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Preprint · June 2023

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ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

60-Day Assessment of Mr. Flush® Cleanser on Hematological indices and Kidney Histoarchitecture in Exposed Wistar Rats

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Authors' contributions
This work was carried out in collaboration among all authors. AuthorsIUP and OS designed the research work, wrote the protocol and first draft of the manuscript. Authors BMI and FTI reviewed and vetted the first draft. Author IUP managed the literature searches, while author WU effected corrections to the first draft. Authors JOJ and IAM performed the statistical analysis. Author WU eviscerated the liver and kidney tissues from all the euthanized experimental rats. All authors made significant financial contributions as well read and approved the final manuscript.

Abstract

Aim: there is a surge in herbal product sales with less adequate information on their safety. The toxicity concerns of Mr. Flush® polyherbal formulation on hematological and biochemical parameters was investigated in Wistar rats.

Methodology: Thirty Wistar rats of both genders were randomly allotted to six groups (5 per group) and treated by oral administration daily thus: Groups 1 and 2-Controls (distilled water, 10 mL/kg), Groups 3-4; 5-6 received the Polyherbal mixture (374, 748) mg/kg, respectively. On 62nd day, animals were euthanized under diethyl ether anesthesia and sacrificed. Blood samples were collected using cardiac puncture for biochemical evaluation. Eviscerated liver and kidneys were weighed and fixed in 10% formalin for histopathological examination.

Results: The LD_{50} of the Polyherbal Formulation was estimated to be 3740.17 mg/kg (mouse per oral). Results were considered significant at $p \leq 0.05$. There was significant increase in blood platelets (PLT) in low dosed and high dosed groups compared with control, WBCs increased in treatment groups compared with controls. Kidney function parameters such as Urea and Creatinine were elevated in all treatment groups compared to control. Histopathology of the kidneys revealed degrees of pathologies such as occluding Bowman's space, widened proximal and distal convoluted tubules, hyperplasia of cortical tissue cells as well as hyperplasia of tubular and connective tissue cells.

Conclusion: There are significant nephrotoxicity concerns with the long-term consumption of this polyherbal formulation. Findings from the study suggests utmost care in the dosage of herbal formulations when consumed and total abstinence where possible.

1. INTRODUCTION

The use of herbal remedies in the treatment of many ailments in Sub-Saharan Africa, especially in Nigeria has been in practice for centuries and recently, is on the rise[1--4]. However, the claims that these herbal preparations are efficacious in the treatment of these ailments are yet to be scientifically elucidated by relevant agencies such as National Agency for Food Drug Administration and Control (NAFDAC). The production process of these crude herbal preparations are most times, devoid of necessary machinery, standards, quality control, thus, may not be hygienic. These herbal concoctions as a result may contain contaminants (heavy metals, polycystic aromatic hydrocarbons and microorganisms) that may pose harm to the consumers[5--8]. There is also the assumption that since these herbal preparations are produced from natural medicinal plants, it would be devoid of any adverse effect when consumed in any amount. Hence, these herbal remedies are without spe[9--12]cific doses and undergo no toxicological evaluation before being introduced into the open market. These disadvantages regardless, have not deterred the consumers of these herbal preparations who believe in their purported efficacy and find it cheaper to buy them from the open market without prescriptions than visiting the hospital or any medical facility to access health care[13--16].

Mr. Flush Cleanser® is a polyherbal product that consists of five different medicinal plant extracts including *curcuma longa, azadirachta indica, garcinia kola, nigella sativa,* and *moringa oleifera.* It is licensed by the National Agency for Food and Drug Administration and Control (NAFDAC) under the registration number A7-1516L.

It is widely distributed and consumed among the inhabitants of North-Central Nigeria as it is acclaimed to be efficacious in the treatment of a plethora of ailments which include diabetes, waist pain, malaria, typhoid, piles, eye infections, low sperm count, vaginal discharge and so on[17--20].

There is a dearth of information on the toxicological evaluation of herbal preparations distributed and consumed in North-Central Nigeria (Nasarawa State) both in animal and human studies. Therefore, this study was designed to evaluate the 60-day exposure of Mr. Flush® in Wistar rats.

2. MATERIALS AND METHODS

2.1 Preparation of Stock solution

The herbal formulation was bought from a Pharmacy (major distributor) in Mararaba (Batch number B-22344, NAFDAC registration number A7-1516L), Nasarawa

State and the stock concentration was determined as follows:

Aliquots of 10 mL herbal solution was poured into four crucibles and were evaporated to dryness in an oven (Griffin Britain) with temperature set at 70 °C to determine the marc. The initial weights of the crucibles were determined using a weighing balance. The stock concentration was determined by taking the average of the differences between the weight of the crucibles and weight of marc in 1 mL of the solution (test sample) using the procedure below:

Weight of crucible = A (g)

Weight of crucible + marc = B (g)

Weight of marc = B – A (g)

The final doses administered in mL were calculated using the formula:

Concentration of drug used = $\sum$B – A (g) / N (mL) = X g/mL

2.2 Experimental Animals

Swiss albino mice and Wistar albino rats of both genders were obtained from and kept at the Department of Pharmacology and Toxicology Animal House of the Faculty of Pharmaceutical Sciences, Bingham University, Nasarawa State, Nigeria. The animals were maintained under standard environmental conditions and fed with standard NIHOR-branded rodent feed (Super Feeds, Nigeria Ltd). The animals were kept at room temperature in cross-ventilated rooms, without illumination at night to achieve the 12 h light/ 12 h dark period. The animals were acclimatized to the laboratory environment for one week before the commencement of the study. All animals were given access to food and water *ad libitum*.

2.3 Determination of Acute Toxicity

The oral median lethal dose (LD_{50}) of the polyherbal mixture was determined according to the modified method of Lorke (Lorke, 1983). A total of twenty (20)Swiss albino mice weighing between 20 – 25grams were fasted overnight. The animals were divided into five groups of three animals per group and administered orally,various doses of the polyherbal mixture as shown in Table 1. The animals were observed for cardinal signs of toxicity and mortality within 24 h. The estimated LD_{50} was used to select the appropriate doses to be administered during the 60-day sub-chronic toxicity studies. The LD_{50} was calculated using the formula:

$LD_{50} = \sqrt{AB}$

Where A = maximum dose with 0% mortality and

B = minimum dose with 100% mortality

2.4 Experimental Design

A total of 30 adult Wistar rats of both genders (15 each) were weighed and randomly allotted to six groups of five animals each and treated as shown in Table 2. The animals were treated daily using oral gavage for 60 days of the test period[21--24]. Rats in different groups were observed closely for any behavioral changes, feeding and drinking habits, as well as body weight and general morphological changes. At the expiration of the test period, the animals were euthanized under diethyl ether (Sigma Aldrich, USA) anesthesia and sacrificed. Blood samples were collected using cardiac puncture technique into EDTA and plain sample bottles for hematologic and biochemical (Full blood count, creatinine, blood urea and liver enzymes) investigations respectively. The liver and kidneys were eviscerated for internal macroscopic and histopathological examinations.Animal handling and care was conducted in complete compliance with the Guide for the Care and Use of Laboratory Animals[25].

2.5 Hematological Analysis

Blood samples were analyzed using the automated hematological analyzer (HEMA-D6031, China). Parameters analyzed included hemoglobin (Hb) concentration, pack cell volume (PCV), red blood cell count (RBC), White blood cell count (WBC), platelet (PLT), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC).

2.6 Biochemical Analysis

Serum creatinine:was assayed based on the reaction of creatinine with an alkaline solution of sodium picrate to form a red complex[26]. Serum creatinine and blood urea nitrogen (BUN) levels were measured as markers of kidney function. Except otherwise stated, these biochemical investigations were done using automated analyzers and Fortress Diagnostic Kits® according to standard procedures of manufacturer's protocols at Bridge Bio-Tech Ltd, Ilorin, Nigeria.

2.7 Histopathological Examination

After the collection of blood from the diethyl ether euthanized and sacrificed rats, the liver and kidneys were immediately excised, freed from adventitia, blotted with tissue paper, weighed, sectioned and fixed in 10% formalin for histological studies. The fixed sections were passed through xylene, alcohol and water to ensure that the tissues were totally free of wax and alcohol. Each section was then stained with hematoxylin and eosin for photo-microscopic assessment using light microscope at a magnification of 40. To minimize bias, the pathologist was denied knowledge of the doses and treatments given to the different groups of experimental rats[27].

2.8 Statistical Analysis

Data generated was statistically analyzed using SPSS version 21. Statistical significance between the groups were analyzed by means of one-way analysis of variance (ANOVA). Results were presented as Mean ± S.E.M. and values less than ($p < 0.05$) were considered significant**.**

2.9 Limitations

This present study was not followed by a 14-day reversibility study (where exposure of the lower dosed animals to the polyherbal mixture must have been discontinued and all parameters determined in the main work, repeated) due to set limits.

3. RESULTS

3.1 Acute Toxicity Test

Using the modified Lorke's method (Lorke, 1983), the LD_{50} value of Mr. Flush® cleanser was estimated to be 3740 mg/kg body weight (oral).

3.2 Effect of Treatment on Body Weight

The results of the effect varying doses of the polyherbal mixture on body weights of the rats are presented in table

3.1. During the period of the experiment, the low dose groups showed gradual increase in their body weight from start to end of the study while the high dose groups experienced a decline in body weight during the last two weeks of the study. However, there was no statistically significant ($p<0.05$) weight difference between control groups and treatment groups administered with the polyherbal mixture.

3.3Effect of Treatment on Hematological Parameters

The results of the effects of Mr. Flush® Cleanser on hematological indices of Wistar rats after 60 days treatment is as shown in table 3. There was no statistically significant difference ($p>0.05$) in the levels of packed cell volume, red blood cells, hemoglobin, neutrophils, monocytes, basophils, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration of animals in the treatment group when compared with the control group. However, there was a significant increase ($p<0.05$) in the platelet count and white blood cell count respectively in the treatment groups compared with control. The high dose male (HDM) showed increased white cell count compared to control at a significant level ($p<0.05$). There was also increased production of platelets in low dose treatment groups (LDM and LDF) administered with the polyherbal formulation ($p<0.05$), but this increase was highest in the high dose male (HDM) and high dose female (HDF) treatment groups.

3.4 Biochemical Parameters

The biochemical indices for liver and kidney function are as represented in table 4. there was no significant change in serum levels of total and direct bilirubin in the various treatment groups when compared with control. However, there was significant changes ($p<0.05$) in alkaline phosphatase (ALP), aspartate transaminase (AST)and urea in the LDM, HDM and HDF groups compared to control. The same was not true for creatinine levels which were insignificant across all treatment groups except the high dose female (HDF) group ($p<0.05$).

3.5 Histopathological Assessment

Histological examination of the kidney of rats (both genders) in the control groups showed normal and well-preserved histoarchitecture of renal cortex with the urinary tubule, proper orientation of layers of urinary ducts and a clear Bowman's capsule. Low dose groups of experimental rats (LDM and LDF) showed no pathological changes either when compared with control.However, in the High dose groups, some pathological changes and mild inflammation around the renal cortex were seen. This was more evident in the HDF group as seen in figure 1.

Histological examination of the liver of rats (both sexes) in the control groups also showedwell preserved cellular architecture of the ductal cells, normal orientation of layers of blood vessels, well-spaced hepatocytes and presence of kupffer cells within the sinusoids. The LDM and LDF groups presented with well-preserved cellular structure, ductal spaces and normal hepatic architecture when compared with the control group.

Table 1. Experimental design for acute toxicity testing of the polyherbal mixture

S/N	Treatment	Dose (mg/kg)	Observed duration
1	Group 1	1000	24 h
2	Group 2	1500	24 h
3	Group 3	2000	24 h
4	Group 4	2500	24 h
5	Group 5	3000	24 h
6	Group 6	4000	24 h

Table 2. Experimental Design

Group	Number of Animals	Treatment	Dose
CM	5	Distilled water	10ml/kg
CF	5	Distilled water	10ml/kg
LDM	5	Mr. Flush Cleanser®	374.17mg/kg
LDF	5	Mr. Flush Cleanser®	374.17mg/kg
HDM	5	Mr. Flush Cleanser®	748.50mg/kg
HDF	5	Mr. Flush Cleanser®	748.50mg/kg

CM=control males, CF=control females, LDM=low dosed males, LDF=low dosed females, HDM=high dosed males and HDF=high dosed females

Table 3. Effect of polyherbal mixture on hematological indices of Wister rats

TREATMENT	PCV (%)	RBC (x 10^3/L)	WBC (x 10^9/L)	PLT (X10^9/L)	HB (g/dl)	Neutrophils (%)	Monocytes(%)	Eosinophils (%)	Basophils (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)
CM	50.6±0.1	5.1±1.7	4.5±1.0	201.0±0.1	13.3±0.9	20.3±1.4	6.0±0.4	1.7±0.5	0.0±0.0	92.7±1.5	31.8±1.1	30.9±0.9
CF	55.3±2.2	5.4±0.8	4.1±2.1	221.7±2.2	16.5±0.0	24.7±2.0	5.7±0.1	2.3±1.3	0.1±0.0	98.0±1.1	33.4±1.7	31.0±1.2
LDM	46.7±2.4	4.9±1.1	6.1±2.4	275.6±2.1*	13.1±0.6	27.0±3.2	6.3±0.2	1.3±1.2	0.0±0.0	87.0±2.8	31.6±2.1	30.1±2.1
LDF	44.3±0.3	5.1±1.4	9.7±0.9	294.3±1.4*	13.3±1.2	26.3±1.9	4.7±1.7	1.0±0.3	0.0±0.0	87.3±2.2	32.4±1.3	30.8±2.6
HDM	42.7±2.1	4.3±1.2	12.3±1.6*	428.0±1.1*	13.5±1.4	34.3±1.2	6.3±1.6	2.0±3.3	0.1±0.0	84.3±1.4	29.4±0.1	32.9±1.6
HDF	41.0±0.9	4.3±2.2	7.2±2.0	511.67±1.8*	14.5±1.8	37.7±0.5	4.3±1.1	2.0±1.2	0.0±0.0	84.0±0.9	28.6±2.3	31.6±2.8

Values are expressed in Mean±SEM*, **, ***, **** indicates significant differences at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively

Table 4. Weekly changes in weight in animals administered with varied doses of Mr Flush® cleanser.

MR. FLUSH®	CM	CF	LDM	LDF	HDM	HDF
WK0	192.1±4.9	202.7±3.8	205.8±8.8	186.7±9.4	200.9±7.3	205.3±9.9
WK1	198.4±6.2	210.6±3.2	209.4±8.9	193.7±8.3	203.5±6.7	208.6±9.8
WK2	205.5±6.6	215.7±2.8	215.4±9.0	199.2±8.7	209.7±7.1	212.0±9.5
WK3	211.3±5.6	220.8±2.6	219.4±8.5	203.1±8.6	212.7±6.3	216.3±9.7
WK4	215.9±6.0	226.2±1.1	221.1±7.2	206.3±6.7	215.9±6.5	218.0±9.5
WK5	221.0±5.4	230.5±0.5	226.0±6.7	212.8±5.6	220.1±6.2	221.3±9.3
WK6	226.3±4.6	234.1±1.1	233.3±5.4	218.5±5.4	239.0±2.9	224.2±9.0
WK7	233.0±3.8	237.8±9.1	237.6±5.3	228.9±2.0	242.7±2.9	226.8±8.5
WK8	238.7±3.7	236.2±7.0	238.7±2.8	236.0±3.8	245.1±3.3	237.5±5.1
WK9	246.1±2.8	242.2±5.8	243.8±2.1	240.1±2.3	247.4±3.2	239.1±4.9

Values are expressed in Mean±SEM*, **, ***, **** indicates significant differences at p<0.05, p<0.01, p<0.001 and p<0.0001 respectively.

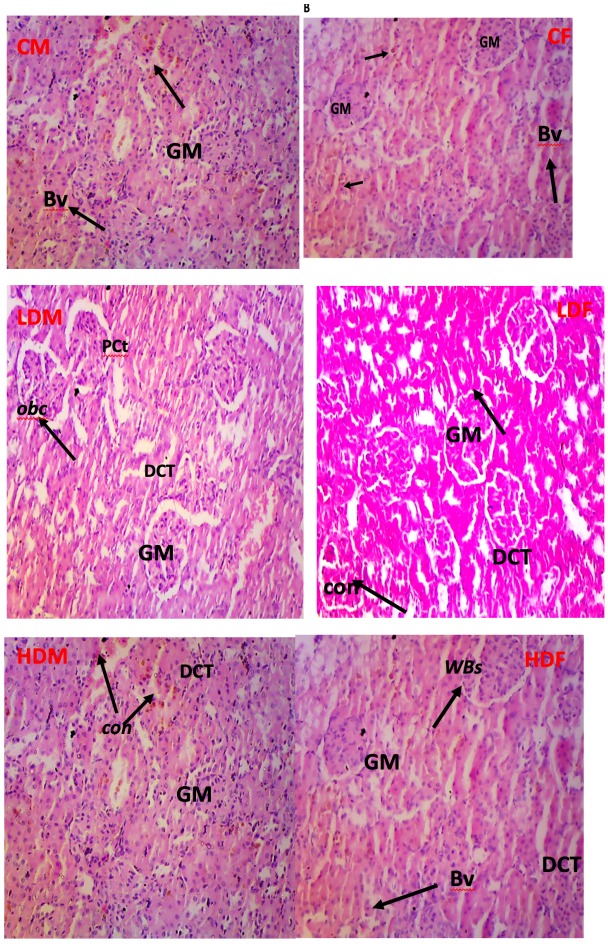

Figure 1 Photomicrographs of kidney sections from controls and Mr. Flush® exposed Wistar rats.

CM = Control males, HDM = High dose males, LDM = Low dose males, CF = Control females, HDF = High dose females, LDF = Low dose females,Obc=obstructed convoluted tubule Widened proximal tubules, Con=congestion, WBs = Widened bowman space, GM = glomerulus,Bv=blood vessel, PCT = Proximal convoluted tubule, DCT = Distal convoluted tubule x 40 magnification.

4. Discussion

In growing animals, gradual weight gain over a considerable length of time is seen as an indicator of good health and nutrition[28--31]. It is generally expected that with appropriate balance in diet, physical activity and good homeostasis, living systems are expected to maintain a steady healthy weight throughout life[32--34]. Fluctuations in mean body weight (excessive weight loss or gain) is seen as indicator of disease or a state of unwellness. In this present study, animals administered with both low doses and high doses of Mr. Flush® showed variations in weight gain over the period of study. This suggests that there is an interaction between the phytochemical constituents of polyherbal mixture and the intrinsic determinants of weight in the animals although the results were not conclusive as aberrations in weight were deemed statistically non-significant. The results from this study are supportive of data according to Adeyemi et al[35] who equally observed changes in weight of animals treated with a certain polyherbal formulation. The aberrations in weight of animals in the treatment groups were credited to the antilipidemic effects of the herbal formulation.

Herbal plants has been used for various medicinal and health benefits. Medicinal plants are often consumed locally without a graded dose or expected duration of use[36--38]. Blood is widely used in toxicological evaluations as a good indicator of pathological and physiological changes in living organisms[39--40]. Changes in hematological parameters such as hemoglobin (Hb), hematocrit (PCV), red blood cell (RBC), white blood cell (WBC), blood platelets (PLT), differentials such as mean corpuscular volume, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are commonly examined to assess toxicity in living organisms. Fluctuations in these hematological parameters is usually indicative of anemia, as well as other hemoglobinopathies[41]. This implies therefore that hematological indices are important for the morphologic classification of anaemia. In the present study, animals treated with selected doses of Mr. Flush did not show any significant changes in PCV, and hematocrit, suggestive of a non-toxic effect on the bone marrow of these animals when compared with the control group. WBCs are known to be the body's defense system against infections and invading pathogens[41--42]. Proliferation of white cells are usually suggestive of an immune response to infection. In this study, white cell count was increased significantly in the high dosed groups (HDM and HDF) compared with their respective controls. This effect may suggest the polyherbal formulation has an inductive effect in the hematopoietic cell lines of the bone marrow[43].Blood platelets are responsible for blood clotting in animals. Low platelet count suggests that the process of blood clothing would take much longer in such an animal and as a result, there would be much blood loss should in any case of trauma arise in such an animal[44]. In this study, treatment groups administered with both low doses and

high doses of the polyherbal mixture showed significant increase in their platelet count all through the period of study especially the High Dose female group (HDF). This may justify the use of this poly herbal mixture in the treatment of wounds, sores and menorrhagia in women. This positive effect on platelet count could be attributed to the phytochemical constituents of the polyherbal mixture. For example, green plants such as *Mangifera indica* leaves are known to be very rich in vitamin C, carotenoids, folates and other polyphenolic compounds which are essential for clotting and wound healing. According to Nse-abasi[45], the major function of white blood cells and its differentials are for humoral immunity, to fight infections and defend the body by phagocytosis against invading organisms[46]. A low WBC count in humans is indicative of a decrease in natural immunity and exposure to evading pathogens. This is seen in people infected with HIV and tuberculosis. In this study, Wistar rats treated with selected doses of Mr. Flush® did show slight increase in WBC count especially in the high dosed groups (HDM and HDF). This increase however was not statistically significant when compared with the control groups (CM and CF). This effect suggests that the polyherbal mixture may not be toxic to white blood cell lines.

Blood urea Nitrogen (BUN) and creatinine which are end products of protein metabolism are good indicators of renal function[47--50]. Urea itself is a waste product of protein metabolism that is normally transferred from the blood to kidneys. Creatinine is a metabolite of muscle creatine whose amounts is usually proportional to muscle mass and size. Increased serum levels of both urea and creatinine are usually indicative of kidney damage or a loss in kidney function[51--54]. In this study, serum levels of urea and creatinine were elevated significantly in rats administered with low dose and high dose of the herbal mixture, Mr. Flush® in comparison to the control and the high dose groups. The nephron is the functional unit of the kidney. Structurally, it comprises of the coiled renal tubule and a vast network of peritubular capillaries and the Bowmans capsule which is responsible for urine formation as well as regulation of glomerular filtration rate (GFR)[55--58]. The rate of urine formation is directly proportional to the GFR (Newman and Price, 1999). The structural appearance of the Bowmans capsule resembles a coiled funnel with a filter paper attached to it[59--61]. Interestingly, histological examination of the kidney tissues of Wistar rats treated with selected doses of the polyherbal mixture showed some signs of vascular congestion, which maybe as a result of ischemia or poor tissue perfusion within the kidney tissue owing to hyperemia. This finding was equally corroborated by Udom et al.[62] and Etuk et al.[63] who reported the presence of hyperemia in the kidneys of animals administered with doses of a particular polyherbal mixture for 60 days. The poor perfusion within the peripheral tissues of the kidney could trigger an inflammatory response from the leukocytes as well as migration of other local tissue factors (cytokines and interleukins) giving rise to congestion and renal damage.Therefore, the kidneys are organs of concern with regards to sub-chronic toxicity of Mr. Flush®. However, reversibility studies were not carried out to ascertain if the renal damage were reversible on withdrawal of the polyherbal mixture.

CONCLUSION

Mr. Flush® was found to be safe, with good hematological potentials, supporting label and popular claims. The product was also found to have tendency cause damage to vital organs at high dose and over a considerable period of time.

Conflict of Interest

It is worthy of note that the products employed in this research are common products in our area of research and country. There is no conflict of interest whatsoever between the authors and manufacturer of the products especially as the authors do not intend to use these products as an avenue for any litigation but for the advancement of scientific knowledge. Also, the research received no funding from any external body (the manufacturers of the test substance inclusive) rather it was funded by personal efforts of all the authors.

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