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Immunomodulatory Activities of Yoyo Bitters: Recommended Dose Precipitated Inflammatory Responses in Male Wistar Rats

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Abstract: This study investigated the immunomodulatory capabilities of the sub-chronic administration of Yoyo bitters in male Wistar rats. Eighteen rats weighing 86.2 ± 4.43 g were randomly picked into three equal groups. The rats were acclimatized for 14 days, after which 0.308 and 0.462 mL kg^{-1} b.wt. of Yoyo bitters were administered once daily to groups B and C respectively for 56 days, while group A received distilled water. The feed intake, body weight, blood glucose, interleukin 2 (IL-2), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), haematological parameters, serum lipid profile and uric acid, liver reduced glutathione and malodialdehyde were determined. The feed intake, body weight and blood glucose concentrations were reduced ($p < 0.05$) at the doses. No changes were recorded in the concentration of serum IL-2 ($p > 0.05$), but IL-6 decreased ($p < 0.05$) in group B and increased ($p < 0.05$) in group C, while TNF- α were increased ($p < 0.05$) dose dependent. The haematological parameters were decreased at all the doses ($p < 0.05$), except the ESR, WBC and lymphocytes that were increased ($p < 0.05$) and platelets in group C ($p < 0.05$). The serum total cholesterol, TAG, LDL-C and atherogenic index were decreased ($p < 0.05$) and HDL-C increased ($p < 0.05$) in group B only. Serum uric acid was reduced ($p < 0.05$) in group B, but increased in group C with the concentration of liver MDA ($p < 0.05$). The study, therefore, established that a dose lower than the manufacturer's recommended dose presented the desired immunomodulatory activities and the habitual use of Yoyo bitters at the adult recommended dose calls for caution.

Key words: Yoyo bitters, lower than, immunomodulatory, habitual, adult, recommended dose, caution

INTRODUCTION

The onset of almost all infectious and degenerative diseases, such as flu, bacterial infections, auto-immune disorders, tumour cells formation, AIDS etc are preceded or accompanied by inadequate immune response, due to the long term breakdown of the immune system (Van de Perre, 2003; Spelman *et al.*, 2006). The various human activities in civilized environments and the lifestyles expose humans to assaults by toxins such as industry wastes, vehicle emission, paints, insecticide and herbicides, work pressure, inadequate rest, social activities like smoking, alcoholism, grilled meats and canned food products. Indulgence in the activities cumulates in poor immune function that account for various clinical conditions such as premature aging, infections, chronic and degenerative diseases (Van de Perre, 2003; Spelman *et al.*, 2006). In addition, in developing countries, people hardly visit the hospital for medical check-up unless they feel ill or disease conditions

become obvious that self medication cannot contain anymore, due to lack of finance and the long wait before the doctor is accessed.

Immune modulating pharmaceutical drugs are prescribed by medical practitioners only in established clinical conditions, as there were no true immune modulating pharmaceutical drugs, due to their low efficacy and vast adverse effects like central obesity, hyperglycemia, osteoporosis, indiscriminate killing of all dividing cells, increasing the risk of opportunistic infections, sacrificing the "self"- "not self" regulatory mechanisms of lymphocytes etc. (Spelman *et al.*, 2006). However, some botanical preparations are alleged as immunomodulators by their abilities to alter and balance the activities of the immune cells through the dynamic regulation of the information molecules such as cytokines (Spelman *et al.*, 2006; Oyewo and Akanji, 2011).

It is thus, very imperative to always consider strengthening the immune system by the use of botanicals, to prevent the progression/management of

infective and degenerative diseases and also avoid unnecessary use of immune modulating pharmaceutical drugs. Unfortunately, there is limited scientific evidence regarding the efficacy to back up the continued therapeutic application of most of these herbal remedies. Therefore, there is the need for the scientific evaluation of immunomodulatory potentials of these herbal bitters. Recent ethnobotanical survey conducted by our group in Kwara and Oyo State region of Nigeria, recorded high frequency for the use of Yoyo bitters, as blood purifier and immune boosters. Consequently, this study was carried out to scientifically investigate the effect of the repeated administration of Yoyo bitters on the immune functions in male Wistar rats.

MATERIALS AND METHODS

Herbal bitters: Yoyo bitters was a product of Abllat Company Nigeria Limited, 12 Ajayi Close, Ikotun-Ijegan Road, Ikeja, Lagos State.

Blood glucose glucometer: Accu-chek active glucometer and test strips, products of Roche Diagnostic GmbH, D-68298 Mannheim, Germany were used for the fasting blood glucose level estimation.

Quantitative assay kits: The kits for the determination of rat Interleukin-2 (IL-2), Interleukin-6 (IL-6) and Tumour Necrosis Factor-alpha (TNF- α) were products of RayBiotech, Inc. USA, while those for Total Cholesterol, Triacylglyceride, High Density Lipoprotein Cholesterol (HDL-C) and Uric Acid were products of LABKIT, CHEMELEX, S.A. Pol. Canovelles-Barcelona, Spain. Reduced Glutathione assay kit was a product of BioAssay Systems, Hayward, USA.

Other reagents: All the chemicals and reagents used in the study were of analytical grade and were purchased from the British Drug House (BDH) Poole England and Sigma Aldrich Chemical Co. Inc., Milwaukee, Wis., USA.

Laboratory animals: Eight to ten weeks old male Wistar rats of average body weight of 86.2 ± 4.43 g were obtained from the Animal Care Facility II, Ladoke Akintola University of Technology (LAUTECH), Osogbo, Osun State. The rats were fed with rat pellet (product of Bendel Feeds and Flour Mills Ltd, Ewu, Edo State, Nigeria).

Methods

Experimental animals and procedure: The eighteen male Wistar rats were randomly grouped into three, comprising of six rats per group. The rats were housed in cages made

of wooden frames and metal netting and were fed *ad libitum* with rat pellet and tap water with 12-hours light/dark cycle. The cages were cleaned every morning and disinfected at intervals of 3 days. The rats were allowed to acclimatize for 14 days before extract administration was commenced. Calculated doses of the herbal bitters in mL kg^{-1} b.wt. as instructed in the manufacturer's pamphlet were administered to male Wistar rats as illustrated:

- Group A: Control, received 1.0 mL distilled water
- Group B: Received 0.308 mL kg^{-1} b.wt. of Yoyo bitters (YB)
- Group C: Received 0.462 mL kg^{-1} b.wt. of Yoyo bitters (YB)

The feed intake of the rats were monitored daily and prior to the administration of the herbal bitters and every interval of 7 days, the fasting blood glucose levels and the body weights of the animals were recorded. Administration of the herbal bitters was performed orally once daily between 7:20 pm \pm 30 min, using metal cannula attached to a 1.0 mL syringe. Administration lasted for 56 days, after which the rats were fasted for 12 hours and the blood glucose level and body weights determined. This study was conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH, 1985).

Haematological analysis: The haematological parameters were analysed by the automated haemology analyzer (SYSMEX K2X1: SYSMEX CORPORATION, JAPAN).

IL-2, IL-6 and TNF-alpha determination: The serum level of IL-2, IL-6 and TNF- α was determined by *in vitro* enzyme linked immunosorbent assay (ELISA) kit, using colourimetric reaction method as instructed in the kit manual with cat # ELR- IL2-001, ELR- IL6-001 and ELR- TNF alpha-001, respectively.

Blood glucose determination: The blood glucose concentration was determined by glucose oxidase reaction, using Accu-chek active glucometer and test strips. Glucose oxidase chromogen indicators and non-reactive agents are contained in the reagent pad to which about 2 μL of whole blood was applied.

Serum lipid profile determination

Total cholesterol: The serum total cholesterol level was determined by cholesterol oxidase-peroxidase (CHOD-POD) enzymatic colourimetric reaction, according to the method as described by Naito (1984a).

Triglyceride (TAG): The serum triglyceride level was determined by glycerol oxidase-peroxidase (GPO-POD) enzymatic colourimetric reaction, according to the method as described by Fossati and Prencipe (1982).

High density lipoprotein cholesterol (HDL-C): The serum HDL-C cholesterol level was determined by precipitation and cholesterol oxidase-peroxidase (CHOD-POD) enzymatic colourimetric reaction, according to the method as described by Naito (1984b).

Low density lipoprotein cholesterol (LDL-C) and very low lipoprotein cholesterol (VLDL-C).

The VLDL-C cholesterol and LDL-C were determined by computation, according to the methods described by Friedewald *et al.* (1972).

Serum uric acid determination: The serum uric acid level was determined by uricase-POD enzymatic colorimetric reaction, according to the method described by Schultz (1984).

Liver reduced glutathione determination: The levels of reduced glutathione (GSH) were determined using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) enzymatic colorimetric reaction, according to the method described by Baker *et al.* (1990).

Liver malondialdehyde determination: The concentration of thiobarbituric acid reactive substances, malondialdehyde (MDA) was determined using the method of Fraga *et al.* (1988).

Statistical analysis: This research work was Completely Randomised Design (CRD) and the results were expressed as mean of 5 replicates \pm standard error of mean (SEM). Results were analyzed using statistical package for social sciences (SPSS) 16.0 for Window software. Results were subjected to one way analysis of variance (ANOVA) to test the effect of each dose level on the parameter under investigation at 95% level of confidence. The Duncan

Multiple Range Test (DMRT) was conducted for the pairwise mean comparisons, to determine the significant treatment dose at 95% level of confidence. Values were considered statistically significant at ($p < 0.05$) and denoted by different alphabets (Mahajan, 1997).

RESULTS

The results were expressed as mean of five determination \pm standard error of mean (SEM) and significantly different ($p < 0.05$) mean values were denoted by alphabets.

Rat behaviour and morphology: Obvious signs of toxicity in terms of the physical appearance and behavioural changes were recorded on the 28th day of administration at 0.462 mL kg⁻¹ b.wt. of Yoyo bitters (Fig. 3), in which the rats were lean and less active. At 0.308 mL kg⁻¹ b.wt. of Yoyo bitters, the furs of the rats were full with no obvious signs of toxicity (Fig. 2), but, when compared to the control rats (Fig. 1), however, they were aggressive when disturbed.

Feed intake, body weight and blood glucose levels of male rat: The effects of the repeated administration of the herbal bitters on the feed intake (a measure of appetite), body weight and blood glucose concentrations of rats are presented in Table 1, Fig. 4, 5, respectively. The rats administered the herbal bitters consumed less standard rat pellets ($p < 0.05$) in an almost dose dependent manner. The body weight and blood glucose levels were reduced ($p < 0.05$) in a manner similar to that obtained in the feed intake.

Serum concentrations of IL-2, IL-6 and TNF- α , uric acid and lipid profile and liver reduced glutathione and malondialdehyde levels.

The effect of the repeated administration of the herbal bitters on serum concentrations of interleukin-2 (IL-2), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF- α), uric acid and liver reduced glutathione and



Fig. 1: A rat representing the control group



Fig. 2: A rat representing the 0.308 mL kg⁻¹ b.wt. group



Fig. 3: A rat representing the 0.462 mL kg⁻¹ b.wt. group

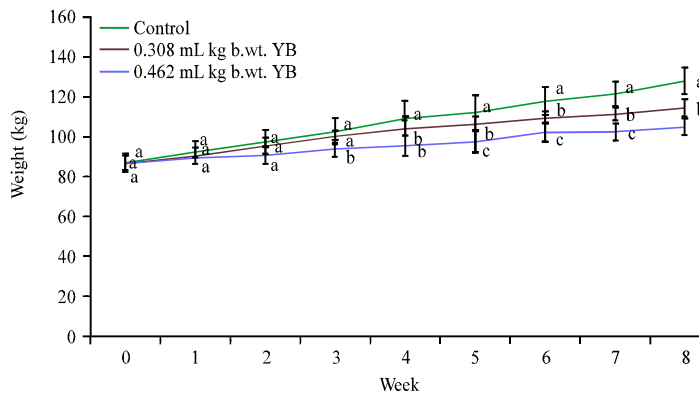


Fig. 4: Body weights of rats administered with the herbal bitters, values are Means±SEM, n = 5 *Values bearing different alphabets are significantly different (p<0.05) YB (Yoyo bitters)

Table 1: Feed intake of the rats following the administration of the herbal bitters

Week (g)	Control	Doses (BW)	
		0.308 (YB)	0.462 (YB)
0	100.71±5.55 ^a	98.47±3.39 ^a	101.35±4.34 ^a
1	107.40±4.75 ^a	118.56±4.87 ^b	110.14±3.59 ^a
2	112.33±3.89 ^a	101.01±5.22 ^b	98.22±4.11 ^b
3	109.26±5.07 ^a	96.67±4.98 ^b	93.07±3.57 ^b
4	114.53±7.22 ^a	98.08±3.33 ^b	90.84±4.22 ^c
5	110.59±4.19 ^a	97.77±4.04 ^b	90.16±3.21 ^c
6	102.99±6.22 ^a	93.92±5.07 ^b	82.55±5.32 ^c
7	118.65±4.92 ^a	90.59±5.35 ^b	72.57±4.43 ^c
8	122.44±5.38 ^a	79.38±3.87 ^b	69.6±3.48 ^c

Values are means±SEM; n = 5, Values bearing different alphabets are significantly different (p<0.05), g (gram), BW (mL kg⁻¹ b.wt.), YB (Yoyo bitters)

malondialdehyde concentrations in male rats are presented in Table 2. The administration of Yoyo bitters did not affect the serum IL-2 concentrations, while significant decrease (p<0.05) was obtained in serum IL-6 concentration at 0.308 mL kg⁻¹ b.wt. and significant increase (p<0.05) at 0.462 mL kg⁻¹ b.wt. The result of serum of TNF-α concentrations indicated significant increases (p<0.05) in a dose dependent manner (Table 2). Serum total cholesterol, triacylglyceride, very low density lipoprotein cholesterol (VLDL-C), low density lipoprotein cholesterol (LDL-C) and the atherogenic index (LDL-/HDL-C) levels were reduced significantly (p<0.05) in the

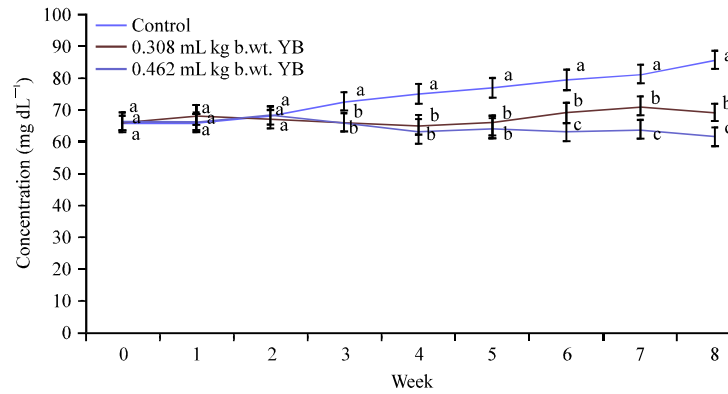


Fig. 5: Effect of herbal bitters on fasting blood glucose of male rats, values are Means±SEM, n = 5, *Values bearing different alphabets are significantly different (p<0.05), YB (Yoyo bitters)

Table 2: Effects of the administration of the bitters on serum cytokines, lipid profile, uric acid and liver reduced glutathione and malondialdehyde concentration

Parameters	Control	Doses (BW)	
		0.308 (YB)	0.462 (YB)
(pg mL ⁻¹)			
IL-2	28.22±2.17 ^a	29.06±1.58 ^a	32.00±3.11 ^a
IL-6	320.41±11.54 ^a	277.75±8.55 ^b	351.45±11.25 ^c
TNF-α	441.32±12.45 ^a	540.50±11.52 ^b	625.35±13.26 ^c
(mg mL ⁻¹)			
Total cholesterol	215.82±6.11 ^a	189.06±8.22 ^b	174.55±7.25 ^c
Triacylglyceride	205.62± 4.72 ^a	187.53±6.45 ^b	181.86±7.14 ^b
HDL-C	45.21± 2.19 ^a	61.05±4.21 ^b	47.55±3.02 ^a
VLDL-C	43.45± 1.55 ^a	38.62± 1.83 ^b	35.45± 1.06 ^b
LDL-C	144.22±5.15 ^a	135.56±4.24 ^b	120.14±3.44 ^c
LDL-C/HDL-C (NA)	3.94± 0.15 ^a	2.14±0.36 ^b	2.86±0.54 ^c
Uric acid	5.25±0.86 ^a	3.65±0.75 ^b	6.64±0.65 ^c
GSH (mg mL ⁻¹)	1.48±0.22 ^a	1.59±0.25 ^a	1.41±0.42 ^a
MDA (U)	5.19±0.55 ^a	4.81±1.02 ^a	6.25±0.89 ^b

Values are means±SEM; n = 5, Values bearing different alphabets are significantly different (p<0.05), Key: BW (mL kg⁻¹ b.wt.), YB (Yoyo bitters), na (not applicable) and U (nmole/mg of protein)

Table 3: Effects of repeated administration of herbal bitters on haematological parameters in Wistar rats

Parameters	Control	Doses (BW)	
		0.308 (YB)	0.462 (YB)
PCV (%)	38.8±2.03 ^a	35.11±1.65 ^b	34.85±1.82 ^b
RBC (10 ¹²) L ⁻¹	6.46±0.49 ^a	5.22±0.22 ^b	5.08±0.51 ^b
Hb (g dL ⁻¹)	12.88± 0.68 ^a	11.05±0.82 ^b	10.35±1.16 ^b
ESR (mm ³ h ⁻¹)	2.7±0.50 ^a	2.9±0.25 ^b	3.0±0.75 ^b
MCV (fL)10 ¹⁵	52.55±2.85 ^a	48.20±1.46 ^b	43.75±2.45 ^c
MCH (10 ⁻¹²)	0.14±0.01 ^a	0.11±0.01 ^b	0.10±0.02 ^b
MCHC (g L ⁻¹)	2.99± 0.21 ^a	2.26± 0.17 ^b	2.06± 0.14 ^b
WBC 10 ⁹ (L ⁻¹)	4.15±0.49 ^a	5.75±0.62 ^b	5.95±0.68 ^b
Lymphocyte (%)	32.4±4.22 ^a	38.8±2.40 ^b	39.6±3.48 ^b
Neutrophil (%)	52.9±4.01 ^a	50.8±2.25 ^a	53.5±3.20 ^a
Monocyte (%)	9.2±0.97 ^a	9.2±1.15 ^a	9.3±0.46 ^a
Eosinophil (%)	1.25±0.25 ^a	1.50±0.25 ^a	2.25±0.50 ^b
Basophil (%)	1.00± 0.00 ^a	1.00± 0.00 ^a	3.00± 0.50 ^b
Platelets(μL)10 ⁵	475.0±8.23 ^a	482.5±6.18 ^a	550.5±9.25 ^b

Values are Means±SEM; n = 5, values bearing different alphabets are significantly different (p<0.05), Key: BW (mL kg⁻¹ b.wt.), YB (Yoyo bitters)

rats administered with Yoyo bitters. The administration of 0.308 mL kg⁻¹ b.wt. of Yoyo bitters resulted in significant decrease (p<0.05) in serum high density lipoprotein cholesterol (HDL-C) and uric acid concentrations (Table 2). However, at 0.462 mL kg⁻¹ b.wt. of Yoyo bitters, the serum uric acid and liver MDA levels were significantly increased (p<0.05). The administration of the

herbal bitters did not affect significantly (p>0.05) the concentration of reduced glutathione in the liver.

Hematological parameters: The trends obtained in the haematological parameters in male rats following the administration of Yoyo bitters are depicted in Table 3. Administration of Yoyo bitters resulted in significant

decreases ($p < 0.05$) in the Packed Cell Volume (PCV), Red Blood Cell (RBC) and Haemoglobin (Hb) counts, mean corpuscular volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC). Significant increases ($p < 0.05$) were recorded in the Erythrocyte Sedimentation Rates (ESR), Total White Blood Cell (WBC), Lymphocytes (L) and platelets counts (Table 3).

DISCUSSION

Herbal supplements have received massive attention as alternative medicines to the modern clinical therapy in recent times, leading to the subsequent increases in their demand and usage (Sushruta *et al.*, 2006). The effect of the administration of Yoyo bitters on the morphological appearance and physical activities of the rats (lean structure, drowsiness and sunken eyes) indicated that the herbal bitters might be toxic at $0.462 \text{ mg kg}^{-1} \text{ b.wt.}$ (Fig. 3).

The pattern obtained in the feed intake following the administration of the Yoyo bitters may indicate an alteration in the appetite of the rats or the interference with the metabolism of ingested food substances. This may be consolidating the Manufacturers claim that “the ingestion of Yoyo bitters speed up digestion of food substances and also, the removal of waste from the bowel, thereby regulating the absorption of food nutrients”. This observation is further strengthened by the trends obtained in the body weight of rats following the administration of the herbal bitters (Fig. 4). Previous study by Patrick-Iwuanyanwu *et al.* (2012), using Baker Cleanser Bitters, reported a similar pattern in the body weight of rats administered the herbal bitters. Although, in this study, the drop in body weight of the test rats compared to the control rats may not necessarily be regarded as a disadvantage, as Yoyo bitters is alleged by the Manufacturer to help detoxify the blood and balance immune functions in users. Thus, this body weight reducing property may suggest Yoyo bitters as an immune modulating regime. This is in agreement with the previous reports that immune modulating regimes often possess body weight reducing or maintenance properties (Pond, 2005; Oyewo and Akanji, 2011). In addition, immune disorders are frequently reported in individuals with high body mass index (Digirolamo, 1994). However, chronic body weight loss (cachexia) should be examined closely and not regarded as advantage always, as it could be due to systemic inflammatory reactions and sepsis that was caused by leaky gut. A close examination of the pattern of the body weight (Fig. 4), even at $0.462 \text{ mg kg}^{-1} \text{ b.wt.}$, indicated a steady gain in weight in the rats administered with the herbal bitters, thereby, not suggesting a case of chronic weight loss.

The serum IL-2 concentrations in rats administered the herbal bitters (Table 2) suggest that the herbal bitters had no marked effect on the stimulation of IL-2 biosynthesis. It is possible that there was no need for the upward/downward regulation of the serum concentration of IL-2 in rats, since IL-2 is necessary for the development of T cell immunologic memory and to discriminate between self and non-self (Beadling and Smith, 2002; Thornton *et al.*, 2004). The result obtained in the serum IL-6 concentration at $0.308 \text{ mL kg}^{-1} \text{ b.wt.}$ of the herbal bitters suggested that the expression and release of IL-6 in muscles and tissues was inhibited, thereby, indicating no tissue damages and inflammatory processes. However, at $0.462 \text{ mL kg}^{-1} \text{ b.wt.}$ of the bitters, localized tissue damages arising from systemic inflammatory response might be indicated. The increase recorded in the serum TNF- α concentration (Table 2) in rats administered with the herbal bitters might imply that the herbal bitters triggered systemic inflammation response and/ or the stimulation of the acute phase reaction, which could be due to infection or tissue damage. TNF- α regulates the growth of normal cells, induces the apoptosis of abnormal or infected cells and activates neutrophils via systemic (Pinckard *et al.*, 1997). On the contrary, high serum levels of TNF- α has been implicated in increased risk of mortality, heart disease, septic shock, dehydration, anorexia, net catabolism, weight loss, anaemia, hepatosplenomegaly, autoimmune disorders and increased risk of cancer (Cesari *et al.*, 2003; Dubinski and Zdrojewicz, 2007). Therefore, the result of the serum TNF- α concentration in rats administered with the herbal bitters supported the result of the feed intake (Table 1), body weight (Fig. 4) and serum IL-6 (Table 2), all precipitating the induction of systemic or localized inflammatory responses by the ingestion of the herbal bitters.

The trends obtained in the PCV, RBC, Hb, MCV, MCH, MCHC and ESR in rats administered with Yoyo bitters (Table 3) indicated the lyses of erythrocytes that could be caused by the chemical constituents of the herbal bitters or the induction of system inflammation in rats. Leukocytes are responsible for clearing off injured or dead cells and tissues in body and fighting infection and their counts are increased/decreased in the blood during infection and/inflammation. Therefore, the increases in the white blood cell count and lymphocytes in rats administered the herbal bitters (Table 3) supported the induction of systemic or localized inflammatory responses by the ingestion of the herbal bitters. The trend obtained in the platelets counts in rats administered with $0.462 \text{ mL kg}^{-1} \text{ b.wt.}$ of the herbal bitters (Table 3), however, did not support the anaemic capability of the herbal bitters, but may indicate an increased level of tissue healing processes or systemic inflammatory

response. This is so because innate immune cells (macrophages and neutrophils) stimulate the production of blood platelets via increased production of adenosine diphosphate (ADP) and the inhibition of nitric oxide (NO) production when there is tissue injury or damage (Ford and Giles, 2000). The result of the blood platelets count $0.462 \text{ mL kg}^{-1} \text{ b.wt.}$ of the herbal bitters supported the trends obtained in the serum IL-6 and TNF- α concentrations (Table 2). This is because increased IL-6 and TNF- α concentrations are reported to induce the release acute phase proteins such as C-reactive protein, fibrinogen etc in blood (Oyewo *et al.*, 2012).

A critical examination of the pattern presented in the blood glucose levels in rats administered Yoyo bitters (Fig. 5) indicated a blood glucose maintaining capabilities of the herbal bitters. This result supported the claim of the manufacturer, as provided on the information sheet. The probable mechanism of the reduction in blood glucose level-body weight loss; could be through the prevention of absorption of glucose in the gut and/or increased insulin secretion by pancreatic stimulation (Borhanuddin *et al.*, 1994). However, the prevention of the absorption of blood glucose is more likely because of the result of the feed intake in rats administered the herbal bitters supported the reduced blood glucose levels (Table 1). This might be due to slow metabolism of ingested food substances and their enhanced removal from the gastrointestinal tract or the depression of appetite. In addition, some of the constituents in the herbal bitters such as *Aloe vera*, *Citrus aurantifolia* etc were indicated to contain alkaloids and flavonoids (Miyagi *et al.*, 2000). Alkaloids and flavonoids have been reported to prevent the absorption of dietary glucose in the gastrointestinal tract (Price *et al.*, 1987; Khanna *et al.*, 2002).

The maintenance/regulation of the blood glucose levels obtained in the Yoyo bitters administered rats supported the immunomodulating activities, as some immune modulating regimes have been reported to possess blood glucose reducing or maintenance properties (Spelman *et al.*, 2006; Oyewo and Akanji, 2011). That is, increase in blood glucose level stimulates the pancreas to release insulin by exocytosis into blood so as to drop the level of blood glucose (Berg *et al.*, 2001). However, insulin has been reported to inhibit the release of growth hormones, which in turn depresses the immune system, by reducing the "phagocytic index" of the innate immune cells (Volk *et al.*, 1993; Digirolamo, 1994). In addition, it proportionally reduced the ability of cell-mediated immune cells to capture bacteria and increased the incidence of degenerative diseases (cancer) (Langley-Evans and Carrington, 2006).

The reduction in the serum total cholesterol and triacylglycerol concentrations in rats administered with Yoyo bitters might have resulted from the presence of alkaloids and flavonoids in the bitters that are known to inhibit the absorption of dietary lipid in the small intestine (Evers, 2008) and/or the inhibition of cholesterol biosynthesis in the liver. The trends obtained in serum low density lipoprotein cholesterol (LDL-C) concentration is consistent with the cholesterol-lowering capability of the herbal bitters, which is possibly by enhanced reverse cholesterol transport and bile acid excretion, through the inhibition of production apo B, needed for LDL-C production, transport and binding (Turner *et al.*, 2004). The result of the atherogenic index (Table 3) suggested that the administration of the herbal bitters reduced the risk of lipid related inflammatory diseases. This supported the manufacturer's claim that the herbal bitters reduced blood lipid levels, as stated on the information sheet. The result of the serum HDL concentrations in rats administered $0.308 \text{ mL kg}^{-1} \text{ b.wt.}$ of the herbal bitters suggested a possible boost of HDL-C biosynthesis in the liver that can be promoted by the presence of flavonoids (Renaud *et al.*, 1999). Thus, more cholesterol would be transported from peripheral tissues to the liver for excretion and could be the reason for the reduced levels in the serum cholesterol concentration in the rats administered with Yoyo bitters.

The reduction in the serum uric acid concentrations at $0.308 \text{ mL kg}^{-1} \text{ b.wt.}$ of Yoyo bitters indicate that there were reduced tissues degradation that could caused by protein turn over due to trauma, oxidative stress, localized inflammation etc. (Berg *et al.*, 2001; Champe *et al.*, 2005). However, the opposite is the case at $0.462 \text{ mL kg}^{-1} \text{ b.wt.}$ of the herbal bitters (Table 2). Uric acid is a very strong reducing agent *in vivo* and contributes over half the antioxidant capacity of blood plasma (Xiang *et al.*, 2001; Baillie *et al.*, 2007). In oxidative stress conditions, the plasma uric acid concentrations are increased so as to augment the drop in reduced glutathione concentrations in the liver (Xiang *et al.*, 2001). The increase in the serum uric acid concentration at $0.462 \text{ mL kg}^{-1} \text{ b.wt.}$ of Yoyo bitters supported the trends obtained in the serum IL-6 and TNF- α concentration in rats administered the herbal bitters (Table 2). Uric acid is a known endogenous adjuvant that drives immune responses in the absence of microbial stimulation (Shi *et al.*, 2003). This might be as a result of oxidative stress that precipitated inflammatory response by the stimulating the increased production of IL-6 and TNF- α so as to attract leukocytes to the site of inflammation. An increase in the plasma level of uric acid concentrations has been implicated in the stimulation of localized inflammatory responses that has been identified

as having a key role in the innate immune response through interleukin-mediated inflammation via activation of the NOD-like receptor protein (NLRP)-3 inflammasome, a multimolecular complex whose activation appears to be central to many pathological inflammatory conditions (Choi *et al.*, 2001; Short *et al.*, 2005).

The administration of Yoyo bitters had no effects on the recovery of reduced glutathione (GSH) in the liver in male rats (Table 2). Although, this is not in agreement with the result obtained in the serum uric acid concentrations and may further suggest that oxidative stress was not too apparent in the rats because the concentration of uric acid in the blood was reported to be tightly regulated by the level of glutathione in the liver (Xiang *et al.*, 2001). However, the result of the liver malondialdehyde (MDA) concentration (Table 2) at 0.462 mL kg⁻¹ b.wt. of Yoyo bitters supported the result of the serum uric acid concentration, which further indicated the induction of oxidative stress in the male rats. This is in agreement with the report of Adeyemi *et al.* (2012) that the administration of Yoyo Bitters caused lipid peroxidation in male rats.

Oxidative stress is a key factor that compromises the immune system through the induction of inflammatory responses by free radicals. The oxidative stress indices employed in this study (serum uric acid, liver reduced glutathione and malondialdehyde) supported the immunomodulatory activity of the herbal bitters at 0.308 mL kg⁻¹ b.wt. of Yoyo bitters. Although, it is most likely that the sub-chronic administration of the Yoyo bitters at the recommended dose (0.462 mL kg⁻¹ b.wt.) did induce oxidative stress in rats, which stimulated the activities of the immune system by the increased serum uric acid concentration, which later induced inflammatory responses via the release of TNF- α .

CONCLUSION

From the foregoing, the study has established that the sub-chronic administration of Yoyo bitters at a dose lower than the Manufacturer's recommended dose for the adult human produced the better immunomodulatory activities. However, the daily use of Yoyo bitters, even below the recommended dose calls for caution due to the suggested inflammatory responses.

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