{2+}) ions did not show any alteration in all the treatment groups when compared to untreated control (Table 6)

Table 4: Effect of bioactive sub fraction of *Lawsonia inermis* leaves on sperm morphology of Wistar rats.

Index	Control	2.5 mg/kg	5 mg/kg	10 mg/kg)
Total abnormal	14.34±1.04	13.79±1.13	14.17±0.59	**12.07±0.61[a]**
Rudimentary tail	0.48±0.23	0.49±0.25	0.48±0.28	0.48±0.27
Tailless head	0.99±0.24	1.08±0.37	0.98±0.24	1.06±0.17
Headless tail	1.08±0.37	1.16±0.37	1.06±0.37	1.24±0.26
Bent tail	3.04±0.34	2.32±0.37	2.77±0.32	**1.97±0.23[b]**
Curved tail	2.79±0.40	2.66±0.64	2.86±0.09	2.22±0.41
Curve mid-piece	2.64±0.54	2.57±0.15	2.86±0.09	2.47±0.23
Bent mid-piece	2.88±0.47	2.90±0.38	2.68±0.26	2.47±0.23
Looped tail	0.56±0.27	0.49±0.25	0.48±0.28	0.56±0.31

Data rep. as Mean ±SD: n=5 ; [a b]Significant [a]p≤0.05 [b]p≤0.01

Table 5: Effect of bioactive sub fraction C of *Lawsonia inermis* Linn leaves on haematology of Wistar rats following sub-chronic administration.

Parameter	Control	2.5 mg/kg	5 mg/kg	10 mg/kg)
PCV (%)	33.67±3.05	32.67±4.73	33.33±2.89	31.00±4.58
RBC×10^6/µl	4.82±0.51	4.65±0.79	4.74±0.46	4.36±0.75
HB (g/dl)	11.10±1.02	10.73±1.57	10.97±0.92	10.53±1.00
MCV (fl)	69.67±0.57	67.33±3.06	70.33±0.58	71.33±2.31
MCH (pg)	22.03±0.31	23.30±0.20	23.17±0.29	23.43±0.51
MCHC (g/dl)	32.80±0.26	32.80±0.10	32.87±0.12	34.23±2.14
WBC×10^3/µl	9.63±1.46	9.39±0.71	8.03±1.34	9.09±1.06
Lymph ×10^3/µl	5.50±1.01	5.57±0.53	5.46±1.28	5.45±0.41
Neutro ×10^3/µl	3.95±0.68	3.66±0.71	2.38±0.76	3.46±0.93
Mono×10^3/µl	0.23±0.12	0.13±0.06	0.11±0.05	0.16±0.12
Platelet ×10^5/d/µl	2.97±0.18	3.17±1.57	3.01±0.14	**4.33±0.43[a]**

Data rep. as Mean ±SD: n=5; [a S]ignificant [a]p ≤0.001

Table 6: Effect of bioactive sub-fraction following sub-chronic administration of *Lawsonia inermis* leaves on serum chemistry of Wistar rats.

PARAMETER	Control	2.5 mg/kg	5 mg/kg	10 mg/kg
T. Protein(g/dl)	8.47±1.41	6.67±0.31	**6.10±0.89[a]**	7.30±0.30
Albumin(g/dl)	5.00±0.87	3.70±0.10	4.47±1.25	5.07±0.55
Globulin (g/dl)	2.80±0.20	2.97±0.29	2.37±0.45	2.23±0.25
ALT (mmol/l)	13.63±1.19	11.67±0.57	**14.13±1.93[b]**	12.73±0.83
AST (mmol/l)	6.55±1.75	**8.23±3.64[b]**	5.48±8.62	7.12±2.59
ALP (mmol/l)	22.30±4.58	23.47±2.39	**19.17±2.08[a]**	20.63±2.56
Urea (mmol/l)	7.53±1.16	**4.40±0.53[b]**	**4.08±0.71[b]**	**4.77±0.68[b]**
Creatinine. (µmol/l)	167.70±8.02	**95.40±3.56[c]**	**107.10±9.68[c]**	**101.80±3.74[c]**
Potassium ion (K^{2+})	5.67±0.15	4.33± 1.12	5.20±0.61	5.23±0.59
Chloride ion (Cl^-)	98.03±1.46	**85.53±2.41[a]**	97.80±4.48	91.67±6.69
Sodium ion (Na^+)	149.50±1.31	**137.90±3.02[c]**	**140.90±1.44[b]**	**141.10±0.76[b]**
Bicarbonate ion (HCO_3^-)	18.73±0.38	21.17±2.02	**22.27±1.76[a]**	19.17±0.90
Calcium ion (ca^{2+})	0.60±0.17	0.66±0.21	0.67±0.25	0.63±0.25

Data rep. as Mean ±SD: n=5; [a b c] Significant [a]p≤0.05 [b]p≤0.01 [c]p≤0.001

DISCUSSION

Reports on toxicological research lay emphases on hepatotoxicity but other organs like kidney, heart, testes and spleen are usually affected when exposed to crude extract from medicinal plants (Ekor, 2013). The body and relative organ weight assessment is one of the index factors used during treatment with definite compound from pure fraction and this proffers information on the well-being of the animals. A significant reduction in body mass or alteration in weight may point to various responses such as treatment induced toxicity (Rocha *et al.,* 2012). The result from this

study `showed that sub-fraction of *Lawsonia inermis* did not alter developmental index of the treated rats signifying that there is no systemic toxic effect without clinical signs of toxicity or death.

Sperm dysfunction is the main cause of male sterility and one of the target tissues for toxicity in biological systems with emphasis on sperm parameters (Cyrus *et al.,* 2015). Reports have shown that sperm motility determines fertilization. Sperm motility are directly associated with ability of a fertile male to achieve fertilization and conception in female (Zhou *et al.,* 2008). The sub-fraction caused significant increased sperm count in treated rats and this was pronounced in treatment 10 mg/kg The result further noted significant reduction in total sperm abnormality compared to untreated control rats. This observation is in line with the work of Kefer *et al.* (2009) confirming that fractions of medicinal plant improve sperm morphology.

Hematopoietic system is known to be sensitive to pure compound from fractions of medicinal plant. Results from different research protocols have shown that exposure to compounds, drugs or whole plant significantly alter heamatological indices. It is an important index of toxicity that have high predictive potential for systemic toxicity (Khora *et al.,* 1997). The present result of methanol sub-fraction of *L. inermis* on haematological parameters indicated non-significant alteration in most of the haematological indices. This result specifically showed significant increased platelet count at 10 mg/kg and this may be as a result of healing property attributed to the leaves of *L. inermis*. Serum protein; albumin is synthesized in liver and the level could provide information on essential proteins synthesis (Rasekh *et al.,* 2008). The outcome of this study showed a non-significant increased albumin at 10 mg/kg while 2.5 and 5mg/kg showed non-significant reduction when compared to untreated control. Rats treated with 10 mg/kg showed a non-significant alteration in ALT, AST and ALP compared with untreated rats. This observation affirms that the sub-fraction of *L. inermis* does not affect hepatocellular function. Serum electrolytes, urea and creatinine are regarded as major markers in kidney function (Gowda *et al.,* 2009). Result of this work following sub-chronic treatment with sub-fraction of *L. inermis* showed non-significant alteration in most of these markers especially group dosed with 10 mg/kg. This observation showed that the sub-fraction was not toxic to the kidney.

Conclusion

It was concluded that bioactive sub-fraction (C) of *Lawsonia inermis* was safe at the administered dosages unlike reported deleterious effects of the crude extract on haematology, sperm morphology and immunity in rats.

Conflict of Interest

The authors have no conflict of interest to declare.

Authors' Contribution

AA and OAO planned the experiments. AA., BA. and AGJ carried out the experimental dosing. AOA and BA. collected samples for various test. AA, BA and AOA contributed to the interpretation of the results. AA and OAO took lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and the manuscript.

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