

Bayero Journal of Biomedical Sciences (BJBS)

**Official Publication of Faculty of Basic Medical Sciences,
Bayero University Kano, Nigeria.**



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Recommendations

Should be optional and must be drawn from the study.

Acknowledgement

Optional and should acknowledge support and assistance rendered by individual(s) or organizations.

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2. Editor(s), compiler(s) as author: Garcia LS, Filarial Nematodes In: Garcia LS (editor) Diagnostic Medical Parasitology ASM press Washington DC 2007: pp. 319-356.
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➤ Electronic Sources as reference

Journal article on the Internet: Parija SC, Khairnar K. Detection of excretory *Entamoeba histolytica* DNA in the urine, and detection of *E. histolytica* DNA and lectin antigen in the liver abscess pus for the diagnosis of amoebic liver abscess .*BMC Microbiology* 2007, **7**:41.doi:10.1186/1471-2180-7-41. <http://www.biomedcentral.com/1471-2180/7/41>

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The Editorial Board of Bayero Journal of Biomedical Sciences (Bayero J. Biomed. Sci.), Bayero University, Kano is proud to present its volume 2, issue 1 to the teeming scholars and young scientists across the globe, who like to be abreast with current research findings in the fields of Biomedical Sciences. The Journal is an official biannual publication of the Faculty of Basic Medical Sciences, Bayero University, Kano, Nigeria. It contains well – researched scientific works that cover six different areas; Anatomy, Biochemistry, Microbiology, Nutrition and Dietetics, Pharmacology and Physiology.

The Publication of the journal is to ensure wide dissemination of scientific research findings that will ginger the academic community to promote human development using evidence – based findings and also to create synergy and collaboration in the areas within the scope of the journal. This is no doubt, a noble idea that will advance knowledge in these areas. We will ensure that expected standard is maintained in all the subsequent editions with the help of our highly qualified team of experienced reviewers.

Scientists in the academic environment, research institutes and industries are therefore encouraged to actively participate in disseminating their works through this medium. It is pertinent to thank our team of talented professionals who ensured promptness in the editing processes of the manuscripts sent to them despite their tight schedules. This will encourage patronage of the journal and help in advancing the career of the contributing authors. The Editorial Board members deserve commendation for being resolute in promoting the journal. Thank you for the support, cooperation and encouragement.

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The Pattern of Anthropometry of Children Aged 5-12 Years according to Level of Mother's Education and Urbanisation from Kazaure Emirate, Northern Nigeria

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Abstract

Anthropometry refers to the measurements of different body parameters in order to assess the growth pattern of a given individual. However, this growth depends on many factors in which socio-economic factors (education, urbanism, household size, birth order etc.) are involved. The aim of this study is to investigate the pattern of anthropometry on children aged 5-12 years according to mother's education and level of urbanisation from Kazaure emirate, Nigeria. The study was designed to find the influence of mother's education and level of urbanisation on the measured variables (height, weight, biceps skin fold thickness, triceps skin fold thickness, subscapular skin fold thickness and suprailiac skin fold thickness, mid-upper arm circumference, hip circumference, waist circumference and calf circumference) of children in Kazaure emirate. A total of 659 males and 553 females aged 5-12 years were recruited for the study. Gold standard methods were adopted for measuring height using an anthropometer, weight using digital weighing scale, body circumferences using non-elastic measuring tape and skinfold thickness using Harpenden skinfold calliper, respectively. One-way ANOVA and independent t-test were used to determine the differences using SPSS version 20 and $P \leq 0.05$ was set as level of significance. The results showed sexual dimorphism in all the measured variables ($P < 0.05$) with exception of height and hip circumferences. For the level of mother's education, the body circumferences of all the children showed significance differences ($P < 0.05$) between educated and non-educated mother with exception of hip circumferences (HC). The weight and BMI (body mass index) also showed significant differences ($t=3.04$, $P=0.002$) and ($t=3.20$, $P=0.002$) respectively. For level of urbanisation, significant differences were observed in all the body circumferences ($P < 0.05$) and weight ($t= 4.50$, $P= 0.01$). For skinfold thickness, differences were observed in all the variables ($P < 0.05$). Much of the differences were between the urban and rural areas. In conclusion, the level of mother's education was shown to have less influence on the skinfold thickness (body fat content) as opposed to body circumferences. However, level of urbanisation had influence on both. The result of this work is suggestive of the effect of mother's education on measure of metabolic obesity (body circumferences) and general obesity (skinfold thickness) of the child.

Keywords: Anthropometry, Body circumferences, Skin fold thickness, Urbanisation, Education

Introduction

Anthropometry (measurement of height, weight, body circumferences and skinfold thicknesses etc.) is widely used in surveys as an indicator of nutritional and health status. ^[1,2] In developing countries like Nigeria, the characteristic pattern of poverty, poor maternal education, high rates of

morbidity, and inadequate nutritional intake of both the mother and child combine to produce a pattern of growth characterised by an increased risk of low birth weight, poor growth velocities, and a growth status that gradually falls away from that of children in developed countries. ^[4]

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References

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➤ Books and Other Monographs

1. Personal author(s): Salisu AI. Physiology of Human Menstrual Cycle and Contraception. 1st ed. Lambert Academic Publishing Company. 2013. Page
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➤ Electronic Sources as reference

Journal article on the Internet: Parija SC, Khairnar K. Detection of excretory *Entamoeba histolytica* DNA in the urine, and detection of *E. histolytica* DNA and lectin antigen in the liver abscess pus for the diagnosis of amoebic liver abscess .*BMC Microbiology* 2007, **7**:41.doi:10.1186/1471-2180-7-41. <http://www.biomedcentral.com/1471-2180/7/41>

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Bayero Journal of Biomedical Sciences (BJBS)
(*Bayero J. Biomed. Sci.*)

Editorial

The Editorial Board of Bayero Journal of Biomedical Sciences (Bayero J. Biomed. Sci.), Bayero University, Kano is proud to present its volume 2, issue 1 to the teeming scholars and young scientists across the globe, who like to be abreast with current research findings in the fields of Biomedical Sciences. The Journal is an official biannual publication of the Faculty of Basic Medical Sciences, Bayero University, Kano, Nigeria. It contains well – researched scientific works that cover six different areas; Anatomy, Biochemistry, Microbiology, Nutrition and Dietetics, Pharmacology and Physiology.

The Publication of the journal is to ensure wide dissemination of scientific research findings that will ginger the academic community to promote human development using evidence – based findings and also to create synergy and collaboration in the areas within the scope of the journal. This is no doubt, a noble idea that will advance knowledge in these areas. We will ensure that expected standard is maintained in all the subsequent editions with the help of our highly qualified team of experienced reviewers.

Scientists in the academic environment, research institutes and industries are therefore encouraged to actively participate in disseminating their works through this medium. It is pertinent to thank our team of talented professionals who ensured promptness in the editing processes of the manuscripts sent to them despite their tight schedules. This will encourage patronage of the journal and help in advancing the career of the contributing authors. The Editorial Board members deserve commendation for being resolute in promoting the journal. Thank you for the support, cooperation and encouragement.

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In general, children living in urban environments in developed countries reflect a better growth status than their rural peers. They have greater heights and weights for age and enter pubertal development at earlier ages. [5,6,7] Urban environments in developed countries are associated with higher socio-economic status (SES), greater opportunity for educational attainment, easier access to high quality health services and a lack of seasonal effects on food availability. [8,9,10,11] The rapid urbanisation characteristic of these countries often leads to environmental deprivation through inadequate housing and sanitation, overcrowding, and pollution in urban areas with a consequent diminution in child health and well-being. [3] The use of triceps skinfold in the assessment of nutritional status of children is based on the assumption that, increased subcutaneous fat resulting from either high calorie intake or low energy expenditure reflects a greater calorie reserve. [12]

Materials and Methods

The study was a cross-sectional survey which involved, 1212 primary school children randomly selected from four (4) local governments (Kazaure, Roni, Gwiwa and Yankwashi) of Kazaure emirate. Informed consent was given by each child's guardian and ethical approval was obtained from Ahmadu Bello University Ethical Committee and Jigawa State Ministry of Health Ethical Committee respectively, before the commencement of the study. All subjects within the selected age group were measured respectively according to the standard techniques. [13] An anthropometer (Holtain Ltd., Crymych, Dyfed, UK) was used to measure height, digital weighting scale (Model DS-410, Seiko, Tokyo, Japan) was used for the measurement of weight, anon-elastic measuring tape (Seca 201 Ergonomic Circumference measuring Tape, Amazon, UK) was used for the measurement of mid upper arm, hip, waist and calf circumferences respectively, a Harpenden skin fold calliper (Harpenden Skin fold Calliper, Amazon, UK) was used for the measurement of biceps, triceps, subscapular, and supra iliac skinfold thicknesses respectively. Body Mass Index (BMI) was calculated using the formula weight (Kg)/height (m²). [14]

Height/Stature (HT): The subjects stood up perfectly upright with arms relaxed by the side,

and ankles and knees placed together. The subjects were encouraged to stand as upright as possible and bare footed before measurements were taken. The anthropometer was positioned behind the subjects and the measurement taken to the nearest 0.1cm. [13]

Body weight: This was taken with subjects wearing light clothes and barefooted to the nearest 0.5kg. [13]

Biceps skinfold: This was taken on the left side using the Harpenden calliper. The measurement was done at the midpoint of the upper non-dominant arm anteriorly and recorded to the nearest 0.1mm [13]

Triceps skinfold: This was measured using the Harpenden skin fold calliper at the midpoint of the upper non-dominant arm posteriorly and recorded to the nearest 0.1mm. [13]

Subscapular skinfold: This was measured using the Harpenden skin fold calliper around the inferior angle of the scapula and recorded to the nearest 0.1mm. [13]

Suprailiac skinfold: This was measured using the Harpenden skin fold calliper around the posterior aspect of the crest of the ilium using the Harpenden skin fold calliper and recorded to the nearest 0.1mm. [13]

Mid upper arm circumference: This was measured using the tailor's plastic measuring tape at the midpoint of the upper non-dominant arm, between the acromial process and the tip of the olecranon and recorded to the nearest 0.1mm. [13]

Hip circumference: This was measured with the subject in standing position. The tailor's plastic tape was placed on the tip of the buttock's curvature posteriorly and pubis anteriorly. The measurement was to the nearest 0.1cm. [13]

Waist circumference: This was taken while the subject was in standing position. The tape was placed between the lowest lumbar region and the sacral region posteriorly and pubic region anteriorly. The measurement was to the nearest 0.1cm. [13]

Calf circumference: This was taken with the subject in standing position. The tape was placed on the gastrocnemius muscle posteriorly and the tibia bone anteriorly. The measurement was to the nearest 0.1cm. [13]

Inclusion Criteria

Participant must be:

- i. Public primary school pupil
- ii. Between 5-12 years of age
- iii. Physically/Mentally fit
- iv. From Kazaure Emirate
- v. Hausa ethnic group

Exclusion Criteria

Participant must not be:

- i. Private primary school pupil
- ii. Below 5 years or above 12 years of age
- iii. Physically/ Mentally unfit or deformed
- iv. From any other Emirate than Kazaure Emirate
- v. Of any ethnic group other than Hausa

Brief History of Kazaure Emirate

Kazaure is located in the northern part of Kano state among the 27 local governments of Jigawa State. It lies between longitude 12° 30' to 12° 45' and latitude 8° 15' to 8° 30' North and East respectively. It covers a land area of about 1780

kilometres square. It is bordered to the north by Daura (Katsina state), west by Ingawa (Katsina state), east by Babura (Jigawa state) and to the south by Dambatta (Kano state).^[15,16]

The area belongs to the Sudan savannah vegetation. Rainfall begins between May and June and ends around September and October. The main annual rainfall is about 600mm with the highest input during the months of July and August. Mean annual temperature is about 26 °C but mean monthly value ranges between 22 °C in the coldest months (December and January) and 31 °C in the hottest months of April and May.^[15]

The major occupation of the people is agriculture and their staple foods are millet, guinea corn, maize, wheat and rice.^[15] The emirate has a population of 459,589.^[17] Kazaure emirate encompasses the four local government councils namely, Kazaure, Yankwashi, Roni and Gwiwa local government.

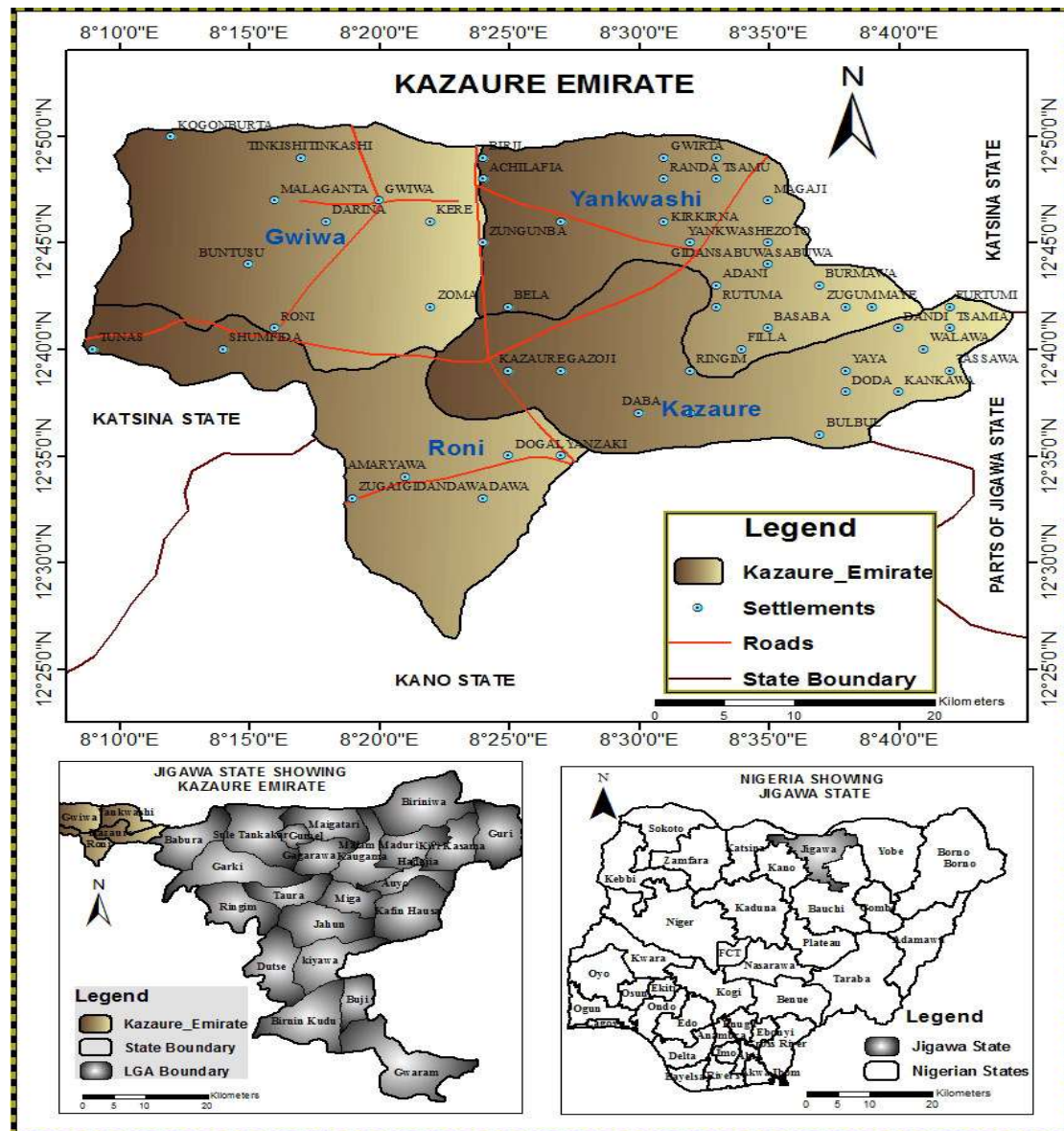


Figure 1: Map of Kazaure emirate showing the location of study

Statistical Analysis The data were expressed as Mean $\pm$ SD. Differences were analysed using independent *t*-test and one way analyses of variance followed by Benferoni post hoc test. The analyses were carried out using statistical package for social students (SPSS) version 20. $P \leq 0.05$ was considered as level of significance.

Results

Table 1 shows that males had higher mean values of age, height, weight, BMI, WC and CC than females

with statistical significant difference $p=0.006$ for age, <0.001 for weight, 0.01 for BMI, 0.03 for WC and 0.003 for CC, respectively except in height which was not statistically significant. However, the females had higher mean values of MUAC, biceps skinfold, triceps skinfold, subscapular skinfold, suprailiac skinfold and HC than the males with statistical significant difference $p<0.001$, <0.001 , <0.001 and <0.001 respectively except in MUAC and HC which were not statistically significant.

Table 1: Mean and standard deviation of the study variables of Kazaure Emirate children

Parameters	Male (n=659) Mean±SD	Female (n=553) Mean±SD	t- value	p- value
Age (yrs.)	9.26±2.34	8.89±2.36	2.74	0.006
Height (m)	1.48±0.05	1.47±0.51	0.05	0.96
Weight (Kg)	25.35±5.85	24.07±5.53	3.89	<0.001
BMI (kg/m ²)	15.09±1.36	14.88±1.56	2.53	0.01
MUAC (cm)	17.31±1.73	17.32±1.88	0.15	0.88
Biceps skfd (mm)	3.23±1.23	3.72±0.78	7.97	<0.001
Triceps skfd (mm)	5.64±1.44	7.08±1.64	16.28	<0.001
Subscapular skfd(mm)	4.83±0.79	5.97±0.43	8.32	<0.001
Suprailliac skfd (mm)	7.08±2.73	8.71±2.38	11.00	<0.001
Hip circumference (cm)	61.97±5.95	62.00±6.01	0.09	0.93
Waist circumference(cm)	56.41±4.71	55.82±4.46	2.23	0.03
Calf circumference (cm)	23.85±2.52	23.44±2.17	2.98	0.003

Table 2 shows that the children of educated mothers had higher mean values of age, height, weight, BMI, MUAC, HC, WC and CC with statistical significant difference $p=0.002$, 0.001 , 0.03 , 0.001 and 0.006 respectively except in age, height and HC which were not statistically significant than children of non-educated mothers.

Table 3 shows that the children of educated mothers had higher mean of biceps skinfold, triceps skinfold, subscapular skinfold and suprailliac skinfold thicknesses than children of non-educated mothers though not statistically significant.

Table 4 shows the mean age for urban children was higher than that of rural, and that of rural children higher than the semi-urban but all with no statistical significant difference. Similarly, the mean height of rural children was higher than that of semi-urban and that of semi-urban higher than that of urban though with no statistical significant difference. However,

the mean weight of urban children was shown to be higher than that of both semi-urban and rural children respectively with statistical significant difference ($p<0.01$). The mean BMI of urban, semi-urban and rural children showed no statistical significant difference between them. Furthermore, the mean MUAC of urban and semi-urban children was higher than that of rural children with statistical significant difference $p=0.004$. Moreover, the mean HC, WC and CC of urban children were higher than those of semi-urban and rural children with $p<0.001$, <0.001 and 0.002 respectively.

Table 5 shows the mean biceps and triceps skinfolds of urban and semi-urban children to be higher than those of their rural peers with statistical significant difference $p=0.009$, <0.001 respectively. Similarly, the mean subscapular and suprailliac skinfolds of urban children were higher than those of semi-urban and rural children with statistical significant difference $p=0.003$, <0.001 respectively.

Table 2: Mean and standard deviation of BMI and body circumferences of Kazaure Emirate children according to level of mother's education

Parameters	Non-educated mothers (n=689) Mean±SD	Educated mothers (n=524) Mean±SD	t- value	p- value
Age (yrs.)	9.01±2.30	9.20±2.42	1.37	0.17
Height (m)	1.28±0.37	1.73±0.31	1.60	0.11
Weight (Kg)	24.33±5.30	25.33±6.23	3.04	0.002
BMI (kg/m ²)	14.88±1.32	15.15±1.61	3.20	0.001
MUAC (cm)	17.21±1.66	17.44±1.97	2.19	0.03
Hip circumference (cm)	61.70±5.25	62.36±6.80	1.90	0.06
Waist circumference(cm)	55.76±4.19	56.63±5.07	3.24	0.001
Calf circumference (cm)	23.50±2.07	23.88±2.70	2.74	0.006

Table 3: Mean and standard deviation of skin fold thickness of Kazaure Emirate children according to level of mother's education.

Parameters	Non-educated mothers (n=689) Mean±SD	Educated mothers (n=524) Mean±SD	t- value	p- value
Biceps	3.44±0.74	3.48±1.41	0.68	0.50
Triceps	6.29±1.63	6.32±1.77	0.32	0.75
Subscapular	5.35±2.55	5.36±2.32	0.07	0.95
Suprailiac	7.85±2.20	7.80±3.24	0.32	0.75

Table 4: Mean and standard deviation and analysis of BMI and body circumferences of Kazaure Emirate children according to level of urbanisation

Parameters	Urban (n=125) Mean±SD	Semi-urban (n=520) Mean±SD	Rural (n=566) Mean±SD	F- value	p- value
Age (yrs.)	9.26±2.47 ^a	8.96±2.30 ^a	9.17±2.38 ^a	1.41	0.24
Height (m)	1.30±0.13 ^a	1.48±0.64 ^a	1.52±0.47 ^a	0.10	0.91
Weight (Kg)	26.21±6.10 ^a	24.61±5.48 ^b	24.57±5.84 ^b	4.50	0.01
BMI (kg/m ²)	15.24±1.34 ^a	14.98±1.45 ^a	14.96±1.48 ^a	2.00	0.14
MUAC (cm)	17.65±1.67 ^a	17.42±1.69 ^a	17.14±1.92 ^c	5.58	0.004
Hip circumference (cm)	65.51±5.93 ^a	61.73±5.04 ^b	61.43±6.51 ^b	25.61	<0.001
Waist circumference(cm)	58.57±5.03 ^a	55.66±4.52 ^b	56.04±4.42 ^b	20.99	<0.001
Calf circumference (cm)	24.29±2.19 ^a	23.71±2.13 ^b	23.48±2.59 ^b	6.31	0.002

Values with the same superscript, show they are the same or the difference is not statistically significant.

Table 5: Mean and standard deviation and analysis of variance of skin fold thickness of Kazaure Emirate children according to level of urbanisation.

Parameters	Urban (n=125) Mean±SD	Semi-urban (n=520) Mean±SD	Rural (n=566) Mean±SD	F-value	p-value
Biceps	3.61±0.89 ^a	3.52±0.78 ^a	3.36±1.32 ^c	4.70	0.009
Triceps	6.54±1.68 ^a	6.50±1.78 ^a	6.06±1.58 ^c	10.61	<0.001
Subscapular	6.01±5.53 ^a	5.38±2.34 ^b	5.18±1.03 ^b	5.96	0.003
Suprailliac	6.34±1.18 ^a	8.17±2.31 ^b	7.84±3.14 ^b	23.97	<0.001

Where values have the same superscript, shows they are the same or the difference is not statistically significant.

Discussion

In this study, it was observed that males had better growth in terms of height and weight and subsequently in BMI than the females. This may possibly be due to physical activities they exposed themselves than the females while the females had higher subcutaneous fatness than the males which may be connected to their sedentary life and therefore less energy expenditure and this agrees with the earlier reports.^[12]

In this study it was found that children of educated mothers and those in urban area had indicated a better growth pattern than those of non-educated mothers and those from rural area. This may be due to the income from place of work the mother receives either monthly, weekly or hourly, and also

due to availability and accessibility to social amenities such as schools, hospitals and so on. This is in line with reports of earlier studies by.^[5,6,7]

In this study, males were found to have higher anthropometric measurements than the females thereby indicating sexual dimorphism in growth pattern. Similarly, level of mother's education had influence on children's growth and development. Urban environment also had influence on children's growth though urbanisation may lead to environmental deprivation through inadequate housing and sanitation, overcrowding, and pollution in urban areas with a consequent diminution in child health and well-being.

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Prevalence of Hepatocellular Injury associated with Antiretroviral and Antituberculosis Drugs in Patients with HIV/TB Co-infection

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Abstract

The prevalence of hepatocellular injury associated with co-administration of anti-retroviral and anti-tubercular drugs in patients with HIV/TB co-infection was assessed. The study was a retrospective review of medical records of HIV infected and HIV/TB co-infected patients already initiated ART alone or ART/Anti TB drugs respectively. Demographic data, CD4 cell counts and serum alanine transaminase (ALT) levels were recorded. The CD4 cell counts and ALT values were recorded at baseline and at six months after commencement of chemotherapy. The results shows that among the 267 patients studied, 166 (62.17%) were females. The mean age $\pm$ SD was 36.9 ± 10.39 years. Twenty five (9.36%) had raised ALT at baseline. A total of 129 (48.31%) had an abnormal rise in ALT level, out of which 35 (13.11%) had severe (grade 3 and 4) hepatocellular injury. Among the grade 3 and 4 hepatocellular injuries, 7 (7.86%) were in group 1 (Nevirapine-based regimen), 15 (16.49%) in group 2 (HIV/TB co-infection) and 13 (14.95%) in group 3 (Truvada/Efavirenz) treatment groups. Grade 1 and 2 hepatocellular injury occurred in 94 (35.21%) patients. The mean $\pm$ SD CD4 cell count before commencement of treatment was 274.12 ± 204.91 c/mm³, and was 417.86 ± 261.53 c/mm³ (95 % CI 386.3-449.33) at six month. Patient/treatment group was found to be an independent risk factor associated with hepatocellular injury ($p = 0.02$). In conclusion the prevalence and severity of the hepatocellular injury were found to be higher in HIV/TB co-infected patients on concurrent Antiretroviral (Tenofovir + Emtricitabine and Efavirenz) and anti-Tuberculosis drugs (Rifampicin, Isoniazid, Pyrazinamide and Ethambutol).

Keywords: Hepatocellular injury, Antiretroviral, Antituberculosis, HIV/TB Co-infection

Introduction

Tuberculosis (TB) and human immunodeficiency virus (HIV) are important health problem in many developing countries, and the prevalence of these diseases remained high.^[1] TB and HIV have been closely linked since the emergence of HIV infection. They cause an overlapping epidemics and hence increased the burden of disease in sub-Saharan Africa, the region that harbours the majority of the world's HIV/TB co-infected patients.^[2] Worldwide, TB is the most common opportunistic infection in HIV-seropositive patients,^[3] and it remains the most common cause of death in individuals with Acquired Immunodeficiency Syndrome (AIDS).^[4,5] HIV infection has result to a significant rise in the worldwide incidence of TB.^[3,6] It worsen TB epidemics tremendously and also increased the risk

of progression of latent infection to an active disease.^[7,8] Therefore the interaction between HIV and TB infections is bidirectional.^[9] Almost one third of the world's population is infected with *Mycobacterium tuberculosis*, resulting in an estimated 9 million new cases of active TB in 2010.^[10] Globally, 14.8% of TB patients have HIV co-infection, and as high as 50-80% have HIV co-infection in parts of sub-Saharan Africa.^[10] In Nigeria the prevalence of HIV among TB patients increased from 2.2% in 1991 to 19.1% in 2001 and 25% in 2010.^[11] Commencing antiretroviral treatment (ART) for all HIV-TB co-infected patients within a few weeks of TB treatment, irrespective of CD4 cell count had been the goal standard as recommended by World Health Organisation

(WHO), but the optimal/ideal timing of ART is still under debate.^[12] Anti-TB and ARVs drugs have a lot of potential drug interactions and toxicities which most often overlapped and can result into serious treatment complication.^[13] Hepatotoxicity is one of the commonest side effect of both non-nucleoside reverse transcriptase inhibitor (NNRTI) based ART and first line anti-TB drugs and yet no enough information is available about the level of hepatotoxicity in TB/HIV co-infected patients receiving NNRTI-based ART and first line anti-TB drugs.^[1] The aim of this study is to assess hepatocellular injury associated with the concomitant use of anti-retroviral and anti-tuberculosis drugs in HIV/TB co-infected patients.

Materials and Methods.

The study was conducted in Anoury Specialist Hospital Kano in Northern Nigeria. It was established by a non-governmental organisation and it is one of the centres in the state that offers a full HIV and TB support and care. The HIV clinic has 3,445 patients enrolled of which 1,360 were actively on follow-up and 1,247 were on ARVs (both first and second line regimens). The centre received patients both from Kano and other states. The Hospital operates 24 hours a day and throughout the week. It possessed fully functional laboratory for basic investigations such as retroviral screening, LFTs, CD4 cell count and other tests.

The study was a retrospective review of patients' files and the files studied were those of the patients seen in the hospital between 2005 to 2014.

Patients infected with HIV alone and those with HIV/TB co-infection and had been on treatment for at least six months formed the study population. Those excluded were patients with liver diseases due to other factors (e.g. Hepatitis B and C and other drugs), or with an already established chronic liver disease and those with incomplete laboratory results.

Data extraction form was used to obtain all the information necessary for the study. A total of 273 files were retrieved (calculated sample size), but 267 files fulfilled the inclusion criteria and were analysed during the study period. The patients were divided into three groups viz: group one were HIV positives receiving nevirapine-based ART (Zidovudine + Lamivudine + Nevirapine), group two

were HIV/TB co-infected patients receiving concurrent treatment with efavirenz-based ARVs i.e. Truvada (Tenofovir + Emtricitabine) and Efavirenz, and anti TB drugs (Rifampicin, Isoniazid, Pyrazinamide and Ethambutol), or two drugs during continuation phase. While the third group were HIV positive patients receiving Truvada (Tenofovir + Emtricitabine) and Efavirenz only. Demographic characteristics, date of diagnosis and commencement of therapy, drug regimen, Cotrimoxazole preventive therapy, CD4 cell counts (pre-treatment and at six months), ALT (pre-treatment and at six months) were all recorded.

Analysis of the data was carried out using the statistical package for social sciences, version 16 (SPSS 16). Categorical variables, such as gender were expressed in frequencies and percentages, whereas numerical variables, such as patient age, CD4 cell counts, ALT levels were expressed in means and standard deviations. Some of the variables were analysed in each of the three groups using Chi square test to evaluate their association with the development of hepatocellular injury. The independent variables were then evaluated using multivariate logistic regression to identified significant factors in development of hepatocellular injury. A p -value ≤ 0.05 was considered to be statistically significant.

An ethical approval for conducting the study was obtained from the Ethical Committee of Aminu Kano Teaching Hospital and Permission from the hospital was also obtained. All the information obtained remained highly confidential.

Hepatocellular injury is defined as an elevation of alanine amino transferase (ALT) level from the normal range and was categorised according to a modified, standardised toxicity grade scale ^[31]. A normal range of serum ALT was 0-30 μ /l (reference range used in the Hospital). The grading of hepatocellular injury was based on ALT elevation in relation to the Upper limit of normal (ULN): grade 1, 1.25-2.5 x ULN; grade 2, 2.6-5 x ULN; grade 3, 5.1-10 x ULN; grade 4, greater than 10 x ULN, although HIV itself can result in elevation of serum ALT level due to hepatocellular damage^[35]. Because of these reasons classification on the changes relative to the baseline was preferred to avoid selection bias for patients with elevated pre-

treatment serum level higher than the upper level of normal^[1,16] : Grade 0, < 1.25 X Baseline (BL) ALT, Grade 1, 1.25-2.5 X BL, Grade 2, 2.6-3.5 X BL, Grade 3, 3.6-4.9 X BL and Grade 4, > 5 X BL. Patients were also grouped into three based on CD4 count levels: <200c/mm³, 200-350c/mm³ and >350c/mm³.

Results

Out of the 267 patients studied 89 were in group one i.e. those HIV positives on Nevirapine base ART, 91 in group two (HIV/TB co-infected on concomitant ARVs and anti TB), and 87 in group three (HIV positives on Truvada + Efavirenz regimen).

General Characteristics of the Study Population

The mean $\pm$ SD age of all the patients in the three group was 36.90 ± 10.39 years, and 95% of the patients' age were between 20 to 59 years (Tables 1 and 6). There were 166 females (62.2%) and 101 males (37.8%) (Table 1). The mean $\pm$ SD age for group 1 was 31.80 ± 10.46 years, while for group 2 and 3 it was 38.1 ± 8.93 and 40.9 ± 9.59 years respectively (Table1); with statistically significant difference between them ($p = <0.001$). Of the total study participants, 55 (20.6%) were underweight with body mass index (BMI) of $< 18.5\text{kg/m}^2$, 164 (61.4%) had a normal ($18.5\text{-}24.9\text{kg/m}^2$), 35 (13.1%) were overweight ($25\text{-}29.9\text{ kg/m}^2$), while 13 (4.9%) were obese ($\geq 30\text{kg/ m}^2$).

Table 1: General characteristics of the study group

Characteristics	Group1 n=89	Group2 n=91	Group3 n=87
Males	20 (33.47%)	43 (48.25%)	38 (44.68%)
Females	69 (77.53%)	48 (52.75%)	49 (56.32%)
Age, years, mean $\pm$ SD	31 $\pm$ 10.46	38.09 $\pm$ 8.93	40.87 $\pm$ 9.59
Body weight(kg)mean $\pm$ SD ^a	57.2 $\pm$ 15.26	58.37 $\pm$ 12.05	65 $\pm$ 13.73
CD4 cell, c/mm ³ ,mean $\pm$ SD ^a	243.9 $\pm$ 185.5	244.8 $\pm$ 197.4	324.3 $\pm$ 223.9
ALT Level (U/l) means $\pm$ SD ^b	18.91 $\pm$ 9.06	34.75 $\pm$ 48.91	21.75 $\pm$ 9.65

a= 6 month after commencement of chemotherapy.

Group 1= HIV patient on Nevirapine based ART.

Group 2= HIV/TB co-infected patients on Anti-TB and ART.

Group 3= HIV patients on Efavirenz based ART.

n= Number of patients.

Baseline Laboratory Parameters of the Study Population

The mean pre-treatment CD4 cell counts were 274.12 ± 204.91 (95 % CI 249.43 to 298.81), while at six month after commencement of chemotherapy the mean CD4 cell count was 417.82 ± 261.53 (95 % CI of 386.30 to 449.33). There was statistically significant difference between the mean CD4 cell counts of the three groups at six months (Group one 391.30, group two 370.10 and group three 494.90) after initiation of treatment ($F = 5.960$, $df = 2$, $p = 0.03$). The mean baseline serum ALT for the three group was $17.63\text{U/l} \pm 11.86$ SD, and $25.22\text{U/l} \pm 30.09$ SD at six months.

Relationship between Risk Factors and Hepatocellular Injury

Patients group and hepatocellular injury

At baseline, 25 (9.36%) patients had raised ALT. While six months after commencement of therapy, 138 (51.69%) had a normal ALT level, 63(23.60%) developed grade 1 hepatocellular injury, 31(11.61%) with grade 2, 18 (6.74%) with grade 3 and 17(6.37%) with grade 4. Severe hepatocellular injury (Grade 3 and 4) was found to be higher in group two (HIV/TB co-infected) with 16.5%. Concurrent administration of ART and Anti TB was associated with hepatocellular injury. ($\chi^2 = 26.935$, $df = 8$, $p = 0.001$) (Table 2).

Table 2: Relationship between patient group and hepatocellular injury

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Total
Patients	n(%)	n(%)	n(%)	n(%)	n(%)	N
Group 1	57(64.04)	11(12.36)	14(15.73)	2(2.25)	5(5.61)	89
Group 2	34(37.36)	32(35.16)	10(10.99)	11(12.09)	4(4.4)	91
Group 3	47(54.02)	20(22.99)	7(8.05)	5(5.75)	8(9.2)	87
Total	138	63	31	18	17	267

($\chi^2=26.935$, df=8, p=0.001)

Group 1= HIV patient on Nevirapine based ART.

Group 2= HIV/TB co-infected patients on Anti-TB and ART.

Group 3= HIV patients on Efavirenz based ART.

n= Number of patients.

BMI and Hepatocellular Injury

Among the 267 patients, 34 (61.80%) of those that were underweight had normal ALT while 21(38.20%) had raised ALT. While 86 (52.40%) of those with normal BMI had raised ALT. There was

no significant association between BMI and development of hepatocellular injury among the groups ($\chi^2 = 2.525$, df = 3, p = 0.471) (Table 3).

Table 3: Relationship between BMI and patient group

	Under-weight	Normal	Overweight	Obesity	Total
patients	n(%)	n(%)	n(%)	n(%)	
Group 1	23(25.8)	51(57.3)	13(31.7)	2(2.25)	89
Group 2	31(34.1)	47(51.6)	12(13.19)	1(1.10)	91
Group 3	9(10.34)	56(64.37)	16(18.39)	6(6.90)	87
Total	63	154	41	9	267

($\chi^2 = 2.525$, df = 3, p = 0.471).

Group 1= HIV patient on Nevirapine based ART.

Group 2= HIV/TB co-infected patients on Anti-TB and ART.

Group 3= HIV patients on Efavirenz based ART.

n= Number of patients

CD4 Cell Counts and Hepatocellular Injury

One hundred and twenty (44.94%) of the patients had a CD4 cell count of 200 c/mm³ and below before the initiation of chemotherapy, 70 (26.22%) had CD4 cell count between 201-350 c/mm³, while 77 (28.84%) had CD4 greater than 350 c/mm³. The severe hepatocellular injury was found to be higher with 18 (13.14%) among those with CD4 greater

than 350 c/mm³ at 6 month of chemotherapy. There was no statistically significant relationship between the CD4 cell count levels both at pre-treatment and at 6 months and development of hepatocellular injury ($\chi^2 = 3.573$, df = 8, p = 0.899, $\chi^2 = 4.044$, df = 8, p = 0.869) (Table 4 and 5).

Table 4: Relationship between pre-treatment CD4 count level and hepatocellular injury

CD4 in c/mm ³	Grade 0 n(%)	Grade 1 n(%)	Grade 2 n(%)	Grade 3 n(%)	Grade 4 n(%)	Total
≤ 200	57(47.5)	30(25.0)	14(11.67)	11(9.12)	8(6.67)	120
201-350	40(57.14)	14(20.0)	9(12.86)	3(4.29)	4(5.71)	70
>350	41(53.23)	19(24.68)	8(10.39)	4(5.19)	5(6.50)	77
Total	138	63	31	18	17	267

($\chi^2 = 3.573$, df = 8, p = 0.899) n= Number of patients.

Table 5: Relationship between CD4 cell count level at 6 month and hepatocellular injury

CD4 c/mm ³	Grade 0 n(%)	Grade 1 n(%)	Grade 2 n(%)	Grade 3 n(%)	Grade 4 n(%)	Total
≤ 200	24(51.06)	11(23.4)	4(8.51)	5(10.64)	3(6.38)	47
201-350	47(56.63)	16(19.28)	11(13.25)	5(6.02)	4(4.81)	83
>350	67(48.90)	36(26.28)	16(11.68)	8(5.84)	10(7.3)	137
Total	138	63	31	18	17	267

($\chi^2 = 4.044$, df = 8, p = 0.869) n= Number of patients

Age group and Hepatocellular Injury

Out of the 267 patients, 129 (48.3%) developed various grades of hepatocellular injury, among which 124 (46.12 %) were between the ages of 20 and 59 years. Thirty five (13.11%) of them developed severe hepatocellular injury (grade 3 and 4). There was no statistically significant relationship between age group and hepatocellular injury ($\chi^2 = 24.81$, df = 24, p = 0.416). Gender and hepatocellular injury one hundred and sixty six (62.2%) of the patients were females in all the three groups and 80 (48.2%) developed a hepatocellular injury. There was no significant relationship between gender and development of hepatocellular injury ($\chi^2 = 8.63$, df = 4, p= 0.071).

Cotrimoxazole and Hepatocellular Injury

One hundred and seventy three (64.8%) of the study population were on cotrimoxazole prophylaxis (Table 8) and out of which 87 (50.3%) developed various grades of hepatocellular injury. No significant association between cotrimoxazole prophylaxis and development of hepatocellular injury ($\chi^2 = 5.7$, df = 4, p = 0.222). Hepatocellular injury and multiple risk factors Logistic regression analysis showed that concurrent administration of ART and Anti-TB was the only independent risk factor for development of hepatocellular injury with a p-value of 0.02.

Discussion

Commencement of ARVs for all HIV/TB co-infected patients within few weeks of TB treatment has significantly reduced early mortality and improved their cure rate. [14] But as a result of the concurrent therapy a serious adverse event of hepatotoxicity may ensue, because hepatotoxicity may be induced by both NNRTIs and first line anti TB drugs. [1]

The age distribution of the patients in Group One (those on Nevirapine-based ARVs) shows that mean $\pm$ SD of 31 $\pm$ 10.46 years. The result is similar to the findings in New York with mean age of 37yrs. [15] In Group Two (HIV/TB co-infected patients on concurrent therapy) mean $\pm$ SD of 38.09 $\pm$ 8.93 years, which is also similar to the findings of the studies carried out in Ethiopia with 35yrs, [16] India 35yrs, [13] and Thailand 36.8 years, [1] While in Group Three (HIV infected patients on Efavirenz base ART) the mean $\pm$ SD of age was 40.87 $\pm$ 9.59 years. This is similar to that of a study from Ethiopia 34 years, [16] and South Africa 35yrs. [17]

The sex distribution of the patients in the three groups was 166 (62.2%) females and 101 (37.8%) males. This shows that women were more affected than their men counterpart in the study. The result is consistent with findings in Rwanda where 56.7% of

the study population were females.^[18] According to WHO and UNPFA ^[19] women comprises up to 60% of people living with HIV in Sub-Saharan Africa. In developing countries in general, women population are the disadvantaged group when it comes to prevention and treatment of HIV, and one of the commonest factors that make them more vulnerable is sexual violence.^[20,21]

The overall prevalence of hepatocellular injury among the three groups was 48.31 %, and this includes various grades of ALT elevation, that is mild to moderate (grade 1 and 2) and severe hepatocellular injury (grades 3 and 4). Twenty five (28.1%), 42(46.15%) and 27 (31.02%) in groups 1, 2 and 3 respectively developed grade 1 and 2 injuries. Severe hepatocellular injury occurred in 5 (7.86%) in group 1, 15 (16.49%) in group 2 and 13 (14.95%) in group 3. The prevalence rate of severe hepatocellular injury of 16.5 % recorded in this study is somewhat outside the range of the studies that were previously reported of 0.6% to 13%.^[1]

The findings in this study revealed that the prevalence of severe hepatocellular injury (grades 3 and 4 alanitis) among HIV/TB co-infected patients receiving concurrent therapy of 16.5% is similar to that of two studies in Ethiopia with prevalence of 18.4%^[27] and 15%, ^[16] but sharply differ from a study from Botswana 9%^[23] and Thailand 2.9%.^[1]

The difference in the prevalence of severe hepatocellular injury recorded in this study among HIV/TB co-infected patients compared with other studies might be probably due to difference in demographic characteristics of the study population, frequency of monitoring ALT and differences in case definition of hepatotoxicity as well as genetically mediated differences in drug toxicity could also partly contribute.

The prevalence of severe hepatocellular injury of 7.86% and 14.95% among the HIV infected patients on Nevirapine and Efavirenz based ART respectively was found to be within the range of 1-18% of severe hepatotoxicity of previously reported studies in patients on HAART.^[24] The findings of the prevalence of severe hepatotoxicity cellular injury of 7.86% among patients on Nevirapine-based ART was similar to the studies from Amsterdam

6.3%,^[25] USA 15.6 %, ^[26] Adult AIDS Clinical Trial Group Meta-Analysis (AACTG) 8.9% ^[27] but sharply differ from study in Kano 0%,^[24] and New York 1.1%.^[28] While the prevalence of severe hepatocellular injury of 14.95% among the patients on Efavirenz based ART is dissimilar to a study from Adult AIDS Clinical Trial Group Meta-Analysis (AACTG) 8.9%,^[27] Ethiopia 8%,^[16] and also contrast that of South Africa 4.2%,^[17] Kano 3.2%,^[24] New York 1.1%,^[28] and USA 8.0%.^[26]

From this study the prevalence of hepatocellular injury of 14.95% was higher in the group of patients placed on Efavirenz based ART compare with the other group of patients placed on Nevirapine based ART of 7.86%. The available information on the frequency of hepatotoxicity due to Nevirapine and Efavirenz shows that Nevirapine causes hepatotoxicity more frequently than Efavirenz.^[34] The same result was found in Thailand,^[1] where severe hepatotoxicity occurred more frequently in the Nevirapine group than in the Efavirenz group. The findings were different in Hamza *et al* study where severe hepatotoxicity was found to be more in Efavirenz than the Nevirapine-based treatment group. In a similar study in India^[13], it was found that there was no significant difference between the Efavirenz and Nevirapine groups in terms of grade two hepatotoxicity. A study in New York City shows no difference in terms of the rates of severe hepatotoxicity among the Nevirapine and Efavirenz treatment groups.^[28] Friis-Møller *et al*.^[30] study was in agreement that there is no significant difference between the rates of alanitis in patients treated with Nevirapine or Efavirenz.

In this study there was a significant association between the patient group, group 1(HIV infected patients on nevirapine-based ART), group 2 (HIV/TB co-infected patients on concurrent Rifampicin-based ATT and Efavirenz based ART regimen) and group 3 (HIV infected patients on Efavirenz-based ARVs) and development of hepatocellular injury. The prevalence of hepatocellular injury was found to be higher among HIV/TB co-infected patients compare with other two groups. The study in Ethiopia^[16] was in conformity with the findings in this study were treatment group was found to be an independent predictor.

There was no association between age group and hepatocellular injury in the current study, this is in conformity with a study by Hassen *et al.*^[35] Although, it was previously reported that advanced age can be a risk factor,^[31,32,33,34] the age bracket in this study mostly are young and middle age patients with very few in the advanced age.

Gender was not found from this study to have an association with hepatocellular injury. This is similar to the findings of Hassen *et al.*^[35] study and is in agreement with the studies by Lima and Melo.^[36] Previous report suggested that women were more predisposed to the risk of anti-tuberculosis therapy (ATT) drug induced hepatotoxicity.^[35]

From this studies, BMI was found not to be significantly associated with development of hepatocellular injury. A larger proportion of patients had a normal BMI which is an indicator of their nutritional status. The findings were different in Makhoulf *et al.*,^[37] Kalsi *et al.*,^[38] Devarbhavi^[39] and Hassen *et al.*^[35] studies where BMI was found to be significantly associated with hepatotoxicity. The explanation to this is that the nutritional status of the previous study population was poor with lower BMI. Malnutrition status is regarded as one of the risk factor for drug induced hepatotoxicity, and its due to depletion of glutathione stores which makes patients more prone to oxidative injuries.^[35] The other reason might be due to the slower pace at which the liver metabolise drugs.^[37,38,39]

The CD4 cell counts were not significantly associated with development of hepatocellular injury. The mean CD4 cell count of the study groups were found to be higher at both pre-treatment and at six months after initiating therapy. Studies have shown that development of hepatotoxicity was found to be significantly associated with lower CD4 cell count of $\leq 50 \text{ c/mm}^3$,^[35,16] but this is not in keeping with the findings from this study. Genetic predisposition might have also play a significant role.

In this study hepatocellular injury was found not to be associated with the use of cotrimoxazole preventive therapy. This is similar to a study in Ethiopia.^[35]

Limitations

Because of the retrospective nature of the study, LFTs result of TB patients could not be found, as serum ALT was not usually considered as a routine investigation in the management of a patient with Tuberculosis. As a result of this, there was no comparison with a group of TB patients on anti-TB treatment. That would have helped in determining the effect of anti-TB drugs on liver enzymes when administered alone. Some vital information were not found in the patient case files such as history of smoking, and the use of traditional medication. As a result of poor record keeping some of the lab results were unable to trace.

Conclusion

The prevalence and severity of the hepatocellular injury were higher in HIV/TB co-infected patients on concurrent antiretroviral and anti-tuberculosis drugs therapy. Hepatocellular injury was found to be more in HIV infected patients on efavirenz based antiretroviral drugs than in those on nevirapine-based antiretroviral.

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Screening of *Securidaca longipedunculata* Stem Bark Extracts for Activity against Newcastle Disease Virus

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Abstract

Securidaca longipedunculata Fresen (Polygalaceae) also known as “*Sanya*, *Uwarmagunguna*” in Hausa literally meaning the mother of medicines is a multi-purpose plant with a long history of use in African traditional medicine to treat various infections. However, few antiviral researches on this plant were published. Newcastle disease (ND) is a deadly disease of poultry which has no medication currently except vaccination. The disease is a threat to both vaccinated and unvaccinated flocks causing huge economic losses to the poultry industries. Hence, this study was carried out to determine the antiviral activity of *Securidaca longipedunculata* stem bark extracts against Newcastle disease virus. The plant part was extracted with Soxhlet apparatus using methanol and petroleum ether as the extraction solvents. The extracts were subjected to phytochemical screening using standard procedures. Antiviral activity of the extracts was carried out using embryonated chicken eggs treated with 100EID₅₀ / 0.1 ml Newcastle Disease Virus (NDV) pre-treated with *S. longipedunculata* crude extracts at concentrations of 5mg/ml to 40mg/ml based on the toxicity result and embryo survival was observed daily. The result of phytochemical screening revealed the presence of alkaloids, resins, tannin, saponin, flavonoids, steroidal ring, steroidal terpenes, reducing sugar, phenols and cardiac glycoside while anthraquinone being the least present in the methanol extract only. Antiviral activity result showed that embryo survival was directly proportional to increasing extract concentration, just as increase in extract concentration was directly proportional to virus death. These findings have clearly demonstrated that, *S. longipedunculata* stem bark extracts have antiviral activity against NDV with a greater activity by the methanol extract at 40mg/ml with percentage mortality of 10. The petroleum ether showed slight activity at 20mg/ml with percentage mortality of 60mg/ml.

Keywords: *Securidaca longipedunculata*, Extracts, Newcastle Disease, Virus

Introduction

Plant materials remain an important resource to combat serious diseases in the world. Pharmacognostic investigations of plants are carried out to find novel drugs or templates for the development of new therapeutic agents. [1] Among the more than 250,000 species of higher plants, only about 5–10% are chemically investigated. [2] Since many drugs, like quinine and artemisinin, [3] taxol and camptothecin [4] were isolated from plants, and because of increased resistance of many microorganisms towards established drugs, investigation of the chemical compounds within traditional plants is necessary. [5] The genus *Securidaca* comprises about 80 species, characterised by papilionaceous purplish flowers

and mostly scandent shrubs and lianas, which produce compounds known as securixanthones with antimicrobial and antioxidant properties. [6,7,8,9] Although protected under provincial and national legislation, *S. longipedunculata* stem bark and roots are still found amongst the most traded medicinal plants in Africa. [10,11] It has been reported that a spoonful of powdered stem bark is mixed with *Mondia whitei* (stem bark), *Uvaria afzelii* (root bark), *Allium ascalonicum* (bulb) and *Parkia biglobosa* (seeds) and then taken with hot porridge to treat a variety of viral infections. [12] The dried bark is ground into powder and taken orally with cow's milk or porridge for fourteen days to treat dysentery. [13] A decoction from the stem bark may be taken

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orally to treat stomach ache, headaches, inflammation, chest complaints, abortion, jaundice, ritual suicide, constipation, snake bites, infertility problems, epilepsy and venereal diseases.^[14, 15, 16] The powdered stem bark is also mixed with hot water and taken orally to treat syphilis and gonorrhoea.^[17] Newcastle disease (ND) is an acute, rapidly spreading, contagious, nervous and respiratory disease of birds of all ages caused by the avian paramyxovirus serotype 1 (APMV-1).^[18] It is a major viral disease of economic importance in poultry ^[19] and rated as one of the greatest constraints to the developing countries including Nigeria, causing serious threats. ^[20] The disease is caused by an enveloped, non-segmented RNA avian paramyxovirus which is transmitted through exposure to faecal, respiratory discharges or rather discharges from infected birds and through contact with contaminated feeds, water, equipment, poultry attendants and clothing, leading to high morbidity and mortality.^[20, 21]

Over the years, Newcastle disease (ND) has defied all control measures. ^[22] The disease has remained at the forefront of infectious diseases afflicting poultry production after avian influenza. Despite the continuous global use of million doses of ND vaccine annually, the causative pathogen, avian paramyxovirus type 1 also known as Newcastle disease virus (NDV) has continued to evolve causing even a threat not only to the unvaccinated but the vaccinated flocks inclusive.^[22] However, there is currently no means of treatment existing for Newcastle disease except vaccination. ^[23] This is a serious threat to the poultry industries which is causing a huge economic losses to these industries. With the serious threats of Newcastle disease virus in poultry, there is an urgent need to extend the search for antimicrobials toward medicinal plants. *Securidaca longipedunculata*, which has long been used in traditional medicines by locals, and which has many different medicinal uses, ^[24] can serve as a source of new pharmaceuticals in curbing this virus and thus the development of traditional medicines.

Materials and Methods Study Area

The research work was carried out at the Department of Viral Research, National Veterinary Research Institute, Vom, Jos, Plateau State, Nigeria

Collection and Processing of Plant Material

Fresh stem bark of *Securidaca longipedunculata* was collected directly from the plant tree in Doguwa local government area of Kano State, Nigeria in the early morning hours, in the year 2015. The plant part was identified (BUKHAN 0013) at Department of Plant Biology, Bayero University, Kano. The stem bark was then processed by washing with clean water, shade-dried and ground into powder using an electric blender as described by Mukhtar and Tukur.^[25]

Collection of Virus and 9-day Old Embryonated Chicken Eggs for Antiviral Assay

A velogenic strain of NDV was obtained from the Department of Viral Research, National Veterinary Research Institute, Vom, Jos, while embryonated chicken eggs were obtained from Poultry Division, of the same institute.

The research was carried out aseptically in the biosafety cabinet using hand gloves, face mask and laboratory coat. Virkon® (Day – Impex limited, United Kingdom) and 70% alcohol were used as the disinfectants. Penicillin, Streptomycin, Gentamycin and Amphotericin B were the antibiotics and antifungals used they were obtained from National Veterinary Research Institute, Vom, Jos. The embryonated eggs were candled and inoculation sites were marked. The eggs were then placed in egg racks with the inoculation sites uppermost.

Extraction of Plant Materials

A procedure by Redfern *et al.* ^[26] was followed for extraction using Soxhlet apparatus (Pyrex Company, United Kingdom). The solvents used for the extraction were methanol and petroleum ether at (1:1) ratio. One hundred grams (100g) of the stem bark powder was filled in filter paper. Following this, 250ml of the solvent was added to a round bottom flask, which was attached to a Soxhlet extractor and condenser on an isomantle at 40⁰ C for petroleum ether and 65⁰C for methanol. The weighed powder was placed inside the Soxhlet extractor. The side arm was lagged with glass wool. The solvent was heated using the isomantle and began to evaporate, moving through the apparatus to condenser. The condensate then dripped into the reservoir containing the stem bark powder. As the level of solvent reached the siphon, it poured back into the flask and

the circle continued, until the plant material colour turned colourless. This process was done for each of the solvent for nine (9) hours.

The extracts were then placed in a water bath for evaporation of the solvents, leaving behind the crude extracts of the stem bark with percentage yield of 78.5%.

Phytochemical Screening

Phytochemical screening was carried out using the method described by Trease and Evans. [27] Alkaloids, resins, tannins, glycosides, saponins, flavonoids, anthraquinones, cardiac glycosides, steroidal ring, steroidal terpenes, and phenols were identified following the method of Poongothai *et al.* [28]

Determination of EID₅₀ (egg infective dose of NDV in 50% of Embryonated Chicken Eggs)

The egg infective dose (EID₅₀) of the viruses was determined according to the method of Young *et al.* [29] Sterile Bijou bottles were labelled 10⁻⁴ to 10⁻⁹. Sterile phosphate buffered saline PBS (0.9ml) was added to each bottle. A ten-fold serial dilution was carried out by adding 0.1ml of NDV to first bottle (10⁻⁴) and mixed thoroughly. From the content of first bottle, 0.1ml was transferred aseptically to second bottle (10⁻⁵) and mixed thoroughly. This transfer was done into the bottles until the last one (10⁻⁹) when 0.1ml of the content was discarded into a discard jar. Embryonated eggs were divided into eight (8) groups of five (5) for inoculation of the dilutions. First group was inoculated via allantoic fluid with 0.2ml of contents of 10⁻⁴, group two was inoculated with 0.2ml of contents of 10⁻⁵. Same inoculation was done to other groups with corresponding dilutions of virus up to six (6) which was inoculated with 10⁻⁹ of mixture. Uninfected control (PBS only) as well as virus control were included. Infected eggs were sealed and incubated in a humid chamber at 37°C for 24 hrs. They were candled after 24hrs for unspecific death while percentage mortalities after 48hrs and 72hrs was recorded. From this, 100 EID₅₀/ 0.1ml of the virus stock were made for the experiment which was obtained from the index calculated using Reed and Muench formula below.

Dilution 10⁻⁸ is the dilution that produces percentage of infected at dilution immediately above 50% and dilution 10⁻⁹ is the dilution that produces percentage of infected at dilution immediately below 50%. The index was then applied to the dilution that produced the percentage immediately above 50% as shown thus; Dilution 10⁻⁸ + 0.5 = 10^{-8.5} The reciprocal of this dilution is the amount of virus contained in the 0.1ml of the original suspension = 10^{-8.5} EID₅₀/ 0.1ml 100 EID₅₀/ 0. 1ml = 10^{-9.5} Therefore, the infective titre of virus has been calculated to be in dilution 10⁻⁹.

Haem agglutination Test

This was done to confirm the presence of the virus in the virus suspension. A drop of 5% chicken red blood cell was mixed thoroughly with a drop of the virus stock on a slide for formation of agglutination. [29]

Inoculation of Eggs

The method used by Chollom *et al.* [30] was adopted. Nine day old embryonated chicken eggs were labelled according to the extracts and concentrations that were used. A set of plastic egg trays were thoroughly cleaned with Virkon® (Day – Impex Limited, United Kingdom), the eggs were swabbed with 70% alcohol in cotton wool and transferred into the cleaned trays for inoculation.

Toxicity Assay

This was carried out to check the toxicity of the extracts on the chicken embryos. Nine day old embryonated chicken eggs were divided into six groups of five. Group one (1) to four (4) were swabbed with 70% alcohol, punched with eggshell punch and immediately injected with needle attached to 1ml syringe. By keeping the needle and syringe vertical, the needle was placed through the punched hole and penetrated into the allantoic cavity (approximately 16mm) with the extracts at concentrations of 80, 60, 40, and 20mg/ml in that order. The needle was then withdrawn from the egg and the hole was sealed with melted wax. Group five (5) was inoculated with 0.2ml phosphate buffered saline (diluent control) while group six (6) was not injected with any extract (negative control). The eggs were sealed with molten wax and incubated at 37°C for 24 hours. This was done for each of the extracts. Embryo survival was observed after 24 hours. [30]

Antiviral Assay

This was carried out to check the efficacy of the extracts using the procedure of Chollom *et al.*^[30] Group one (1) to four (4) of embryonated eggs were swabbed and placed in the –bio-safety cabinet where they were punched and immediately inoculated with the virus via allantoic route, sealed with molten wax and incubated at 37°C. After 24 hours, the petroleum ether extract at final concentrations of 20, 15, 10 and 5mg/ml while methanol extract at final concentrations of 10, 20, 30 and 40mg/ml were then injected in that order. Group five (5) were inoculated with 0.2ml of 100EID₅₀/ 0.1 ml standard NDV (virus control), group six (6) were inoculated with 0.2 ml extract (extract control). Group seven (7) were inoculated with 0.2ml phosphate buffered saline (diluent control) while group eight (8) were not inoculated with anything (uninoculated control). The eggs were sealed with molten wax and incubated at 37°C for 72 hours. This was done for each extract. Embryo survival was observed daily for 3 days.

Results

The yield of stem bark of *S. longipedunculata* was found to be 16.4grams with methanol as the solvent. Whereas, extract from stem bark petroleum ether yielded the least extract with 9.0grams. All extracts showed slight difference in their colour before and after evaporation of the respective solvents. The extracts also varied in their consistency after evaporation. Methanol extract which was greenish in colour before evaporation became greenish brown sticky solid after evaporation while the pale yellow petroleum ether extract before evaporation turned to yellow powdered solid after evaporation as shown in Table 1.

The egg infective dose in 50% of the embryonated eggs (EID₅₀) of Newcastle disease virus was found to be at dilution 10⁻⁹ as shown in Table 2.

Table 1: Physical appearance and weights of *S. longipedunculata* stem bark crude extracts

Extract	Physical appearance			Weight/grams
	Before evaporation of extract	After evaporation of extract	of	
A	Green liquid	Greenish brown sticky solid		16.4
B	Pale yellow liquid	Yellow powdered solid		9.0

Key: A = *S. longipedunculata* stem bark methanol extract, B = *S. longipedunculata* stem bark petroleum ether extract.

Table 2: EID₅₀ of newcastle disease virus after 72 hours

Dilution	No. of eggs with live embryo	No. of eggs with dead embryo	% Mortality (%)
10 ⁻⁴	1	4	80
10 ⁻⁵	1	4	80
10 ⁻⁶	1	4	80
10 ⁻⁷	1	4	80
10 ⁻⁸	2	3	60
10 ⁻⁹	3	2	40

In the antiviral assay, the toxicity result showed that all the extracts were relatively toxic. Stem bark petroleum ether extract was more toxic in which the highest concentration of this extract the embryonated eggs survived was 20mg/ml. Meanwhile, 40mg/ml concentration of the stem bark extract was the highest concentration at which the embryonated eggs

survived. Only one embryonated egg survived the concentration of the methanol extract while petroleum ether extract did not kill the embryos of the eggs at concentrations of 60mg/ml and 80mg/ml (Table 3).

Table 3: Toxicity assay of *S. longipedunculata* stem bark crude extracts on Newcastle disease virus (No. of eggs inoculated = 5)

Extract(s)	Conc. (mg/ml)	No. of eggs with dead embryo after 24h	No. of eggs with live embryo after 24h
A	20	0	5
	40	0	5
	60	3	2
	80	4	1
B	20	0	5
	40	2	3
	60	3	2
	80	3	2
Dc		0	5
Nc		0	5

Key: A = *S. longipedunculata* stem bark methanol extract, B = *S. longipedunculata* stem bark petroleum ether extract, Dc: Diluent control, Nc: Negative control, h = Hour.

The result of the antiviral activity of *Securidaca longipedunculata* stem bark extracts revealed that, stem bark methanol extract exhibited the highest activity with mortality rates of embryonated eggs of only 10% at 40mg/ml concentration of the extract. Petroleum ether extract had lowest activity with 60% mortality rate of embryonated eggs at 20mg/ml as

shown in Table 4. The result of phytochemical screening revealed the presence of the following bioactive components tested: Cardiac glycoside was present in larger quantity in all the extracts. Glycoside was absent in all the extracts. Anthraquinone was also absent in the petroleum ether extract (Table 5).

Table 4: Antiviral activity of *S. longipedunculata* stem bark crude extracts against NDV

Extract	Conc. (mg/ml)	No. of eggs	Mortality			% Mortality after 72h
			24h	48	72h	
A	10	5	0	5	0	100
	20	5	0	3	1	80
	30	5	0	1	1	20
	40	5	0	1	0	10
Ec		5	0	0	0	0
B	5	5	0	5	0	100
	10	5	0	5	0	100
	15	5	0	3	2	100
	20	5	0	0	3	60
		5	0	0	0	0
Ec		5	0	0	0	0
Vc		5	5	0	0	100
Dc		5	0	0	0	0
Nc		5	0	0	0	0

Key: A=*S. longipedunculata* stem bark methanol extract, B = *S. longipedunculata* stem bark petroleum ether extract, Vc = virus control, Ec = extract control, Dc = diluent control, Nc = negative control, h=Hours

Table 5: Phytochemical constituents of *S. longipedunculata* extracts

Compounds	A	B
Alkaloids	+	+
Resins	+	+
Tannin	+	+
Glycoside	-	-
Saponin	+	+
Flavonoids	+	+
Anthraquinone	+	-
Cardiacglycoside	+++	+++
Steroidal ring	+++	+
Steroidalterpenes	+++	+
Phenols	+	+
Reducing sugar	+	+

Key: A = *S. longipedunculata* stem bark methanol extract, B = *S. longipedunculata* stem bark petroleum ether extract, + = Present, +++ = present in higher amount, - = Absent

Discussion

The phytochemical test of the crude methanolic and petroleum ether stem bark extract of *S. longipedunculata* revealed the presence of alkaloids, resins, tannins, saponins, flavonoids, anthraquinones, cardiac glycosides, steroidal rings, steroidal terpenes, phenols and reducing sugar. Glycosides were found to be absent. The absence of glycosides is relative to its absence in the compounds containing monosaccharides and thus might not enhance antiviral activity in these extracts. This is similar to the study done by Muanda *et al.* [31]

The EID50 revealed at dilution 10-9 is similar to the dilution titre for animal viral research used by the National Veterinary Research Institute, Vom. This is also similar to the work of Chollom *et al.*, [30] where the EID50 was found at dilution 10-9. In the antiviral assay, toxicity result showed higher toxicity of petroleum ether extract than the methanol extract, and this showed increasing toxicity with increasing extracts concentrations, indicating that, the extracts are relatively toxic to the experimental eggs.

This corresponds with the study of Auwal *et al.*, [32] on albino rats. However, antiviral assay of *S. longipedunculata* confirmed that the stem bark methanol crude extract had the highest antiviral properties against ND virus. This was revealed by the inhibition of virus growth at 20, 30 and 40mg/ml. At these concentrations, all the inoculated eggs had live embryos. However, petroleum ether extract of the stem bark revealed slight activity at 20mg/ml.

The methanol extract of the stem bark exhibited a greater activity at 40mg/ml and 30mg/ml with 10 and 20 percentage mortality respectively and also slight activity at 20mg/ml with percentage mortality of 80%. This is not unusual considering the phytochemical composition of the plant and a previous report on the antiviral potentials of the plant against viruses which according to Borikini *et al.*, [12] the stem bark is used to treat a variety of viral infections. These findings are scientific and relevant judging from the performance of the control groups.

The virus control was potent enough to cause embryo death within 24hrs post inoculation just as extract, diluents and uninoculated controls did not interfere with embryo survival signifying the acceptability of the outcome of the tests groups. To the best of my knowledge, this is the first documented animal virus to be confirmed susceptible to the plant. The presence of the phytochemical constituents from the phytochemical screening in the stem bark extracts of *S. longipedunculata* is an indication that this plant would yield drugs of plant origin with pharmacological significance. This is better supported by the fact that, the plant family (Polygalaceae) to which *S. longipedunculata* belongs, is known to be involved in ethnomedicine in the management of some ailments notably epilepsy. [33]

They are also used as anti-snake venom and as purgative. [34] Moreover, the presence of alkaloids, cardiac glycosides, flavonoids, saponins and tannins in this plant is in compliance with an already documented literature which stated that, plants belonging to families Polygalaceae, Moraceae and Cannabinaceae are known to contain triterpenes, resins and higher fatty acids in their stem and root bark. [35] However, results obtained from this study complimented and strengthened the antiviral property of *Securidaca longipedunculata* especially against the Newcastle disease virus.

Conclusion

From the result obtained in this study, *Securidaca longipedunculata* had more extracts in methanol than in petroleum ether. The Egg infective dose (EID₅₀) of Newcastle disease virus was determined

at dilution 10⁻⁹. The plant showed antiviral activity against Newcastle disease virus especially the methanol extract. It showed that the plant had various bioactive compounds in appreciable quantities.

Recommendation

Based on the result obtained in this study, it is recommended that more solvents need to be used to extract various bioactive compounds from this plant. More antiviral activity from this plant especially the stem bark should be explored against Newcastle disease virus and other animal and human viruses of economic and medical importance. There is also a need to investigate the biological activity of the other compounds occurring in the plant extracts. The leaves, roots should also be considered

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Hydrogen Bond Interaction with Trypanosomal Adenosine Kinase; Ornithine Decarboxylase and Triose Phosphate Isomerase could not be involved in the Antitrypanosomal Activity of Stigmasterol: An *in Silico* Study

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Abstract

Stigmasterol has previously been reported to possess antitrypanosomal activity using *in vitro* and *in vivo* models. However, the mechanism of antitrypanosomal activity is yet to be elucidated. In the present study, molecular docking was used to decipher the mode of interaction and binding affinity of stigmasterol to three known antitrypanosomal drug targets viz; adenosine kinase, ornithine decarboxylase and triose phosphate isomerase. Stigmasterol was found to bind to the selected trypanosomal enzymes with minimum binding energy of -4.2, -6.5 and -6.6 kcal/mol for adenosine kinase, ornithine decarboxylase and triose phosphate isomerase respectively. However, hydrogen bond was not involved in the interaction of stigmasterol with all the three enzymes but hydrophobic interaction seemed to play a vital role in the binding phenomenon which was predicted to be non-competitive like type of inhibition. It was concluded that binding to the three selected enzymes, especially triose phosphate isomerase, might be involved in the antitrypanosomal activity of stigmasterol but not mediated via a hydrogen bond interaction.

Keywords: Stigmasterol, Trypanosomes, Molecular docking, Adenosine kinase, Ornithine decarboxylase, Triose phosphate isomerase

Introduction

African trypanosomes are the causative agents of sleeping sickness in humans and nagana in animals. These protozoan parasites belong to the Salivarian group of the genus *Trypanosoma* that are transmitted by the bite of infected tsetse flies (*Glossina* sp.) and live and multiply in the blood and tissue fluids of their mammalian host. Sleeping sickness and nagana disease occur in sub-Saharan Africa.^[1] Millions of people and cattle living in this so-called tsetse belt are at risk of getting infected with trypanosomes.^[2] Both diseases have long caused major health and socio-economic problems in the affected African countries. This is mainly due to the fact that adequate chemotherapies are not available for the treatment of the diseases. All drugs currently used for treatment of sleeping sickness and nagana disease are outdated, require parenteral administration, induce significant toxic side effects, have limited efficacy and are highly prone to drug

resistance.^[3] The use of natural products for the treatment of diseases still holds a strong potential since the influence of natural products upon drug discovery is impressive and a number of clinically active drugs are either natural products or have a natural product pharmacophore.^[4] Luckily, ethnopharmacological reports revealed that a number of African plants contain potent trypanocidal agents.^[5-7] These suggest the need for exploring medicinal plants for new therapeutically active and more potent trypanocides. Hence, our recent research interest is to develop an effective plant-based anti-trypanosome remedy that is devoid of the aforementioned limitations.

Molecular docking is a recent and effective tool for *in silico* screening. It is playing an important and ever increasing role in rational drug design.^[8] Docking is a computational procedure of searching

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how a ligand fits both energetically and geometrically to a protein's binding site. In other words, it is a study of how two or more molecules e.g. ligand and protein, fit together. It is commonly known that molecular binding of one molecule (the ligand) to the pocket of another molecule (the receptor) is responsible for accurate drug activity. Hence, molecular docking proved to be a very efficient tool for novel drug discovery for targeting protein.^[9] Furthermore, protein-ligand molecular docking is used to predict the possible mechanism of action of a compound against a previously identified bioactivity.

Recent review from our research group revealed that 264 African medicinal plants across the African sub region were investigated for *in vitro* and/or *in vivo* antitrypanosomal activities.^[10] Unfortunately however, only 48 bioactive compounds (including stigmasterol) were characterised in isolated forms, to be the bioactive agents using *in vitro* antitrypanosomal models but none of these compounds was tested for *in vivo* antitrypanosomal activity which is usually the next step in drug discovery and development. Thus, in order to fill this important knowledge-gap, we recently subjected one of those compounds (stigmasterol) to a detailed *in vivo* studies and reported that it retarded the proliferation and pathological features of trypanosome infection in rats in addition to the inhibition of trypanosomal sialidase *in vitro* and *in silico*.^[11] Stigmasterol is a phytosterol derived from cholesterol that possesses a 3 β -hydroxy group and a 17 β -aliphatic side chain normally with 8-10 carbon atoms.

In spite of the above-mentioned findings, the precise mechanism of action of stigmasterol against the trypanosome parasites was not identified which is a relevant information to our interest on drug discovery and development against pathogenic trypanosomes. In this study, molecular docking procedure was used to predict the possible mode of interaction as well as binding conformations between stigmasterol and three validated anti-trypanosomal drug targets namely; adenosine kinase; ornithine decarboxylase and triose phosphate isomerase. This would allow us to decipher whether, or not, interaction with this validated drug targets is an important mechanism of the previously observed *in vivo* antitrypanosomal activity of stigmasterol.

Materials and Methods

Acquisition and Preparation of the Proteins

The 3D crystal structures of *Trypanosoma brucei* *brucei* adenosine kinase (PDB ID 4N09 resolved to 2.6 by x-ray diffraction), ornithine decarboxylase (PDB ID 1NJJ resolved to 2.5 by x-ray diffraction) and triose phosphate isomerase (PDB ID 4TIM resolved to 2.4 by x-ray diffraction) were retrieved in pdb format from the protein data bank (www.rcsb.org). Subsequently, Chimera version 1.11.2 was used to remove all the co-crystallized ligands and water molecules from the proteins structures. Thereafter, the Dock Prep tool of the chimera software was used to prepare each of the proteins for docking. All default parameters for the Dock Prep tool in chimera were used.

Acquisition and Preparation of the Ligand

The 3D structure of stigmasterol (CID 5280794) was retrieved in sdf format from the PubChem database and then opened in the Chimera software before subjecting it to the Dock Prep tool. All default parameters for the Dock Prep tool in Chimera were used and the structure was retrieved as a pdb format after the dock prep.

Molecular Docking of the Prepared Proteins and Ligand

The PDB prepared versions of the proteins and ligand were opened in Chimera and subjected to the AutoDock Vina tool^[12] in the same software. The list of the active site residues that were selected for grid generation in *T. brucei brucei* adenosine kinase were Thr264, Asp266, Gly298, Asn222, Gln288 and Ile330 while Asp385, Pro340, Glu384 were used for ornithine decarboxylase and Gly235, Asn11, His95, Ser213, Lys13, Gly173, Gly234 for triose phosphate isomerase. The grid sizes (xyz points) were set as 28.70 x 34.67 x 16.85, 51.62 x 64.92 x 21.93 and 20 x 55.36 x 63.49 for adenosine kinase, ornithine decarboxylase and triose phosphate isomerase respectively while the grid centres were designated at dimensions (x, y and z): 10.61, 0.78 and 16.52 for adenosine kinase, 15.85, -9.84 and 5.38 for ornithine decarboxylase and 23.39, 13.78 and 41.71 for triose phosphate isomerase. For each of the protein, the grid sizes and centres were selected to cover the entire protein while the active site residues for each of the protein were determined from the protein data bank. Other parameters of Auto Dock Vina tool in Chimera were left as default.

AutoDock Vina employs iterated local search global optimizer and all output files were saved in pdbqt format. After the successful docking in vina, the pose with lowest energy of binding was extracted and aligned with the receptor structure for further analysis of hydrogen bond interaction and other predicted (unspecified) interactions between each of the proteins and the stigmasterol.

Results

Multiple docking poses were obtained between stigmasterol and *T. brucei brucei* adenosine kinase but the pose with the minimum binding energy of -4.2 kcal/mol was selected (Figure 1). However, with this docking pose, there was no hydrogen bond interaction between the protein and the ligand but 75 other non-covalent interactions (Table 1) between the duos were observed (Figure 1). On the other hand, hydrogen bond was also not observed between stigmasterol and *T. brucei brucei* ornithine decarboxylase at a docking pose with minimum

binding energy but 52 interactions other than hydrogen bonds were recorded (Figure 2). This observation was similar to the docking analysis between stigmasterol and *T. brucei brucei* triose phosphate isomerase but with 60 non-covalent interactions other than hydrogen bond formation (Figure 3). On the other hand, it was noteworthy that all the docking poses of stigmasterol with the minimum binding energy against all the selected enzyme targets were not obtained around the active site of the enzymes. Comparatively, the affinity of stigmasterol to the selected enzymes was in the order; triose phosphate isomerase > ornithine decarboxylase > adenosine kinase (Table 1). Furthermore, C17 of the stigmasterol seems to mediate more interactions with the selected enzymes targets than other ligand atoms whilst hydrophobic amino acids (Ala and Leu) were more involved in interacting with stigmasterol than other group of amino acid residues of the selected enzyme targets (Table 1).

Table 1: Docking results and binding free energy (Kcal/Mol) of stigmasterol with the selected antitrypanosomal drug targets

	Minimum Binding energy (kcal/mol)	Number of hydrogen bonds	Number of all contacts	Commonest residue involved in all contacts	Commonest ligand atom involved in all contacts
Adenosine kinase	-4.2	0	75	Ala4	C18
Ornithine decarboxylase	-6.5	0	52	Thr270	C17
Triose phosphate isomerase	-6.6	0	60	Leu238	C17

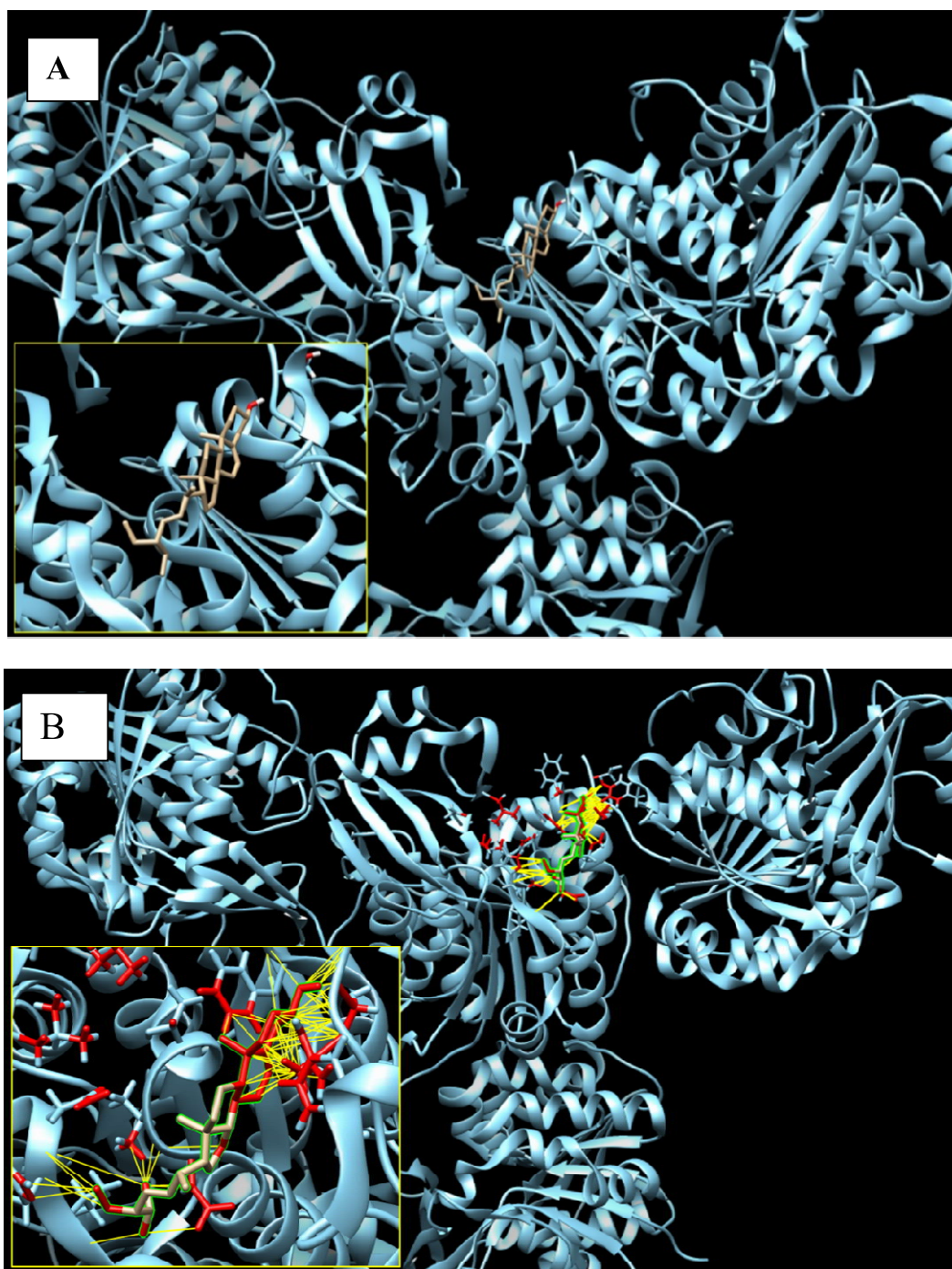


Figure 1: Docking pose with minimum binding energy of stigmasterol on *T. brucei* adenosine kinase showing no hydrogen bond interaction (A) and other potential interactions (B). The binding site has been zoomed out for each protein-ligand interaction and presented at bottom left side of the respective interaction.

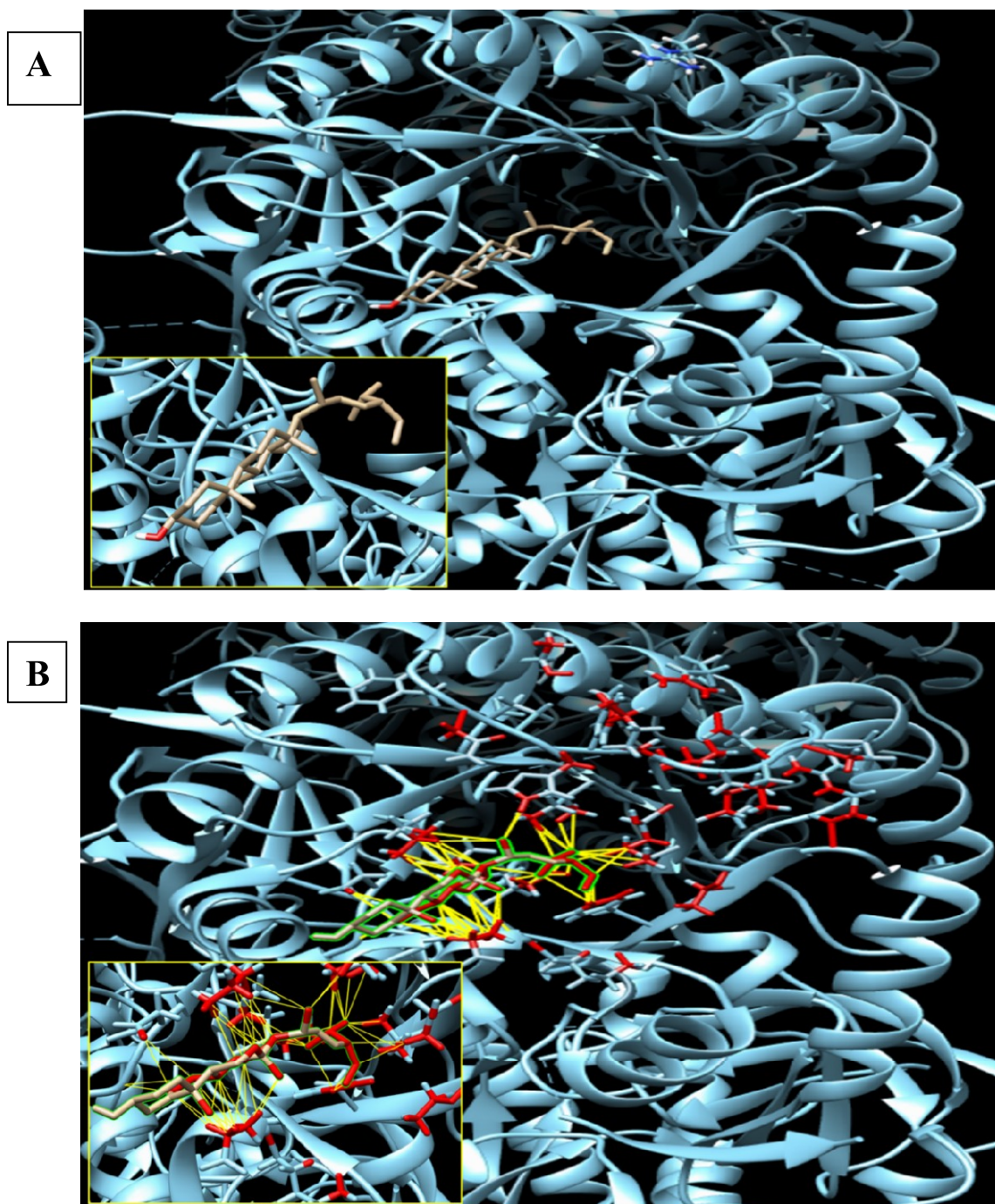


Figure 2: Docking pose with minimum binding energy of stigmasterol on *T. brucei brucei* ornithine decarboxylase showing no hydrogen bond interaction (**A**) and other potential interactions (**B**). The binding site has been zoomed out for each protein-ligand interaction and presented at bottom left side of the respective interaction.

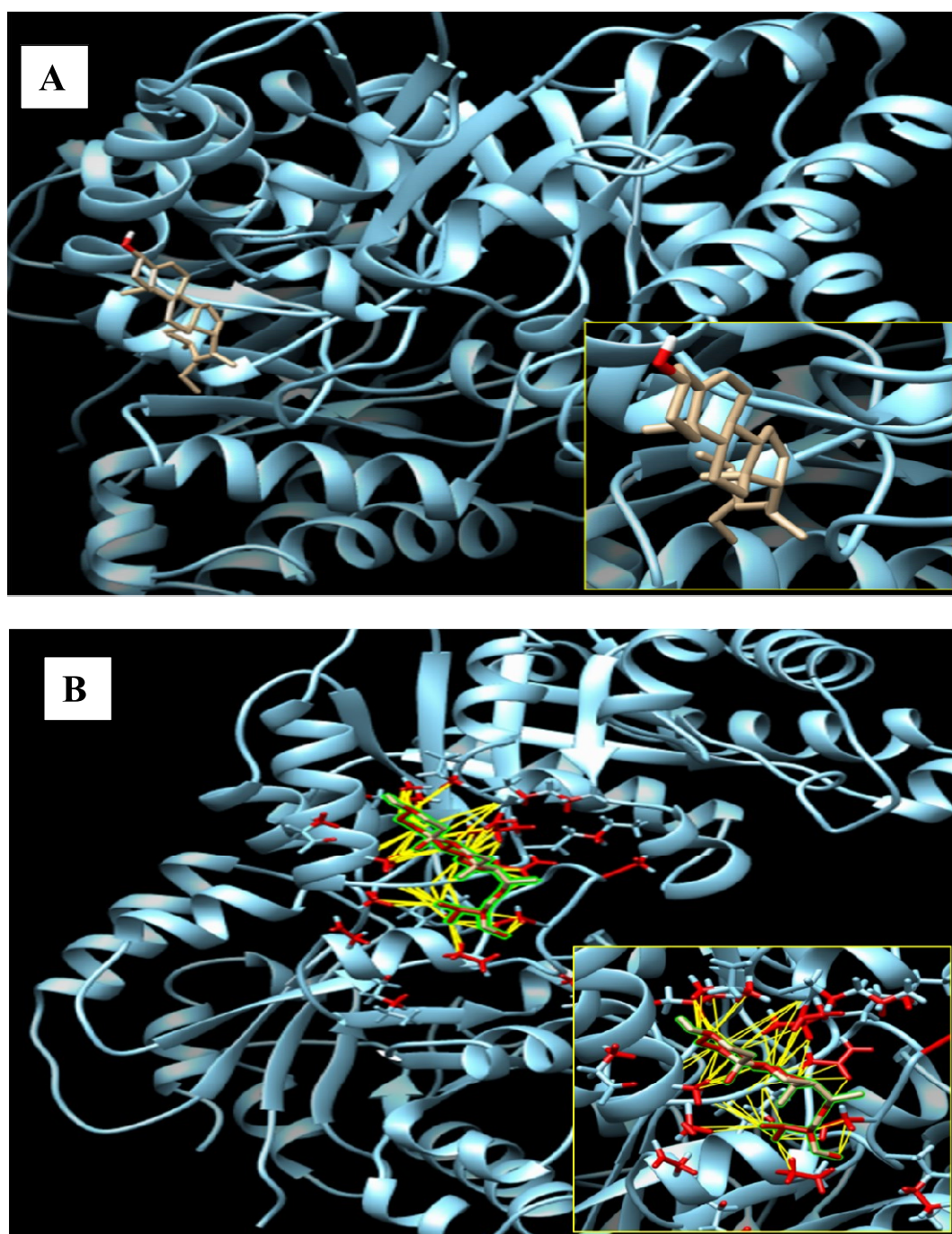


Figure 3: Docking pose with minimum binding energy of stigmasterol on *T. brucei* triose phosphate isomerase showing no hydrogen bond interaction (A) and other potential interactions (B). The binding site has been zoomed out for each protein-ligand interaction and presented at bottom right side of the respective interaction.

Discussion

Pharmaceutical research has successfully incorporated a wealth of molecular modelling methods, within a variety of drug discovery programs, to study complex biological and chemical systems. The integration of computational and experimental strategies is of great value in the identification and development of novel promising compounds. Broadly used in modern drug design, molecular docking methods explore the ligand conformations adopted within the binding sites of macromolecular targets. In the present study, molecular docking studies revealed that stigmasterol binds to 3 crucial drug targets of trypanosome parasites but not through a hydrogen bond interaction. Adenosine kinase is considered as a drug target for chemotherapy of trypanosomiasis because the parasites lack de novo purine synthesis and solely depends on salvage pathway to obtain the purines from their host.^[13] Thus, adenosine kinase is a key enzyme in the purine salvage pathway, catalysing the transfer of gamma-phosphate from adenosine triphosphate to adenosine leading to the formation of adenosine monophosphate^[14] while ornithine decarboxylase catalyses the decarboxylation of ornithine to produce putrescine, an essential precursor for the synthesis of higher polyamines (spermidines and spermines), which are vital for the normal and neoplastic cell growth.^[15] On the other hand, triose phosphate isomerase catalyses the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the glycolytic pathway and structural differences between mammalian and trypanosomal triose phosphate isomerases as well as the dependency of bloodstream form of trypanosomes on glycolytic pathway for energy metabolism make this enzyme a suitable target for the treatment of trypanosomiasis.^[16]

Thus, our observation of a higher binding affinity of stigmasterol towards triose phosphate isomerase seems to suggest that the compound has more inhibitory preference towards glycolytic pathway and energy generation system of the parasites. However, the findings may also suggest that the high hydrophobicity of stigmasterol may restrict reasonable quantity from penetrating into the cells to interact with the triose phosphate isomerase

because the enzyme is not exposed to the external milieu to eventually interact with the stigmasterol.

The observed lack of hydrogen bond interaction between stigmasterol and all the selected enzymes could be attributed to the lack of multiple polar (OH) groups on the molecule and the only available OH group on the molecule was not properly oriented during the minimum docking pose to interact with any polar amino acid on the surface of the enzymes. This is contrary to our previous observation where the OH group of the stigmasterol could align with Asp63 on the surface of *T. rangeli* sialidase.^[11] It seems therefore that the ability of the OH group of stigmasterol to interact with trypanosomal enzymes is dependent on the nature of the enzyme. In general, the foregoing observation of a lack of hydrogen bond interaction of stigmasterol and the selected enzyme targets could not adequately explain our previously observed antitrypanosomal activity of stigmasterol^[11] and it may be that the inhibition of other protein targets and/or other kinds of interactions is the biochemical mechanism of activity of this compound. On the other hand, the failure of stigmasterol to interact with any of the amino acids at the active site indicates that the type of inhibition might be more of non-competitive, rather than competitive.

Analysis of other potential interactions apart from the hydrogen bond revealed that the critical amino acids of the selected enzymes that binds to the stigmasterol are Ala and Leu which are known hydrophobic amino acids. On the other hand, the critical carbon atoms on the stigmasterol that mediate the interactions are C17 and C18 which are highly hydrophobic carbon atoms on the structure of stigmasterol. The foregoing observation might therefore suggest that the interactions between stigmasterol and the three enzymes are mainly mediated via hydrophobic interactions. However, with respect to the number of the non-covalent interactions, adenosine kinase had the highest (75) number which suggests that stigmasterol has higher tendencies of non-covalent interaction with adenosine kinase than does with triose phosphate isomerase, despite the higher binding affinity of the latter.

It was concluded that stigmasterol could not bind to three antitrypanosomal drug targets via a hydrogen bond but possibly through hydrophobic interactions that results in a non-competitive inhibition. Derivatization of C17 and C18 of the stigmasterol molecule for enhanced antitrypanosomal activity will be the subject of our future studies.

Conflict of interest: There is no conflict of interest in the study

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The Effect of Ethanol Leaf Extract of *Combretum micranthum* on Lipid Profile and Liver Enzymes in Alloxan-induced Hyperglycaemic Wistar Rats.

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Abstract

The aim of this research was to investigate the effect of ethanol leaf extract of *Combretum micranthum* on lipid profiles and liver enzymes in alloxan-induced Diabetes in Wistar rats. Diabetes was induced using alloxan (150 mg/kg *i.p.*). The rats were grouped into five groups of five rats each. Group 1 served as the diabetic control, Group 2 received 2 mg/kg glibenclamide, while Groups 3, 4 and 5 were administered 100, 200 and 400 mg/kg body weight of ethanol leaf extract of *C. micranthum* respectively. The route of administration was oral and for a period of 4 weeks. At the end of the experimental period, the rats from each experimental group were sacrificed using light chloroform, and serums were collected for the determination of lipid profile and liver enzymes. As regards to the lipid profile, it showed significant decrease ($p < 0.05$) in serum total cholesterol, triglyceride, low density lipoprotein. However, an increase in the level of high density lipoprotein was observed as compared to the hyperglycaemic control. The extract significantly ($p < 0.05$) reduced liver enzymes (aspartate transaminase, alanine transaminase and alkaline phosphatase as compared with the control. The preliminary phytochemical screening of the extract revealed the presence of tannins, flavonoids, alkaloids, cardiac glycosides, resins and terpenoids. The LD₅₀ was determined to be greater than 5000 mg/kg orally. In conclusion ethanol leaf extract of *C. micranthum* had hypolipidemic effect and also reduced the activity of liver enzymes in alloxan-induced hyperglycaemic rats.

Keywords: *Combretum micranthum*, Hyperglycaemia, Alloxan, Lipid profile, Liver enzymes

Introduction

Diabetes is a common metabolic disorder characterised by hyperglycemia due to an absolute or relative insulin deficiency [1,2]. It affects essential biochemical pathways of the body including carbohydrate, protein, and lipid metabolisms. The World Health Organization (WHO), estimated that there were 171 million people in the world with diabetes in the year 2008 and this is projected to increase by over a 100% to 366 million [2]. Diabetes is associated with reduced life expectancy, significant high mortality and diminished quality of life. In 2005 an estimated 1.1 million people died from diabetes and diabetes complications [3]. Its prevalence is rising globally, including the rural Nigerian populations [4]. *Combretum micranthum* is popularly known as *Sansami* in the Hausa language in Nigeria. It is used traditionally in Senegal and

Mali for fatigue, liver ailments, headache, convalescence, diabetes, blood disease, weight loss, cancer, sleep disorders, and it is especially used for fasting by Mourides in Senegal. It is one of the plants of power in Nigerian medicine and is used to treat liver disorders. *Kinkeliba* means “health tree” and the French import *kinkeliba* and call it “*tisane de longue vie*” or infusion of life [5]. The branches of the tree are quite strong, and are useful material floor building tools, beds, tool handles. A tea produced by steeping the leaves in boiling water was a traditional tonic drink in tropical savannah countries. The aim of this study is to evaluate the effect of ethanol leaf extract of *C. micranthum* on lipid profile and liver enzymes in alloxan-induced hyperglycaemic in Wistar rats.

Materials and Methods

Collection of Plant Materials

Fresh leaves of *C. micranthum* were collected from Hunkuyi town, Kudan local government area of Kaduna state, Nigeria in January, 2016. The plant was authenticated at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. A voucher specimen (No 1520) has been deposited for future reference.

Chemicals / drugs used

Glibenclamide and alloxan were purchased from Sigma Chemical Company (St. Louis U.S.A.)

Preparation of Extract

Combretum micranthum leave were air-dried under shade for twenty one days and then size-reduced into powder with a pestle and mortar. About 150g of the powdered leaves was macerated with 250ml ethanol for 72 hrs with occasional shaking. The extract was concentrated *in vacuo* affording a yield of 18.4%w/w and subsequently referred to as ethanol leaf extract of *Combretum micranthum*.

Preliminary Phytochemical Screening

The screening was carried out in accordance with the standard protocol as described by Trease and Evans [6].

Acute Toxicity Study

The lethal dose (LD₅₀) of the plant extract was determined by the method of Lorke^[7] using 12 mice. In the first phase, mice were divided into 3 groups of 3 mice each and were treated with the extract at doses of 10, 100 and 1000 mg/kg body weight orally. They were observed for 24 hrs for signs of toxicity and death. In the second phase, 4 groups containing one mouse each were injected with four more specific doses of the extract administered orally. The median lethal dose (LD₅₀) was determined as the geometric mean of the highest non lethal dose and the lowest lethal dose of which there is 1/1 and 0/1 survival.

Experimental Animals

Twenty five (25) Wistar rats of both sexes (120-150 g) were obtained from the Animal House of the Department of Human Physiology, Ahmadu Bello University, Zaria, Nigeria. The Rats were maintained on standard laboratory animal feed and water *ad libitum*. The animals were housed in polypropylene cages at room temperature throughout the study and were maintained on standard small animal feeds (Excel feed, Ilorin, Nigeria). This research was carried out in Ahmadu Bello University in accordance with the rules governing the use of laboratory animals as accepted internationally. The “Principle of laboratory animal care” (NIH publication No 85-23)”guideline and procedures were followed in this study (NIH publication reserved 1985).

Induction of Hyperglycaemia

Wistar rats were fasted for about 16-18 hrs, after which hyperglycemia was induced by a single intraperitoneal injection of Alloxan monohydrate dissolved in 0.9% cold normal saline solution at a dose of 150 mg/kg body weight [8]. Alloxan produces fatal hypoglycaemia and to prevent this, the rats were treated with 20% glucose solution orally for 6 hrs. After which they were placed on 5% glucose solution for 24 hrs^[9]. Blood was collected from the tail vein of the rats after 72 hrs of Alloxan injection. The rats having fasting blood glucose level greater than or equal to 200 mg/dl were selected for the study.

Determination of Fasting Blood Glucose Levels

Fasting blood glucose levels were determined by using the glucose oxidase method of Trinder^[10] with ONE TOUCH BASIC® Glucometer (LIFESCAN, Inc 2001 Milpitas, CA 95035, USA) and results were reported as mg/dl^[11]. Blood glucose level was determined by collection of blood sample from the tail artery of the rats. Rat with blood glucose levels greater or equal 200 mg/dl were considered for the study.

Experimental Design

In the experiment, a total of twenty five (25) Wistar rats were used; the animals were randomly divided into six groups of five rats each as follows:

- Groups 1: Hyperglycaemic administered 0.5 ml/kg body weight distilled water oral route for 4 weeks
- Group 2: Hyperglycaemic administered glibenclamide 2 mg/kg body weight oral route for 4 weeks.
- Group 3: Hyperglycaemic administered *C. micranthum* 100 mg/kg body weight extract oral route for 4 weeks.
- Group 4: Hyperglycaemic administered *C. micranthum* 200 mg/kg body weight extract oral route for 4 weeks.
- Group 5: Hyperglycaemic administered *C. micranthum* 400 mg/kg body weight extract oral route for 4 weeks.

Blood Sample Collection and Serum Preparation

All animals were sacrificed using light chloroform and 5mL of blood sample were collected into specimen bottles and allowed to clot and separated by centrifugation at 3,000g for 10 minutes using Centrifuge Hitachi (Universal 32). The supernatant obtained were used for the determination of lipid profile and liver enzymes.

Determination of Serum Liver Enzymes Activities

An activity of serum alanine aminotransaminase was estimated by the method of Tietz^[12] aspartate aminotransaminase was determined by the method of Henderson and Moss^[13] while alkaline phosphatase was determined according to the method of Scherwin.^[14] All the above analysis were done using Elitech Clinical Systems reagent kits.

Determination of Lipid Profile

Assay of Serum Total cholesterol

Total cholesterol was determined spectrophotometrically, using enzymatic colometric assay kits (Randox Laboratories Limited kits, United Kingdom) as follows: The serum level of total cholesterol was quantified after enzymatic hydrolysis and oxidation of the sample as described by the method of Stein^[15]. About 1000 μ L of the reagent was added to each of the sample and standard. This was incubated for 10 minutes at 20-25 °C after mixing and the absorbance of the sample (A_{sample}) and standard (A_{standard}) was measured against the reagent blank within 30 minutes at 546 nm. The absorbance is expressed in mmol/L.

Assay of Serum Triglyceride

Triglyceride level was determined after enzymatic hydrolysis of the sample with lipases as described by

the method of Tietz^[16]. About 1000 μ L of the reagent was added to each of the sample and standard. This was then incubated for 10 minutes at 20-25 °C after mixing and the absorbance of the sample (A_{sample}) and standard (A_{standard}) was measured against the reagent blank within 30 minutes at 546 nm. The value was expressed in mmol/L.

Assay of Serum High-density Lipoprotein Cholesterol

High-density Lipoprotein cholesterol was measured by the method of Wacnic and Albers^[17]. Low-density lipoproteins (LDL and VLDL) and chylomicron fractions in the sample was precipitated quantitatively by addition of phosphotungstic acid in the presence of magnesium ions. The mixture was allowed to stand for 10 minutes at room temperature and centrifuged for 10 minutes at 2000 g. The supernatant represented the HDL-C fraction. The cholesterol concentration in the HDL fraction, which remained in the supernatant, was determined. The value was expressed in mmol/L.

Assay of Serum Low-density Lipoprotein cholesterol

Low-density Lipoprotein cholesterol was measured according to the protocol of Friedewald.^[18] The value was expressed in mmol/L.

Statistical Analysis

Values obtained from each group were expressed as mean $\pm$ SEM. The data were statistically analyzed using ANOVA with Tukey's *post hoc test* to compare the levels of significance between the control and experimental groups. All statistical analysis was evaluated using SPSS version 17.0 software and Microsoft Excel (2007). The values of $p \leq 0.05$ were considered significant.

Results

Table 1: Preliminary phytochemical screening of ethanol leaf extract of *Combretum micranthum*

Constituents	Remark
Tanins	+
Saponins	-
Flavonoids	+
Alkaloids	+
Cardiac glycosides	-
Resins	+
Terpenoids	+
Carbohydrates	-
Anthraquinone	-

Note: + Present, - Absent

The preliminary phytochemical screening revealed the presences of tannin, flavonoid, alkaloid resin and terpenoid as shown in Table 1.

Acute toxicity study (LD₅₀) The sign of toxicity were first noticed after 12-18 hrs of extract

administration. There was decreased locomotor activity, decreased feed intake, and prostration after 12 hrs of extract administration. The median lethal dose (LD₅₀) in rats was calculated to be greater than 5000 mg/kg body weight oral.

Table 2: Effects of ethanol leaf extract of *Combretum micranthum* on serum lipid profile on alloxan- induced Hyperglycaemic Wistar Rats

Treatment Groups (n=5)	Serum Cholesterol (mmol/L)	Total Triglyceride (mmol/L)	Serum High Density Lipoprotein (mmol/L)	Serum Low Density Lipoprotein (mmol/L)
Hyperglycaemic control	5.23 ± 1.19	3.22 ± 0.15	0.44 ± 0.39	1.56 ± 0.21
Hyperglycaemic + glibenclamide 2g/kg	2.26 ± 1.14 ^a	1.67 ± 0.21 ^a	1.63 ± 0.33 ^a	1.02 ± 0.18 ^a
Hyperglycaemic+50 mg/kg <i>Combretum micranthum</i>	2.03 ± 1.01 ^a	1.11 ± 1.13 ^a	1.92 ± 0.23 ^a	0.52 ± 0.15 ^a
Hyperglycaemic+100 mg/kg <i>Combretum micranthum</i> extract	2.16 ± 1.16 ^a	0.92 ± 1.16 ^a	1.18 ± 0.42 ^a	0.49 ± 0.11 ^a
Hyperglycaemic + 200mg/kg <i>Combretum micranthum</i>	2.11 ± 1.23 ^a	0.61 ± 1.17 ^a	1.74 ± 0.13 ^a	0.38 ± 0.14 ^a

Table 2 shows the effect ethanol leaf extract of *C. micranthum* on lipid profile after 4 weeks of administration. Values are mean ± SEM; n=5. Values are statistically significant compared to hyperglycaemic control at: ^a $p < 0.05$ ns= not significant. As regards to the cholesterol, triglyceride and low density lipoprotein levels, there

was a significant decrease as compared with the hyperglycaemic control. However there was a significant increase in the level of high density lipoprotein when compared to the hyperglycaemic control.

Table 3: Effects of Ethanol Leaf Extract of *Combretum micranthum* on serum liver enzymes in alloxan-induced hyperglycaemic wistar rats

Treatment Given	Serum AST (IU/L)	Serum ALT (IU /L)	Serum ALP (IU/L)
Hyperglycaemic control	52.1 ± 1.01	53.0 ± 2.00	62.8 ± 1.21
Hyperglycaemic + glibenclamide(2 mg /kg)	22.0 ± 1.04 ^a	26.4 ± 2.05 ^a	25.2 ± 1.14 ^a
Hyperglycaemic+50 mg/kg <i>Combretum micranthum</i>	24.1 ± 1.03 ^{ns}	24.2 ± 2.31 ^a	28.3 ± 1.31 ^a
Hyperglycaemic +100 mg/kg <i>Combretum micranthum</i>	20.6 ± 1.42 ^{ns}	22.0 ± 2.34 ^a	24.3 ± 1.39 ^a
Hyperglycaemic + 200 mg/kg <i>Combretum micranthum</i>	19.5 ± 1.22 ^{ns}	22.4 ± 2.14 ^a	20.4 ± 1.29 ^a

Table 3 presents the effect ethanol leaf extract of *Combretum micranthum* on liver enzymes after 4 weeks of administration. Values are mean ± SEM; n=5. Values are statistically significant compared to hyperglycaemic control at:^a $p < 0.05$ ns= not significant. AST (aspartate transaminase) ALT (alanine transaminase) ALT (alkaline phosphatase) However there was a significant decrease ($p < 0.05$) in the aspartate amino transferase, alanine amino transferase and alkaline phosphatase levels when compared with hyperglycaemic control.

Discussion

Serum lipid levels are commonly increased in diabetes mellitus and such an elevation represents a risk factor for coronary heart disease [19;20]. The present study showed a decrease in the levels of total cholesterol, triglyceride, low density lipoprotein and an increase in high density lipoprotein in diabetic control animals. This is in agreement with the reports of Fernandes [21;22] who demonstrated increase serum lipids in diabetic in animals.

Diabetes-induced hyperlipidemia is attributable to excess mobilisation fat from the adipose due to under utilisation of glucose [23]. The lack of insulin and elevations of the counter-regulatory hormones glucagon lead to activation of enzymes (hormone-sensitive lipase) that stimulate lipolysis and enhanced release of free fatty acids from adipose tissue [24]. The fatty acids from adipose tissues are mobilised for energy purpose and excess fatty

acids are accumulated in the liver, which are converted to triglyceride [25]. The marked hyperlipidemia that characterises the diabetic state may therefore be regarded as a consequence of unlimited actions of lipolytic hormones on the fat depots [26]. Lowering of serum lipid levels through dietary or drugs therapy seems to be associated with a decrease in the risk of vascular disease in diabetes [26].

The results of the present investigation showed that all doses of the ethanol extract of *C. micranthum* leaves administered to diabetic rats produced a significant effect on serum lipid profiles in alloxan-induced hyperglycaemic rats. It also showed that of administration ethanol leaves extract of *Combretum micranthum* for a period of four weeks significantly decreases serum total cholesterol, triglyceride, low density lipoprotein and an increase in the level of high density lipoprotein. These findings are consistent with those reported by Sevim [27].

This observed improvement in the lipid profile of diabetic treated rats revealed the cardio-protective properties of extract of *C. micranthum* which might be due to the secondary metabolites of the extract. Kwiterovich [28] had reported that about 30% of blood cholesterol is carried in the form of HDL-C. HDL-C function to remove cholesterol atheroma within arteries and transport it back to the liver for its excretion or reutilisation, thus

high level of HDL-C protect against cardiovascular disease. Therefore, the observed increase in the serum HDL-C level on administration of different doses of the extract to hyperglycaemic rats indicates that the extract might have HDL-C boosting effect. Furthermore, the stabilisation of serum triglyceride and cholesterol levels in rats by the extract may be attributed to the secondary metabolites of the plant as revealed in the preliminary phytochemical screening to glucose utilisation and hence depressed mobilisation of fat [29].

These findings suggest that the extract may be useful in reducing the complications of hyperlipidemia and hypercholesterolemia which often co-exist in diabetes^[30]. Serum enzyme measurements are important tool, in clinical diagnosis that provides information on the effect and nature of pathological damage to any tissue^[31]. Liver enzymes are biomarkers of damage to the plasma membrane and endoplasmic reticulum and are often used to assess the integrity of the plasma membrane and tissues after being exposed to certain pharmacological agents^[32]. Result obtained in the present study showed that the activities of serum liver enzymes; aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were significantly increased in the diabetic untreated animals. However, administration of the extract significantly

decreases the levels of liver enzymes when compared with diabetic control. Increase gluconeogenesis and ketogenesis might be due to an elevated activity of transaminase^[33]. Abolfathi^[34] reported that the elevation in markers of liver injury such as ALT, AST and ALP indicate hepatocyte damage in experimental diabetes. And this increase in the levels of these enzymes in diabetes may be as a result of leaking out of these enzymes from the tissue into the blood stream^[35].

Aspartate transaminase and alanine transaminase are released when injury involves organelles such as the mitochondria^[36]. Transaminases mediate the catalysis of amino transfer reactions, and are vital markers of liver injury in clinical diagnostics^[37]. Alkaline phosphatase is a hydrolase enzyme located in the cytoplasm^[38] and is responsible for removing phosphate from nucleotides and proteins released due to hepatic cellular damage. The ability of ethanol leaf extract of *Combretum micranthum* to significantly decreases alanine transaminase, aspartate transaminase and alkaline Phosphatase levels, might suggest its hepato-cellular protective function and this can be attributed to secondary metabolite of the plant as such flavonoid. In conclusion the results of this study indicate that ethanol leaf extract of *Combretum micranthum* had hypolipidemic effect and reduced the activity of liver enzymes in alloxan-induced diabetic rats.

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Effects of Aqueous Extract of Balsam Apple (*Momordica balsamina* L.) on Lactogenesis and Liver Function in Lactating Wistar Rats.

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Abstract

Momordica balsamina L. plant is taken by mothers to boost the production of milk. There is dearth of scientific literature on its lactogenic effects. The plant contains momordicin which is toxic to the liver. This study is designed to evaluate the daily milk yield, serum oxytocin level, liver enzymes and histology of liver cells in lactating female Wistar rats. Twenty five lactating rats were randomly divided into 5 groups of 5 rats each and treated as follows: Group I: received 1ml/kg distilled water; group II: received 5 mg/kg metoclopramide; group III, IV & V: received 200, 400 and 800 mg/kg of *M. balsamina* L. respectively. Administration was done orally, once daily at 18:00 hrs for a period of ten (10) days, during which the milk yield was calculated daily at 18 hrs after gavage. The animals were sacrificed; blood and tissues samples were collected for analysis. *M. balsamina* L. significantly increased milk yield and oxytocin level ($p < 0.05$). It caused a significant increase in serum liver enzymes ($p < 0.05$) with a minor alteration to the histology of liver. It is concluded that aqueous extract of *M. balsamina* L. stimulate increased in milk yield, oxytocin level and liver enzymes with mild hepatotoxicity at higher doses.

Keywords: Galactagogue, Hepatotoxicity, Milk yield, *Momordica balsamina*, Wistar rats.

Introduction

Milk production is a complex physiological process involving the interaction of multiple hormones including prolactin, growth hormone, and oxytocin. Oxytocin is a lactogenic hormone that causes milk letdown through ejection reflex during lactation [1]. *Momordica balsamina* L. (MBL) is an herb with tremendous medicinal value (figure 1). A macerate of the whole plant is also used as a galactagogue,

mostly by Hausa communities in northern Nigeria and Niger republic to enhance lactation [2,3]. The whole plant extract has insecticidal properties [4]. Its leaves were found to be highly haemolytic and hepatotoxic in Wistar rats, while the fruits were observed to be toxic to various organs and tissues of rats at very high doses [5].



Figure 1: Plant of *Momordica balsamina* L. ^[6].

The purpose of this study was to evaluate the galactogenic and hepatotoxic effects of aqueous extract of *M. balsamina* L. in lactating Wistar rats

Materials and Method Chemical and Drugs
Oxytocin ELISA 48 test kit [(GenAsia Biotech Co. Ltd, No. GA-E0504RT)], Metoclopramide [(NAFDAC Reg. No.: 04-6476)], Chloroform Poole, BH15 1TD England. All the drugs and chemicals used are of analytical grade.

Instruments and Devices

Dissection Kits, 120 Standard bottle, 2 ml pipette, Pestle and Mortar, Bench Centrifuge Hawksley [(RegNo: 891481 Ser. No. 07. 4. 26.[®])] Weighing balance [(Salter, HoMedics Group Ltd. Production no. IB-1066-1011-03)].

Plant Collection and Extraction

The samples of *M. balsamina* L. were collected in Sabon Gari local government area of Kaduna State, Nigeria. The plant was identified at the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria with a voucher number of 1139. The *M. balsamina* L. was washed, dried under shed and pounded into powder.

The extraction was done in Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. The powdered (500 g) was sucked in hot water for 3 hrs. The extract was concentrated under reduced pressure to yield greenish brownish semi-solid sticky residue which weighed 35.3 g. The extract was subsequently dried in desiccator and kept in a refrigerator until use.

Acute Toxicity Study (LD₅₀)

The lethal doses (LD₅₀) of the plant extract were determined by method of ^[7] using 13 rats. In the first phase the rats were divided into 3 groups of 3 rats each and were treated with the aqueous extract of the *M. balsamina* L. at doses of 10, 100 and 1000 mg/kg intraperitoneally. They were observed for 24 hrs for signs of toxicity. In the second phase 4 rats were divided into 4 groups of 1 rat each and also treated with aqueous extract of *M. balsamina* L. at doses of 1000, 1600, 2900 and 5000 mg/kg intraperitoneal. The median lethal dose was calculated using the second phase.

Phytochemical Screening

The aqueous extract of *M. balsamina* L. were subjected to preliminary screening, to identify the chemical constituents. The method employed were those described by^[8].

Experimental Animals

A total of twenty five (25) female and thirteen (13) male Wistar rats weighing between 180-240 grams were used for the study. The 25 female Wistar rats were used for the lactogenic study and 13 male Wistar rats were used for the acute toxicity study. They were kept in standard cages under 12 hrs light/dark cycle condition in the Animal House of the Department of Human Physiology, Ahmadu Bello University, Zaria. Their cages were cleaned on regular bases and were fed on commercial feed with tap water provided *ad libitum*. The procedure followed were in accordance with the ethical standards of Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) of Ahmadu Bello University Zaria, Kaduna state, Nigeria.

Experimental Design

Twenty five (25) female Wistar rats aged about 3 months old were housed and mated with the male rats in a stainless steel metal cage. Following birth, the litter's weights were recorded and culled to 4 litters per dam and the day of parturition was designated as day 1 of lactation. The 25 lactating Wistar rats were randomly selected and divided into five (5) groups of five (5) rats each and treated as follows: Group I: distilled water (1ml/kg); group II: metoclopramide (5mg/kg); group III, IV & V: received 200 mg/kg, 400 mg/kg and 800 mg/kg of *M. balsamina* L. respectively. All animals were treated daily, starting from day 4 to day 13 of lactation. Metoclopramide and aqueous extract of *M. balsamina* L. were administered orally daily at 18:00 hrs.

The milk production for each group were measured daily at 18 hrs after administration, starting from day 5 until day 14. The pups were weighed every day during the study period at 07:00 h and their weight recorded as (w1), and then isolated from their dam for a period of 4 hrs^[9, 10]. At 11:00 hr, the pups were weighed again and the weight recorded as (w2); and then re-united with their dam and allowed to feed for 1 hrs and their weight taken again at 12:00 hrs and

recorded as (w3). Milk yield 18 hrs after gavage was estimated as w3-w2 with a correction for weight loss due to metabolic processes using the following formula:

$$\text{Correction for weight loss} = (w2-w1)/4^{[9]}.$$

This value was then multiplied by the number of suckling hours per day and added to the suckling daily body weight gain^[9, 11, 12]. Their weights were measured using an electronic balance (Mettler P3®).

Blood Analysis

On day 14 of the experiment, the animals were euthanized using chloroform in a chloroform chamber and blood samples were collected through cardiac puncture. Blood samples were centrifuged and the sera obtained were used for the analyses of oxytocin using ELISA kit according to manufacturer's instruction. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) were assayed as described by Bergmeyer and Walefield^[13], while serum level of alkaline phosphatase (ALP) was determined as described by Bowers and Mc-Comb^[14].

Oxytocin Assay

Oxytocin analysis was carried out using the oxytocin rat ELISA kit according to the manufacturer's instruction. A rat specific oxytocin ELISA kit was used for the quantitative evaluation of oxytocin. The sera samples was pipetted into an antibody coated microplate, and allowed for two (2) hrs during which the endogenous oxytocin in the sample binds to the antibodies fixed on the inner surface of the wells. After the addition of a second polyclonal horseradish peroxidase-labelled antibody, a sandwich complex which consisted of two antibodies and the rat oxytocin was formed.

The excess enzyme conjugate was washed out and a chromogenic substrate, tetra-methyl benzidine (TMB) was added to the wells and allowed for 30 minutes incubation, during which the substrate was converted to a coloured end product (blue) by the fixed enzyme. The enzyme reaction was inhibited by dispensing hydrochloric acid as the stop solution. The optical density of the colour solution was measured using a microplate reader at 450 nm. A

standard curve was obtained by plotting the concentration of standard versus the absorbance.

The oxytocin concentration of the specimens and controls were determined concurrently with the standards. The lowest detectable level of oxytocin with this test was 20 ng/mL.

Histological Study

Liver tissues were harvested on the day 14 of lactation from the experimental rats and fixed in 10% formal saline. The histological preparation and interpretation was carried out at the Department of Histopathology, Ahmadu Bello University Teaching Hospital Shika, Zaria, Kaduna State, Nigeria. The method of H and E staining technique was employed.

Preliminary Phytochemical Screening of Aqueous Extract of *Momordica balsamina* L.

The yield of the aqueous extract of *Momordica* screening revealed the presence of the following *balsamina* L. was 14.16%. Preliminary phytochemical compounds as shown in (Table 1)

Statistical Analysis

Results were expressed as Mean $\pm$ SEM. All analysis was done using one way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparison using SPSS version 23. Values with ($p < 0.05$) were considered statistically significant.

Results Acute Toxicity Study of Aqueous Extract *Momordica balsamina* L.

The plant is characterised by a very low degree of toxicity. The acute toxicity LD₅₀ of aqueous extract of *Momordica balsamina* L. in Wistar rats was found to be above 5000 mg/kg-1 according to the method of [7].

Table 1: Phytochemical screening of aqueous extract of *Momordica balsamina* L.

Compound	Present
Tannins	+
Flavonoids	+
Alkaloids	+
Anthraquinone	-
Saponin	+
Carbohydrate	+
Cardiac Glycosides	+

+ = Trace.

- = Absent.

Effect of Aqueous Extract of *Momordica balsamina* L. on Daily Milk Yield in Lactating Wistar rats

The highest level of yield in the 400 mg/kg treated group was observed on day 9 of treatment (3.0 ± 0.45).

The highest level of milk yield in metoclopramide treated group was observed on day 7 (3.1 ± 0.44). The least yield was observed in the 200 mg/kg treated group compared to the other extract treated groups.

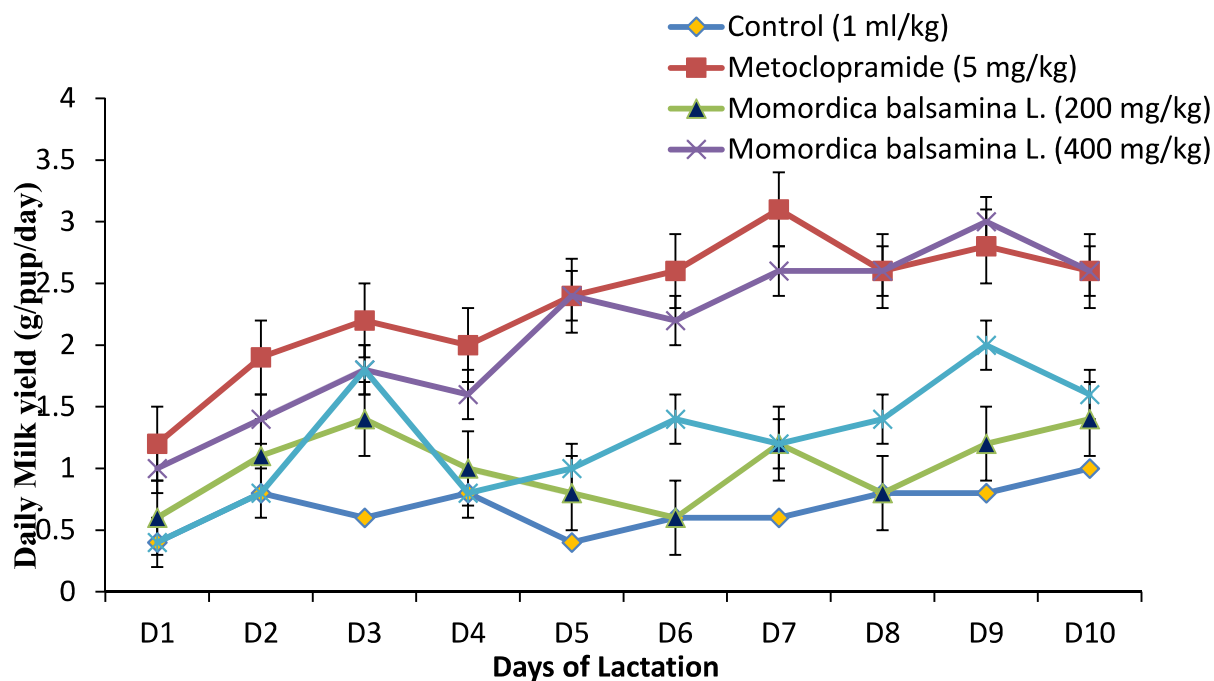


Figure 2: Effects of aqueous extract of *Momordica balsamina* L. on milk production 18 hrs after administration in lactating Wistar rats.

Effect of Aqueous Extract of *Momordica balsamina* L. on Serum Oxytocin Level in Lactating Wistar rats

The serum level of oxytocin hormone in lactating Wistar rats increased significantly ($p < 0.05$) in the

extract treated groups when compared to the normal control as follows: MBL 200 mg/kg (404.80 ± 1.71 vs 309.80 ± 8054), MBL 400 mg/kg (384.60 ± 6.33) and MBL 800 mg/kg (412.40 ± 7.67 vs 309.80 ± 8.54), respectively (Figure 3).

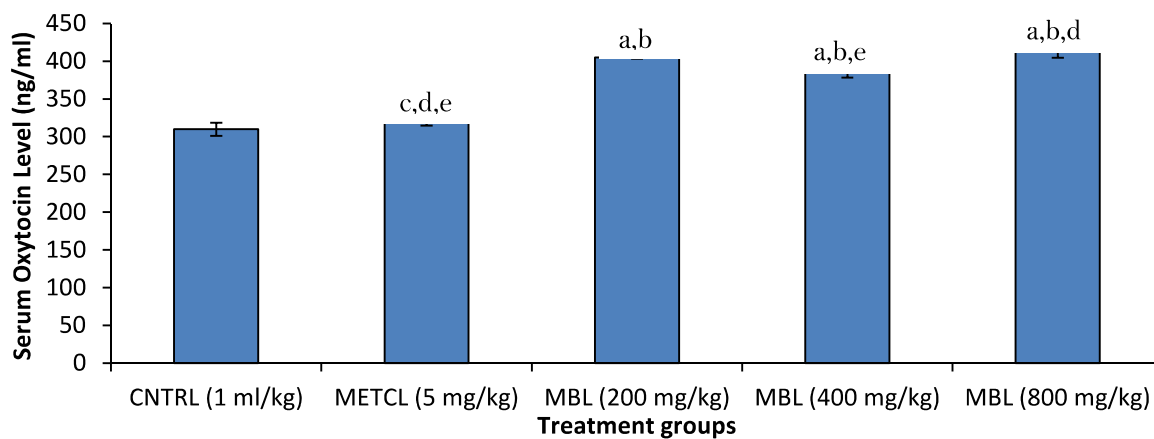


Figure 3: Effects of metoclopramide and aqueous extract of *Momordica balsamina* L. on serum oxytocin level in lactating Wistar rats. CNTRL =Control group (normal saline 1 ml/kg), METCL= Metoclopramide (5 mg/kg), MBL= *Momordica Balsamina* L. (200, 400 and 800 mg/kg). Different alphabetical superscripts indicate significant difference ($p < 0.05$)

Effect of Aqueous Extract of *Momordica balsamina* L. on Serum Liver Enzymes in Lactating Wistar Rats

The serum alkaline phosphatase (ALP) increased significantly ($p < 0.05$) in all the treated groups as follows: 200 mg/kg (128.20 ± 3.09), 400 mg/kg (132.40 ± 2.16), 800 mg/kg (140.40 ± 1.99) and Metoclopramide (129.60 ± 3.44), respectively when compared with the control group (90.20 ± 2.80). Serum alanine amino transferase was significantly higher ($p < 0.05$) in the treated groups when compared to the control as follows:

Metoclopramide (73.40 ± 1.78), 200 mg/kg (76.60 ± 2.20), 400 mg/kg (97.00 ± 2.88) and 800 mg/kg (127.00 ± 4.30) vs control (62.40 ± 1.22), respectively. The serum aspartate amino transferase (AST) in the 400 mg/kg (89.40 ± 3.37) and 800 mg/kg (119.00 ± 1.14) treated groups were significantly higher ($p < 0.05$) compared to control group (60.40 ± 0.68) (Figure 4).

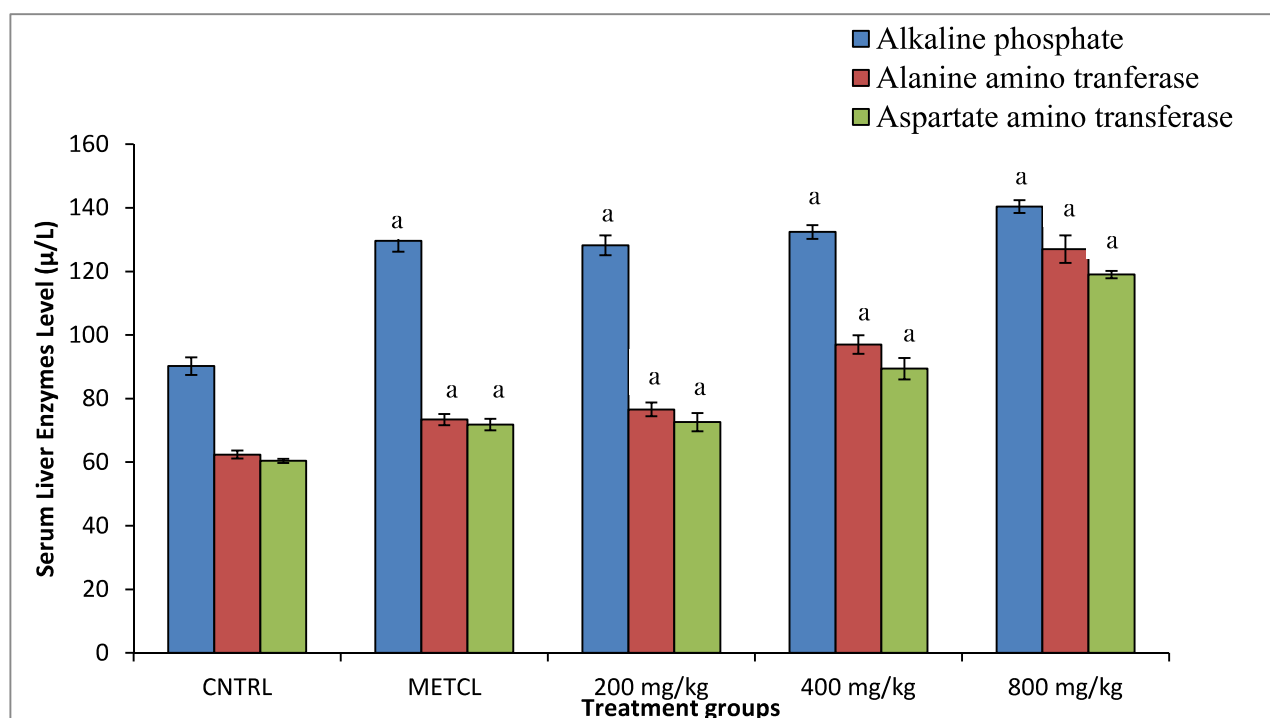


Figure 4: Effect of Metoclopramide and aqueous extract of *Momordica balsamina* L. (MBL) on ALP, ALT and AST levels in lactating Wistar rats. SEM with alphabetical superscript (a) indicate statistically significant difference ($p < 0.05$) compared to the control group.

Effect of Aqueous Extract of *Momordica balsamina* L. on the Histology of the Liver in Lactating Wistar rats

Photomicrograph sections of the liver following the administration of metoclopramide (5 mg/kg) and MBL at varying doses of 200 mg/kg, 400 mg/kg and 800 mg/kg, respectively. The control group showed normal central vein, hepatocellular integrity, kupfer

cells and sinusoidal dilations (Plate A). However, the metoclopramide treated group showed scanty microvascular fatty droplets (Plate B). The extract treated groups showed progressive hepatocellular damage with increasing dose of the extract: 200 mg/kg (Plate C), 400 mg/kg (Plate D) and 800 mg/kg (Plate E), respectively.

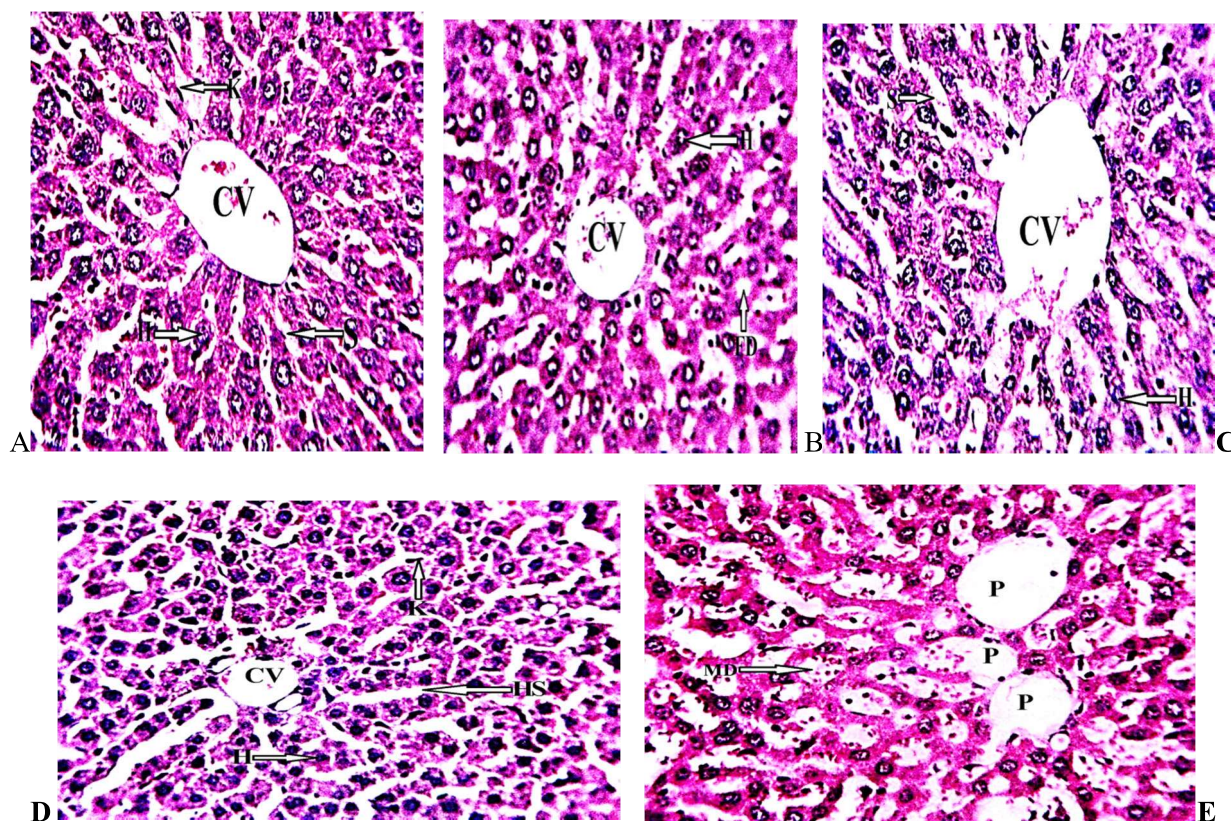


Plate I: Section of the Liver micrograph: **A.** Control Group showing normal Central Vein (CV), Hepatocyte (H), Kupffer Cells (K) and Sinusoid (S). **B.** Metoclopramide (5mg/kg) showing normal Central Vein (CV), Hepatocyte (H), with scanty microvascular fatty droplet. **C.** MBL (200 mg/kg) showing normal Central Vein (CV), Hepatocyte (H) similar with that of the control group with some dilated sinusoid (HS). **D.** MBL (400 mg/kg) showing normal Central Vein (CV), Hepatocyte (H) similar with that of the control group with some dilated sinusoid (HS). **E.** MBL (800 mg/kg) showing the Portal Triad (P), Macrovascular fatty droplet (MD) and bi-nucleated hepatocyte. (H and E Stain, X250).

Discussion

Milk yield was significantly ($p < 0.05$) higher in the *M. balsamina* L. group than in the controls. In addition, Daily milk yield was highest on day 9 of 400 mg/kg of MBL treated group. The MBL extract takes a longer duration to exert its effect when compared to metoclopramide at day 7. Similarly, daily milk yield was found to be higher in MBL 200 mg/kg and 800 mg/kg treated groups

when compared to control. The aqueous fraction of *M. balsamina* L. produced an appreciable increase in oxytocin level when compared to the control and metoclopramide treated groups. The extract exhibited a lactogenic activity by increasing the oxytocin level at medium dose of 400 mg/kg in lactating rats. The presence of cardiac glycosides, flavonoids and saponins in the aqueous extract of *M. balsamina* L. might be responsible for the lactogenic effect. This is in conformity with the findings of [15, 16, 17] who identified the lactogenic activity of various plant extract based on their antioxidant capacity. Flavonoids and saponins aid in the inhibition of xanthine oxidase in formation of superoxides or carry out direct scavenging of reactive oxygen species leading to maximal function of the body systems, therefore an enhancement in lactation.

The mechanism through which *M. balsamina* L. exerted its effect might be by increasing the synthesis of oxytocin in magnocellular neurosecretory cells in the supraoptic nucleus and paraventricular nucleus of the

hypothalamus ^[18]. The oxytocin expression was found not to be dose dependent. However despite the significant increase in the oxytocin level at 800 mg/kg, the milk yield was higher at 400 mg/kg. In this study the extract has no activity at higher doses. The aqueous extract of *M. balsamina* L. showed a potential for hepatotoxicity at higher doses in this study. It increases serum liver enzymes, which is indicative of liver damage ^[19, 20]. This finding is in harmony with earlier researches where it was reported that some herbal medicinal products like *Camellia sinensis* (Green tea extract) are associated with a spectrum of hepatotoxicity events. Increased activity of liver enzymes are suggestive of the degree and type of liver damage ^[21].

The hepatotoxic effect of the MBL was dose dependent with higher liver enzymes level at 800 mg/kg. The effect of MBL on liver damage could be due to its bitter constituents, as it contains momordicin ^[22]. Its major cytotoxic mechanisms may occur via destruction of mitochondrial membranes and the induction of reactive oxygen species formation. This could eventually lead to destruction of hepatocytes cell membranes and subsequent release of liver enzymes into the blood circulation ^[21]. This observation agrees with the findings of ^[5] who reported that the leaves and fruits of MBL extract, produce hepatotoxicity and also induce toxicity in various organs and tissues in

Wistar rats at high doses. However, it is contrary to the findings of ^[23], who showed that extract of MBL have protective effects for both biochemical markers and histological appearance of hepatocytes. The aqueous extract of *M. balsamina* L. showed progressive hepatocellular damage with increase in dose at 200 mg/kg, 400 mg/kg and 800 mg/kg, respectively. The group treated with 400 mg/kg of MBL showed sinusoidal dilatations, whereas the 800 mg/kg MBL treated group showed higher number of sinusoidal dilatations, macrovascular fatty droplets (MD) and binucleated hepatocytes.

Conclusion

It can therefore be inferred that aqueous extract of *M. balsamina* L. stimulates increased milk yield, oxytocin level and liver enzymes activities with mild hepatotoxicity. It is therefore, suggested that the *M. balsamina* L. can be used as a galactagogue at moderately low doses and for a short periods to avoid its possible hepatotoxic effects

Acknowledgement

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Conflict of Interest

We declare there is no any conflict of interest in this work.

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Lactation Profile of Aqueous Extract of *Hibiscus sabdariffa* L. seed in Wistar Albino rats.

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Abstract

With the potential safety challenges and nutritional defects in infant formula, mother's milk is still the best nourishment for infant's survival and growth. In Nigeria, a decoction of *Hibiscus sabdariffa* L. seed is taken to enhance lactation in cases of poor milk production and maternal mortality. The study is designed to evaluate the effect of seed extract of *H. sabdariffa* on milk yield, prolactin level and toxicity. The lactating rats were grouped into seven groups of five rats (n=5), Group I received 1ml/kg normal saline, Group II received metoclopramide (5 mg/kg), Group III, IV, V & VI were administered the extract of *H. sabdariffa* seed (200, 400, 800 and 1600 mg/kg) respectively and Group VII received extract (200 mg/kg) + dopamine (5 µg/kg). All groups received the extract and drug orally for ten days from day 3 – 13 of lactation, except dopamine which was injected intraperitoneally. The measurement of milk production in rats was conducted through direct method by milk yield estimation of pup weight and weight gain. The result showed that acute toxicity LD₅₀ of *H. sabdariffa* seed extract in rats was estimated to be 5000 mg/kg while Sub-chronic toxicity studies conducted on liver enzymes and kidney metabolites were shown to be within the accepted values. The milk yield and prolactin level of the extract-treated rats showed significant increase (P<0.05) when compared to control. *H. sabdariffa* seed extract possess lactogenic activity by enhancing milk yield and serum prolactin level through dopaminergic receptors as dopamine antagonist.

Keywords: Dopamine, *Hibiscus sabdariffa*, Lactation, Prolactin, Milk yield.

Introduction

Hibiscus sabdariffa linn (Malvaceae) is a herb that is cultivated for leaf, fleshy calyx, seed or fiber.^[1] It is a medicinal herb grown in all parts of the world and taken as common local drink popularly known as *zobo* in Nigeria. *H. sabdariffa* is reported to have anti-hypertension effect.^[2,3] *Hibiscus* anthocyanin, a group of phenolic natural pigments present in the dried flower of *Hibiscus sabdariffa* and *Hibiscus rosasinensis* have been found to have cardioprotective,^[4] hypocholesterolemic,^[5] anti-oxidative and hepatoprotective^[2] effects in animals. Delphinidin 3-sambubioside, a *Hibiscus* anthocyanin induces apoptosis in human leukemia cells through oxygen reactive species-mediated mitochondrial pathway.^[6] Polysaccharides from *H. sabdariffa* flowers stimulate proliferation and differentiation of human keratinocytes.^[7] *Hibiscus* protocathechuic acid has inhibitory and inductive effect on tumour promotion in mouse skin and in human leukemia cells

respectively.^[8] *H. sabdariffa* has also been reported to be antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, diuretic, emollient, purgative, refrigerant, sedative, stomachic and tonic.^[4] The World Health Organization recommends that all infants worldwide be exclusively breastfeed for the first 6 months of life, with continued breastfeeding up to 2 years of age.^[9] However, more than half of mothers had an early weaning, the most important reason being hypogalactia.^[10] Lactating effect of herbs, seeds and micro-nutrients has been reported in many plants (*Asparagus racemosus*, fennel seed, Grape sap, milk thistle and goat's rue).^[11] In Nigeria, a decoction of the seed is given to enhance or induce lactation in cases of poor milk production, poor letdown and maternal mortality.^[12] The study is designed to evaluate the effect of aqueous extract of *H. sabdariffa* seed on milk yield, serum prolactin level and its long term effect in animal models.

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Materials and Methods

Chemicals and Drugs

Prolactin ELISA kit (ALPCO Diagnostics® Limited, 29-AH-R011 US) Metoclopramide (NAFDAC Reg. no. 04-5946) and Dopamine (Aldrich Chemical Co.).

Extraction of Aqueous Extract of *Hibiscus sabdariffa* L. Seed

H. sabdariffa seed were washed thoroughly, shade dried and ground into coarse powder. Maceration method was used for aqueous extraction of the seed. One hundred grams (100 g) of the powdered *Hibiscus sabdariffa* L. seed were soaked and the mixtures were then continuously shaken for hours with mechanical shaker. The mixtures were further macerated and the supernatant liquid (extract) was filtered through a plug of cotton or glass wool. The process was repeated for complete extraction and the extract were then poured into evaporating dish to evaporate the solvent in the extract over the hot water bath at the temperature of 40°C – 45 °C which yielded (32%) yellowish golden extract weighing 31.25 g.

Phytochemical Analysis

The aqueous extract of *Hibiscus sabdariffa* L. seed was subjected to preliminary Phytochemical screening to identify the chemical constituents. The methods of analysis employed were those described by Brain and Turner.^[13]

Experimental Animals

Adult Wistar Albino rats (180 – 240 g) of either sex were used for the acute toxicity studies while male rats were used for the sub-chronic toxicity profiling. The animals were obtained from the Animal House of the Department of Human Physiology, Faculty of Basic Medical Sciences, Ahmadu Bello University Zaria, Nigeria. They were fed with standard feeds (Vital Feeds, Jos, Nigeria) and had free access to tap water *ad libitum*. They were also maintained under standard conditions of humidity, temperature and approximate 12 hr light/darkness cycle. The animals were acclimatized for a week before the commencement of the study. A standard protocol was drawn up in accordance with Ahmadu Bello University, Zaria, Nigeria and the Good Laboratory Practice (GLP) Regulations of the WHO,^[14] “Principles of Laboratory Animal Care”.

Experimental Design

Thirty five female albino rats were obtained from the Animal House of Department of Human Physiology, Ahmadu Bello University, Zaria. Following birth, the litters' weights were recorded and culled to 6 litters per dam. The thirty five lactating rats were randomly divided into four main groups (control, metoclopramide-treated, extract-treated and extract plus dopamine-treated groups). Control, metoclopramide-treated and extract plus dopamine-treated groups consisted of five rats each (n=5), while the extract-treated group was sub-divided into four sub-groups of five rats each (n=5). Accordingly, the extract was administered in different doses: 200, 400, 800 & 1600 mg/kg respectively. All groups had received the extract and the drug for 10 days from day 3 to day 13 of lactation.^[15] The extract and the drugs were administered orally except for dopamine which was injected intraperitoneally. The animals were then euthanized on day 14 and heart blood samples were analysed using prolactin (ELISA) kit.

Evaluation of Milk Yield of Aqueous Extract of *Hibiscus sabdariffa* L. Seed

All the animals were treated daily, starting from day 3 of lactation. Metoclopramide and aqueous extract of *Hibiscus sabdariffa* L. were orally administered each day at 18:00 hr. Milk production was measured from day 4 to day 13 of lactation. Milk yield and body weight of dams and weight gain of pups were measured each day with an electronic balance (Mettler P3) accurate to 0.01 g. Milk production for Groups III & IV that were administered 200 and 400mg/kg of the extract *Hibiscus sabdariffa* L respectively was estimated 18 hr after gavages. The pups were weighed every day during the study period at 07:00 hrs (W_1), then isolated from their dams for a period of 4 hr ^[16, 17, 18]. At 11:00 hr, the pups were weighed (W_2), returned to their dams and allowed to feed for 1 hr, and their weight taken at 12:00hrs (W_3). Milk yield 18 hrs after the gavages was estimated as $W_3 - W_2$ with a correction for weight loss due to metabolic processes in the pups as $(W_2 - W_1)/4$.

Measurement of Prolactin

The animals were then euthanized on day 14, at the termination of the experiment; they were anaesthetized by chloroform inhalation in a closed chamber and thereafter sacrificed. Blood was aspirated and immediately centrifuged to obtain serum for the determination of prolactin. The analysis was conducted in the Department of Chemical Pathology Ahmadu Bello University Teaching Hospital, Shika, Zaria. The prolactin kit species specific enzyme-linked immunosorbent assay [(ELISA) kit ALPCO Diagnostics® Limited, 29-AH-R011 US] in micro-plate was designed for the quantitative evaluation of rat prolactin according to manufacturer's manual.

Data Analysis All data are expressed as mean $\pm$ standard of error mean (Mean $\pm$ S.E.M.). The data obtained were analysed using one way analysis of variance (ANOVA) and student-Newman Keul's test *post hoc* test for multiple comparisons. The ($P < 0.05$) was considered significant.

Results

Phytochemical Analysis

The preliminary Phytochemical analysis of the aqueous seed extract of *H. sabdariffa* revealed the presence of varying amount of alkaloids, saponins, tannins, anthraquinones, cardiac glycosides, cardenolides, flavonoids and phlobatanins in various concentrations as indicated in table 1.

Table 1: Phytochemical Analysis of aqueous extract of *Hibiscus sabdariffa* L. seed

Extract constituents	Concentrations
Alkaloids	++
Anthraquinones	+
Cardenolides	+
Cardiac glycosides	++
Deoxy sugar	+
Flavonoids	+
Phlobatannins	+
Saponins	+++
Steroidal ring	++
Tannins	+

+++ = High concentration was recorded if a definite heavy precipitate or flocculation observed;

++ = Moderate concentration was recorded if a definite turbidity, but no flocculation observed.

+ = Low concentration was recorded if the reagent produced only a slight opaqueness.

- = Not detected ^[19].

Acute and Sub-chronic Toxicity Studies

The acute toxicity LD₅₀ of *H. sabdariffa* seed extract in albino rats was found to be above 5000 mg/kg according to the method of Lorke.^[20] The plant is characterised by a very low degree of toxicity. In sub-chronic toxicity of *Hibiscus sabdariffa* L. the index of chronic studies was done by accessing the liver enzymes and kidney metabolites as indicators for liver and renal function tests.^[21] In liver function tests

the mean values of the following enzymes Aspartate aminotransferase (AST; SGOT), Alanine aminotransferase (ALT; SGPT), Alkaline phosphatase, Bilirubin and Albumin was within the normal ranges as indicated in table 2. Also, the levels of urea and creatinine, as indicators for renal function were within the accepted values as shown in table 3.

Table 2: Effect of aqueous extract of *Hibiscus sabdariffa* L. seed on Liver enzyme in lactating Wistar Albino rats

Treatment (mg/kg)	ALT (SGPT) (IU/L)	AST (SGOT) (IU/L)	ALK (IU/L)	BIL (IU/L)	ALB (IU/L)
Normal range Enzymes ^[22]	16 – 40	6 – 25	21 – 92	4 – 17	30 – 52
Normal saline 1ml/kg	28.25±1.7	23.50±2.2	61.5±3.8	7.2±1.1	41.25±2.7
Aq. Ext. HSL.					
10	24.75±0.8*	21.00±0.9	65.5±3.3	8.5±2.1	42.0±2.6
100	23.75±0.7*	23.00±1.4	63.5±3.8	8.6±2.2	40.75±2.3
500	23.00±0.9*	21.25±1.4	68.5±4.1*	9.1±1.2	42.02±1.8
1000	35.75±1.4*	22.00±0.9	76.5±3.5*	9.5±1.5	41.25±2.2

* = 0.05; HSL = *Hibiscus sabdariffa* L.**Table 3:** Effect of aqueous extract of *Hibiscus sabdariffa* L. seed on Urea and Creatinine in lactating Wistar Albino rats

Treatment (mg/kg)	Urea (IU/L)	Creatinine (IU/L)
Normal range Enzymes ^[22]	2.5 – 6.5	9 – 126
Normal saline 1ml/kg	4.33±0.2	65.25±2.5
Aq. Extract HSL		
10	4.10±0.4	62.25±2.9
100	4.85±0.5	75.75±2.1*
500	4.98±0.2	82.00±4.3*
1000	3.75±0.2	71.00±4.5*

* = 0.05; HSL = *Hibiscus sabdariffa* L.**Milk Production**

Milk production of both groups receiving 200 and 400 mg/kg of aqueous extract of *H. sabdariffa* seed was significantly ($P<0.05$) higher than that of the control group figure 1 (a). Milk yield increased from 1.01±0.2, 1.22±0.3 and 1.14±0.2 g/pup/per day to about 1.91±0.1, 2.78±0.3 and 2.44±0.2 g/pup per day for the control and aqueous extract of *Hibiscus sabdariffa* L. group respectively.

The significant ($p<0.05$) differences observed for 200 mg/kg started from day 2 until the end of treatment day 10. The significant ($p<0.05$)

difference observed for 400 mg/kg begins from day 5 to the end of treatment day 10. Milk yield increased from 1.01±0.2, 0.82±0.3 g/pup/day to 1.91±0.1, 2.6±0.4 g/pup/day for the control and metoclopramide group respectively. It recorded a significant ($p<0.05$) difference from day 3 to the end of treatment day 10. The mean milk yield was 1.53±0.4, 2.32±0.3 and 1.84±0.4 g/pup per day throughout the experimental period respectively figure 1 (b).

Evaluation of Prolactin Level

The result obtained in this study for ten (10) days showed that the seed extract of *H. sabdariffa* have increased serum prolactin level significantly ($P < 0.05$) in lactating albino rats. The various concentrations of the extract (200, 400, 800 & 1600mg/kg) reported marked ($p < 0.05$) increase in prolactin level (43.52 ± 0.2 , 45.76 ± 0.3 , 50.56 ± 0.3 & 53.12 ± 0.2 ng/mL) as compared to the control group (38.72 ± 0.2 ng/mL). It is noticed that the increase is dose-dependent table 4. With regard to metoclopramide effect, it showed an appreciable ($P < 0.05$) increase in serum prolactin level (54.98 ± 0.3 ng/mL) when compared to control group

(38.72 ± 0.2 ng/mL) table 4. Prolactin level (53.12 ± 0.2 ng/mL) of the highest dose of aqueous extract *H. sabdariffa* when compared to prolactin level (54.98 ± 0.3 ng/mL) of metoclopramide showed no significance difference ($p > 0.05$).

This implies that the potency of the extract at the highest dose is almost the same with metoclopramide. The dose 200 mg/kg of aqueous extract *Hibiscus sabdariffa* L. was observed to have the highest milk yield. When 5 μ g/mL of dopamine was added to 200 mg/kg of aqueous extract *H. sabdariffa* L., it completely inhibited the effect of the extract as shown in figure 2.

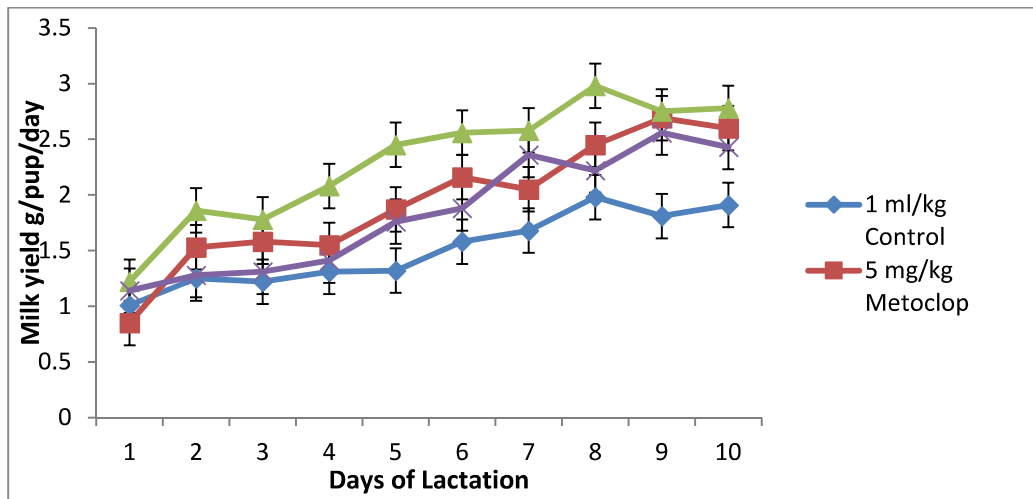


Figure 1a: Effect of aqueous extract of *Hibiscus sabdariffa* l. on milk production 18 hours after administration.

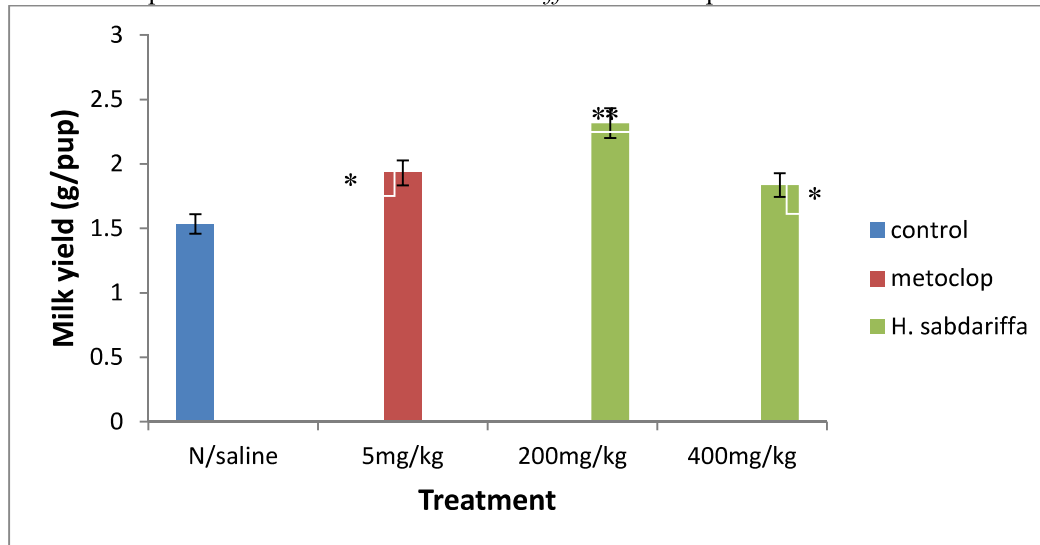


Figure 1b: Mean milk production per day for the effect of aqueous extract of *Hibiscus sabdariffa* L.

Table 4: Effect of aqueous extract of *Hibiscus sabdariffa* L. seed on serum prolactin levels in lactating Wistar Albino rats

Treatment (mg/kg)	Prolactin (ng/ml)
Normal saline	
1ml/kg	38.72±0.2
Metoclopramide	
5	54.98±0.4*
Aq. extract of HSL.	
200	43.52±0.3*
400	45.76±0.2**
800	50.56±0.4**
1600	53.12±0.2**

* = 0.05; ** = P<0.01; HSL = *Hibiscus sabdariffa* L.

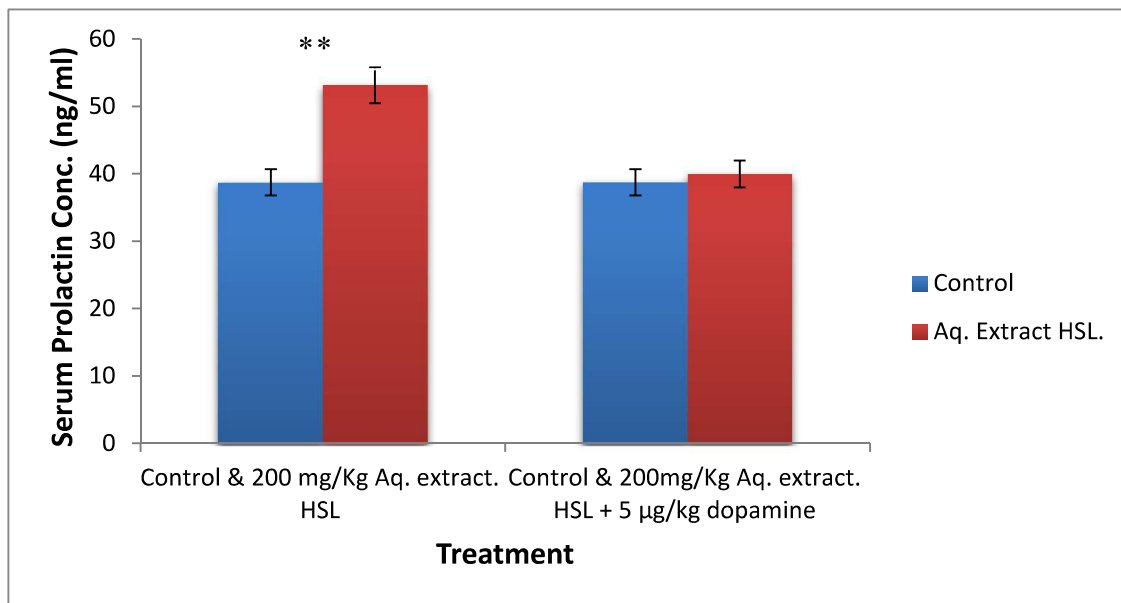


Figure 2: Effect of dopamine and aqueous extract of *Hibiscus sabdariffa* L. seed on serum prolactin level in lactating Wistar albino rats.

Discussion

Safeguarding the quality and quantity of breast milk positively, impacts the newborn with good outcome and development. The result of the present study showed that, the aqueous seed extract of *Hibiscus sabdariffa* produced an appreciable increase in serum prolactin level when compared to the control in a dose-dependent manner. The diverse effects of prolactin on the mammary gland include growth and development of the mammary gland, synthesis of milk and maintenance of milk secretion.^[23, 24] The seed extract of *H. sabdariffa* exhibited a lactogenic

activity by increasing the serum prolactin level and milk yield in lactating rats. The lactogenic activity displayed by the 200 mg/kg of aqueous seed extract *H. sabdariffa* was observed to have the highest milk yield, because from day 2 – 10 of lactation, milk yield was significantly increased 18 hours after gavages. The mean milk production per dam per day 2.3 g/pups/day (2.39 ml) was very significant as compared to control 1.51 g/pups/day (1.56 ml). In the dose of 400 mg/kg of extract, significant increase in milk yield started from day 5 – 10 of lactation. With

the potential security challenges and nutritional defects in powdered infant formula, breastfeeding is more beneficial for the newborn's survival.^[10] Prolactin is best known for the multiple effects it exerts on the mammary gland^[24] however, the activity seems to be higher at low dose. Human breast milk is widely accepted to be the optimal source of nutrition for the newborn infant, containing all the proteins, lipids, carbohydrates, micronutrients and trace elements required for growth, development and immune protection.^[25] It has been reported that some herbs affect milk production either by stimulating prolactin or mammary gland development. (*Asparagus racemosus*, *Acacia nilotica*, *Gunnera perpensa*, *Octopus vulgaris* and *Carica papaya*).^[16, 24, 26, 27, 28] The presence of steroidal saponin and sapogenin constituents may contribute in the lactogenic effect of aqueous seed extract *H. sabdariffa* ^[12, 26, 27, 28] The mechanism through which *H. sabdariffa* exerted its effect might be through dopaminergic influence, as dopamine receptor antagonist, since dopamine blocked the effect of the extract. The acute toxicity LD₅₀ of *Hibiscus sabdariffa* L. seed extract in albino rats was found to be above 5000 mg/kg according to the method of Lorke.^[20] Sub-chronic toxicity studies of *H.*

sabdariffa conducted on liver enzymes and kidney metabolites as indicators for liver and renal function tests showed liver enzymes and kidney metabolites to be within the accepted values when compared to control. Thus, it can be concluded from this study that the aqueous seed extract of *H. sabdariffa* is safe even at a dose of 1.6 g/kg and it is found to possess lactogenic activity by enhancing milk yield and prolactin level through dopaminergic receptors as dopamine antagonist.

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Impairment in Renal Function among Petrol Vendors in Kano, North-Western-Nigeria.

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Abstract

Petrol hawkers and pump attendants are exposed to petrol fumes, vehicular exhausts and other ambient roadside pollutants which have both been reported to cause significant human cardiopulmonary morbidity, endocrine and metabolic dysfunctions as well as renal dysfunction among animals. There is a dearth of information defining the nephrotoxic tendencies of petroleum exposure among humans. This study assessed some serum biochemical markers of renal function and a urinary marker of renal dysfunction among petroleum hawkers and pump attendants. Data was analysed using SPSS version 20.0, mean of variables were compared using one-way analysis of variance and independent sample t-test, where applicable, with significance level set at $p < 0.05$. The results show a significant increase in serum urea, creatinine and potassium with significantly lower serum levels of sodium, chloride and bicarbonate among hawkers when compared to controls. Equally, they were found to have significantly higher serum levels of urea, creatinine and potassium but lower levels of serum chloride and bicarbonate than a cohort of pump attendants. On the other hand, pump attendants were found to have significantly higher serum levels of urea, creatinine and potassium and significantly lower serum levels of sodium and chloride relative to the controls. For both exposure groups, derangement of serum analytes was more pronounced among those with long term exposure. Also, proteinuria was much higher among petrol hawkers (54%) and pump attendants (38%) as compared to the controls (12%). Our finding suggests that exposure to petroleum predisposes to renal dysfunction which could possibly be *accentuated* by road-side pollution.

Keywords: Renal impairment, Renal function, Petrol vendors, Petroleum exposure

Introduction

Petrol is a complex mixture of low molecular mass volatile organic compounds (VOCs) obtained from the fractional distillation of crude oil which is formed by the effect of heat and pressure on the remains of tiny sea plants and animals that have decomposed over millions of years beneath thousands of kilometres of sand.^[1] It comprise about 60-70% alkanes, 6-9% alkenes and 25-30% aromatics.^[2] In Nigeria, 6-8% is the regulated limit for benzene as against the 1-5% standard in Europe and the USA.^[3] One (1) parts per million (ppm) is the weighted average regulations limit for an 8 hour work day exposure for benzene.^[4] However, between 20 and 200 ppm has been reported near petrol pumps during vehicular refueling.^[5]

Petrol hawkers and pump attendants, while on duty, are in constant exposure to petrol fumes, and other

toxic compounds and particles from vehicular exhaust and ambient roadside pollutants.^[6] These particles are smaller in diameter with consequent ability to remain airborne and has large surface area that allows for carriage of toxic compounds and metals deeper into the lungs.^[7]

Following exposure, the fumes are rapidly absorbed and widely distributed throughout the body^[3] where the benzene component-which is the most important component of the vapor-undergoes primary metabolism in the liver to a variety of hydroxylated and ring-opened products which undergo further metabolism in various tissues leading to the generation of reactive oxygen species (ROS), cytotoxicity,^[8] inflammatory responses,^[5] long term immune,^[9] metabolic and endocrine dysfunctions.^[10] Despite these reported systemic dysfunctions and a

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good deal of epidemiological and experimental evidence implicating hydrocarbon exposure in inducing glomerulonephritis and/or worsening its course in experimental animals as well as humans, there is still a dearth of information defining the nephrotoxic tendencies of petroleum exposure among petrol hawkers and pump attendants. Renal dysfunction, although not uncommonly asymptomatic,^[11] may be characterised by abnormal serum urea, creatinine, electrolytes and/or proteinuria.^[11,12] We therefore aimed to evaluate the effects of petroleum exposure on some serum markers of renal function, urinary marker of renal dysfunction and to also examine the relative effect of duration and type of job on possible exposure-related-outcomes on renal function among petroleum hawkers and petrol pump attendants.

Materials and Methods

This retrospective cohort study was conducted in Kano metropolis after obtaining ethical clearance from Kano State Ministry of Health's Research and Ethics Committee (Ref: MOH/Qff/797/T.I./39). One hundred and fifty (150) participants (age, sex, weight and height matched) were divided into three equal groups. Group one comprises of petrol hawkers while groups two and three were petrol pump attendants and bankers respectively. A multistage sampling technique was employed to generate the required sample size from along the five major roads (Gwarzo Road, Zaria Road, Maiduguri Road, Hadejia Road and Katsina Road) linking Kano metropolis with neighboring states. Informed Consent was obtained from each of the study participants after a brief explanation of the intent, content and extent of the research and attending to

their fears and questions. Brief medical history and general physical examinations were conducted on each target subject. Those who spent less than one year in the business, are less than 18 years of age or more than 40 years old, and those found to have an eventful medical history or physical examination findings were excluded from the study. Recruited subjects were handed over a clean sample bottle for next day's early morning urine sample collection after a brief explanation on how to do so. From a peripheral vein in the cubital fossa, five millilitres (5 ml) of blood were drawn from the participants for immediate centrifugation to obtain a serum which was used for the estimation of analytes.

Serum urea and creatinine were estimated using auto-analyzer based methods,^[13] whereas serum K⁺, Na⁺, HCO₃⁻ and Cl⁻ were determined using appropriate diagnostic kits.^[11] Semi-quantitative method was used to estimate proteinuria using urinary dipstix while weight and height of subjects were assessed using a weighing scale and stadiometer respectively.

Data was analysed using the statistical package for social sciences (SPSS) version 20.0, summarised using percentages and mean $\pm$ standard error of mean and was presented in tables. One-way analysis of variance (one-way ANOVA) and independent sample student t-test were used to compare means of groups for statistical associations at 95% confidence interval. Bonferonni multiple comparison test was used to determine significance between and within groups while Pearson's chi-square test was used to ascertain statistical associations between categorical variables.

Results and Discussion

Table 1: Serum renal function markers among petrol vendors

Variables	F- Value	Petrol Hawkers	Pump Attendants	Controls
Urea	F=65.6	11.00 $\pm$ 0.84 ^a	5.35 $\pm$ 0.17 ^{a,b}	3.22 $\pm$ 0.10 ^{a,b}
Creatinine	F=181.2	105.04 $\pm$ 3.71 ^c	58.22 $\pm$ 2.20 ^{c,d}	36.76 $\pm$ 1.24 ^{c,d}
Potassium	F=55.5	4.71 $\pm$ 0.14 ^e	3.97 $\pm$ 0.08 ^{e,f}	3.18 $\pm$ 0.07 ^{e,f}
Bicarbonate	F=18.1	20.96 $\pm$ 0.37 ^g	22.98 $\pm$ 0.39 ^{g,h}	24.30 $\pm$ 0.43 ^g
Sodium	F=11.4	132.34 $\pm$ 0.65 ⁱ	133.62 $\pm$ 0.47 ^j	136.10 $\pm$ 0.57 ^{i,j}
Chloride	F=25.8	91.72 $\pm$ 0.94 ^k	95.58 $\pm$ 0.49 ^{k,l}	98.32 $\pm$ 0.40 ^{k,l}

Variables are in mmol/l; values are presented as mean $\pm$ SEM. Values within the same row bearing similar superscripts are statistically significant (p<0.05).

Table 2: Burden of proteinuria among petrol vendors.

Proteinuria	Petrol hawkers	Pump attendants	Controls	Total
Present O (E)	27 (17.3) 54%, SR=2.3	19 (17.3) 38%, SR=0.4	6 (17.3) 12%, SR= -2.7	52(52)34.7%
Absent O (E)	23 (32.7)	31 (32.7)	44 (32.7)	98(98)
Total O (E)	50 (50.0)	50 (50.0)	50 (50.0)	150

O=observed counts, E=expected counts, SR=standardized residual

X²=19.839, P<0.001**Table 3:** Serum renal function parameters among petrol hawkers in relation to their duration of exposure.

Variables	1-5 years exposure	>5 years exposure	T-Value	p-Value
Urea	6.06±0.34	14.58±0.98	-8.18	0.000
Creatinine	82.19±2.95	121.59±3.72	-8.30	0.000
Potassium	3.91±0.13	5.30±0.14	-7.28	0.000
Bicarbonate	22.76±0.52	19.66±0.36	4.91	0.000
Sodium	133.38±0.66	131.59±0.99	1.50	0.140
Chloride	95.57±0.86	88.93±1.27	4.32	0.000

Variables are in mmol/l; values are presented as mean±SEM.

Table 4: Serum renal function parameters among petrol pump attendants in relation to their duration of exposure.

Variables	1-5 years exposure	>5 years exposure	T-Value	p-Value
Urea	4.39±0.18	5.69±0.19	-5.02	0.000
Creatinine	47.92±2.86	61.84±2.56	-3.62	0.001
Potassium	3.54±0.15	4.12±0.09	-3.36	0.003
Bicarbonate	25.77±0.99	22.00±0.25	3.70	0.003
Sodium	136.54±1.06	132.59±0.40	3.48	0.003
Chloride	99.54±0.79	94.19±0.40	6.05	0.000

Variables are in mmol/l; values are presented as mean±SEM.

From Table 1, it is observable that both petrol hawkers and pump attendants have a significantly higher serum levels of urea, creatinine and potassium as well as lower levels of sodium and chloride compared to controls. On the other hand, petrol hawkers show a significant increase in serum urea, creatinine and potassium with lower serum levels of bicarbonate and chloride compared to pump attendants signifying more adverse outcome of exposure among petrol hawkers than pump attendants. Similar profile could also be found for proteinuria (Table-2). Even though a point prevalence of 12% proteinuria is recorded among the controls, a statistically significant (X²=19.84; p<0.001) proteinuria was found to associate more with petroleum hawkers than pump attendants with point prevalence and standardized residuals of 54%,

38% and SR=2.3, SR=0.4 respectively, signifying a relationship between exposure to petroleum and renal dysfunction.

Renal dysfunction of any kind, affects all parts of the nephron, however, the net biochemical features would depend on the proportion of glomeruli to tubules affected and on the number of nephrons involved.^[14] The reported higher serum levels of urea, creatinine and potassium as well as the lower serum level of bicarbonate among petrol hawkers and pump attendants compared to controls demonstrate a dominance of glomerular damage over renal tubular dysfunction. This is possibly because petrol may cause oxidative and cell mediated glomerular cell injury associated with cytokine driven proliferation of parietal epithelial

cells, increase in post glomerular resistance and decrease in glomerular filtration rate (GFR).^[15,16] This will result in retention and thus elevated serum levels of urea and creatinine because the rate of filtration had failed to balance that of production.^[14] Similarly, the reduced GFR leads to reduced sodium delivery to the distal nephron where it is exchanged for hydrogen and potassium ions. Consequently, serum bicarbonate levels will decrease due to reduced reclamation as a result of reduced secretion of hydrogen in exchange with sodium. Also, reduction in sodium delivery to the distal nephron results in decreased potassium secretion, and hence hyperkalaemia. Similarly, the decreased delivery of sodium to the macula densa activates the renin-angiotensin-aldosterone system which although increases distal tubular reabsorption of sodium-via the aldosterone mechanism-but causes increased antidiuretic hormone (ADH) secretion which dilutes the plasma and reduces plasma sodium concentration, hence the observed hyponatraemia among the petroleum exposed groups.^[14]

On the other hand, the higher burden of proteinuria among petrol hawkers and pump attendants may be a pointer towards proximal tubular dysfunction. This is because; although Petrol may cause oxidative and cell mediated cytoskeletal injury of foot processes, loss of size and disruption of charge barriers of the glomerular basement membrane which causes loss of proteins into the bowman's space,^[15] proximal tubular reabsorption of low-molecular-weight proteins may counter-balance the glomerular protein loss. Therefore, depending on degree of the glomerular injury, proteinuria may occur due to failure in reabsorption of the low-molecular-weight proteins (such as α_1 microglobulin and retinol binding protein), leakage of enzymes and proteins such as *N*-acetyl- β -D-glucosaminidase (NAG) from an injured proximal tubular cell.

From Tables 3 and 4, both petrol hawkers and pump attendants, show a time dependent nephrotoxic manifestations signifying the progressive nature of renal damage associated with petroleum exposure. This could possibly be due to an increasing number of nephrons damaged by continued petroleum exposure. Also, the nephrotoxic manifestations were more apparent among petroleum hawkers than a cohort of pump attendants possibly because petrol hawkers could be more exposed to the ambient

roadside pollutants which have been implicated in cumulative adverse health effects.^[17] Also petrol hawkers' attitudinal use of an oral means to manually siphon fuel from larger stock containers while dispensing to motorist or smaller containers may cause accidental swallowing that could increase exposure load and hence more consequences than their pump attendants counterpart.^[6]

Our overall finding of renal dysfunction (dyselectrolytaemia, uremia, raised serum creatinine and proteinuria) among petroleum exposed individuals is in consonant with findings by other researchers. For example, Mahmood *et al.*,^[13] reported a significant increase in serum creatinine and urea levels among gasoline workers in Sulaimani city, Kurdistan. Our observed time dependent nephrotoxic manifestations among both petrol hawkers and pump attendants corroborates the findings of Ogunneye *et al.*,^[18] who also reported an increase in serum levels of urea and creatinine among pump attendants in Ibadan in a time dependent manner. Similarly, Nwanjo *et al.*^[19] reported increased serum levels of urea and creatinine as well as proteinuria among petrol pump attendants in Owerri city, however, the findings were significant only among those exposed to petroleum for 6 – 10 years. Our findings of renal dysfunction among those exposed to petrol for lower than six (6) years may be due to compounding effects of roadside pollutants that were reported to be over 8% higher in northern cities than cities in the south.^[20] In addition, the higher prevalence of risk factors for kidney diseases in our study area as highlighted by Nalado, *et al.* ^[21] could have played a significant additive role to the early nephrotoxic features observed in our study.

In conclusion, our finding shows that petrol hawkers and pump attendants in Kano metropolis have biochemical evidence of impaired renal function (increased serum levels of creatinine, derangement of serum electrolytes, uremia and proteinuria), which is more pronounced among petrol hawkers and those with longer term exposure. To safeguard the health of these categories of workers, a massive awareness campaign should be launched by the relevant stakeholders as it may go a long way to promote positive attitudes toward the use of personal protective equipments. Also, enforcement (by regulatory agencies) of pre-employment and

periodic medical checkups will promote early identification and referral of affected petrol vendors in order to stop mild systemic impairment from progressing to chronic or end-organ damage. In the same vein, creation of more youth empowerment

programs, employment opportunities; improved social amenities such as electricity and enforcement of regulations/guidelines will be of importance in reducing the menace of roadside petroleum hawking.

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Effects of Methanolic Leaf extract of *Vitex simplicifolia* on Antioxidant Vitamins, SOD, Liver glycogen content and Lipid Profile in Alloxan induced-diabetic Wistar rats

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Abstract

The effects of oral administration of methanolic leaf extract of *Vitex simplicifolia* on vitamins A, E and C, SOD and lipid profile levels in Alloxan induced diabetic Wistar rats were investigated. The study was conducted with 30 Wistar rats, assigned into six groups of five rats each, and daily administration of aqueous leaf extract of *Vitex simplicifolia* for 21 days was done. Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection (i.p) of 150 mg/kg body weight of Alloxan monohydrate. Group 1 was the normal control group, 2 was the diabetic control, group 3 was administered 10mg/kg of glibenclimide (positive control) and groups 4, 5, and 6 were administered 250, 500 and 1000mg/kg body weight of aqueous leaf extracts of *Vitex simplicifolia* respectively. There were no significant changes ($p < 0.01$) in the levels of total cholesterol (TC), triglyceride (TAG), low density lipoprotein - cholesterol (LDL-C), high density lipoprotein - cholesterol (HDL-C), SOD, vitamins A, E and C for the animals administered with the extract compared with the control groups. The result of this study suggests that methanolic leaf extract of *Vitex simplicifolia* has no antioxidant and hypolipidemic effect.

Keywords: *Vitex simplicifolia*, SOD, Lipid profile, Antioxidant vitamins

Introduction

Diabetes is a major degenerative disease in the world today, affecting at least 150 million people and having complications which include hypertension, atherosclerosis and microcirculatory disorders.^[1] The prevalence of diabetes is on the increase globally and in African communities due to the ageing of the population and drastic lifestyle changes accompanying urbanisation and westernisation.^[2] Also, studies from five West African communities in Nigeria and Ghana have identified genes within populations that create susceptibility to diabetes.^[3] The prevalence of diabetes mellitus in Nigeria is 1.9% with more than 1.5 million cases.^[4] Hence, it represents a growing burden on health care systems of African countries, most of which already face difficult economic conditions. The disease remains incurable and can only be controlled with drugs; hence, a scrupulous control is needed to help reduce

hyperglycaemia and the risk of long-term complications, which are known to be the major causes of morbidity and mortality.^[3]

Oxidative stress plays a pivotal role in cellular injury from hyperglycemia. High glucose level can stimulate free radical production. Weak defence system of the body becomes unable to counteract the enhanced reactive oxygen species (ROS) generation and as a result, a condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress.^[5] A certain amount of oxidative stress/ROS is necessary for the normal metabolic processes since ROS play various regulatory roles in cells.^[6] Due to oxidative stress the metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as

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well as in the myocardium.^[7] Oxidative stress acts as mediator of insulin resistance and its progression to glucose intolerance and in the development of diabetes mellitus, subsequently favouring the appearance of atherosclerotic complications, and contributes to rise in many micro- and macrovascular complications.^[8]

An antioxidant is a molecule that inhibits the oxidation of other molecules. It terminates chain reactions by removing free radical intermediates, and inhibits other oxidation reactions. Antioxidant defense mechanisms involve both enzymatic and non enzymatic strategies. Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Other antioxidants include lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoid, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B₁, B₂, B₆, B₁₂). They work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydrogen peroxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation^[9] and inhibit Fenton and Haber-Weiss-type reactions; vitamins A and E scavenge free radicals.^[10] Extensive studies of pharmacological interventions based on biological antioxidants have been carried out.^[11] Discrepancies in observed biomarkers for oxidative stress continue to be seen in the many review, especially in the activities of SOD, catalase, and glutathione peroxidase in experimentally diabetic animals.

In Nigeria, reference available from the indigenous traditional healers indicated that a decoction of the chopped stem barks and leaf of *Vitex simplicifolia* is prepared and taken orally for treatment of diabetes and other disease conditions. The plant extracts have been used as medication for infertility, liver disease, anodyne, stiffness, hypertension, cancer, febrifuge, as tonic galactagogue to aid milk production in lactating mothers, sedative, digestive regulator and treatment of eye, kidney and as supplement for lack of vitamin A and B.^[12,13]

Although parts of this plant are used by traditional healers of various ailments, there is paucity of scientific reference to establish the scientific basis of its use and like many other herbal remedy, there is little or no information about its possible side effects or toxicities.

This study, therefore, was aimed at determining the hypolipidemic and antioxidant effects of methanolic leaf extract of *Vitex simplicifolia* on alloxan induced diabetic Wistar rats. This is important since science requires the validation of drugs by medicinal practitioners and drug regulatory authorities demand that all potential drugs should pass through a rigorous series of study and scrutiny.^[14]

Materials and Methods

Collection and Preparation of Plant Material

Fresh leaves of *Vitex simplicifolia* were collected from the botanical garden of Bayero University, Kano State, Nigeria, with the assistance of Herbarium keeper. The leaves were authenticated in the Department of Biological Sciences, Bayero University Kano (Herbarium number 242). The leaves were dried under room temperature and then grinded using pestle and mortar to a semi powdered form.

Experimental Animals

Thirty (30) adult Wistar rats weighing between 160 - 240g were obtained from the Animal House of Department of Physiology, Bayero University, Kano and kept in cages at a room temperature for two (2) weeks to acclimatize and allowed access to food and water *ad libitum*.

Plant Extract

Vitex simplicifolia leaves (500g) were soaked in 2.5 litres of methanol at room temperature in a conical flask. The content of the flasks were shaken and the top was covered with aluminium foil and kept at room temperature for 48hrs after which the extracts were obtained by filtration using a Whatman No 1 filter paper. The extracts were concentrated using vacuum evaporator.

Acute Toxicity Studies and Determination of LD₅₀

The lethal dose was determined by Lorke's method.^[15]

Phase 1: Nine Wistar rats were used. They were divided into three groups of three animals each. Each groups of animals were administered different doses (10, 100, and 1000 mg/kg) of the extracts and then observed for 24 hrs to monitor their behaviour as well as mortality.

Phase 2: Three animals were used. They were divided into three groups of one animal each. The animals were administered higher doses (1600, 2900 and 5000 mg/kg of the extracts and observed for behaviour as well as mortality.^[15] Then LD₅₀ is calculated by the formula: $LD_{50} = \sqrt{(D_0 \times D_{100})}$ D₀ = Highest dose that gave no mortality, D₁₀₀ = Lowest dose that produce mortality.

Induction of Diabetes Mellitus

Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection (i.p) of 150 mg/kg body weight of Alloxan monohydrate.^[16] Hyperglycaemia was confirmed by the elevated blood glucose levels, determined after 72hrs and then confirmed after the 7th day of injection. The rats found with elevated glucose level of 400 mg/dl and above were used for the study.^[17]

Experimental Design

A total of thirty (30) Wistar rats were used for the study. The rats were divided into six (6) groups of five (5) each. Extracts were administered orally using 1ml syringe. Group 1 - Normal untreated rats (Animal control) Group 2 – Diabetic untreated rats (Diabetic control) Group 3 – Diabetic rats administered 10 mg/kg of Glibenclamide^[18] Groups 4,5 and 6 - Diabetic rats administered 250, 500 and 1000 mg/kg body weight of aqueous leaf extract of *Vitex Simplicifolia* respectively.

Sub-Chronic Studies/ Collection and Treatment of Samples

The extracts were reconstituted in distilled water, and administered orally on daily basis for 21 days. At the end of 21 days, the animals were anaesthetized using chloroform and bled by cardiac puncture 24 hrs after the last treatment. The blood samples were collected in specimen bottles, allowed clotting and the serum separated by centrifugation at 3000rpm for 10 minutes and then subjected to biochemical parameters analysis.

Biochemical Analysis

The serum levels of total cholesterol, triacylglycerol and HDL-C were determined by enzymatic method described by Stein,^[19] while the serum levels of LDL-C was measured according to protocol of Friedewald.^[20] Liver glycogen was determined using the destruction by Alkali method.^[21] Vitamins A,C and E were determine by the methods of Bessey, Roe and Kuether, and Fabianek^[22,23,24] respectively, while SOD was determine using the method of McCord and Fridorich^[25]

Phytochemical Screening

Test for anthraquinones was conducted using the method of Felgils^[26] and Test for alkaloids and Terpenes using the method of Sofowora^[12] while Test for saponins, flavonoids and tannins were done using the method of Earl^[27]

Statistical Analysis

The results obtained are presented as Mean± standard error of mean (SEM). A one way analysis of variance (ANOVA) was used for the data analysis. Significant differences between groups were detected in the ANOVA using Bonferini test at P values less than 0.05 and 0.001.Using SPSS 20 Soft ware package for windows.

Results

Table 1 and 2 below indicate the result of acute toxicity studies showing the LD₅₀ of aqueous extract of *Vitex simplicifolia* leaf is greater than 5000 mg/kg body weight.

Table 1: Phase I LD₅₀, (Oral) of the methanolic leaf extract of *Vitex simplicifolia*

Group	No. of Animals	Doses (g/Kg)	No. of Death
1	3	10	0
2	3	100	0
3	3	1000	0

Table 2: Phase II LD₅₀ (Oral) of the methanolic leaf extract of *Vitex simplicifolia*

Group	No. of Animals	Doses (mg/Kg)	No. of Death
1	1	1600	0
2	1	2900	0
3	1	5000	0

Table 3: Results of phytochemical screening of the methanolic extract of the *Vitex simplicifolia* leaf

Phytochemicals	Qualitative
Alkaloids	Present
Cardiac glycosides	Present
Phenols	Absent
Flavonoids	Present
Saponins	Present
Tannins	Present
Steroids	Absent
Terpenoids	Absent
Phytates	Present
Oxalates	Present
Cyanates	Present

Effects on Lipid Profile and Liver Glycogen Content

The effect of daily doses of methanolic leaf extract of *Vitex simplicifolia* on lipid profile of alloxan induced diabetics rats is presented in Table 5. There was no significant ($p < 0.05$) change in the serum level of total cholesterol (TC), triglyceride (TAG), low density lipoprotein cholesterol (LDL-C) and high

density lipoprotein – Cholesterol (HDL-C) for the animals administered with the extract compared with control groups. The liver glycogen content is significantly lower in the diabetic control compared to the normal control and significantly higher in test groups compared to the control groups.

Table 5: The effect of methanolic fraction of leaf extract of *Vitex simplicifolia* on liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.280 ± 0.09	0.24 ± 0.01	0.12 ± 0.05	0.20 ± 0.03	0.02 ± 0.03
2	1.960 ± 0.24	0.27 ± 0.01	0.11 ± 0.03	0.12 ± 0.02	0.10 ± 0.01
3	2.880 ± 0.02	0.14 ± 0.11	0.077 ± 0.03	0.13 ± 0.06	0.07 ± 0.04
4	3.323 ± 0.63	0.23 ± 0.21	0.127 ± 0.04	0.13 ± 0.02	0.06 ± 0.02
5	2.450 ± 0.05	0.24 ± 0E7	0.116 ± 0E-7	0.12 ± 0E-7	0.068 ± 0E7
6	2.240 ± 0E7	0.23 ± 0.0E7	0.042 ± 0.02	0.21 ± 0.01	0.151 ± 0.03 ^{ab}

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grps 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values are presented as mean ± standard error of mean. ^a = significantly different (P < 0.05) from the Normal control, ^b = significantly different (P < 0.05) from the Negative control.

Effects on SOD and Antioxidant Vitamins

The effect of daily doses of methanolic leaf extract of *Vitex simplicifolia* on SOD and Vitamins A,C and E in alloxan induced diabetics rats is presented in Table 6. There is significant decrease in vitamin A and no significant (p<0.05) change in superoxide dismutase (SOD), Vitamin C and E for the animals administered with the extract compared with the control groups

Table 6: The effect of methanolic fraction of leaf extract of *Vitex simplicifolia* on liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.280 ± 0.09	0.24 ± 0.01	0.12 ± 0.05	0.20 ± 0.03	0.02 ± 0.03
2	1.960 ± 0.24	0.27 ± 0.01	0.11 ± 0.03	0.12 ± 0.02	0.10 ± 0.01
3	2.880 ± 0.02	0.14 ± 0.11	0.077 ± 0.03	0.13 ± 0.06	0.07 ± 0.04
4	3.323 ± 0.63	0.23 ± 0.21	0.127 ± 0.04	0.13 ± 0.02	0.06 ± 0.02
5	2.450 ± 0.05	0.24 ± 0E7	0.116 ± 0E-7	0.12 ± 0E-7	0.068 ± 0E7
6	2.240 ± 0E7	0.23 ± 0.0E7	0.042 ± 0.02	0.21 ± 0.01	0.151 ± 0.03 ^{ab}

Group 1: Normal control, Group 2: Negative control, Group 3: Positive control, Groups 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values

are presented as mean ± standard error of mean. ^a = significantly different (P < 0.05) from the Normal control, ^b = significantly different (P < 0.05) from the Negative control.

Discussion

The result of acute toxicity study indicated that the LD₅₀ of the methanolic leaf extract of *Vitex simplicifolia* is greater than 5000mg/kg body weight. Thus, the non-lethal effects produced with the high dose of this extract are an indication that the leaf extracts of *Vitex simplicifolia* is relatively safe on acute oral exposure. It can therefore be concluded that *Vitex simplicifolia* leaf extract is non-toxic, which is in agreement with the report of Abdelmagid

[28] on essential oil of the leaves of *Vitex simplicifolia* and with Bruce,^[29] American Society for Testing and Materials,^[30] Aditya and Ravi,^[31] Kingsley^[32] and Ravichandra,^[33] that any chemical substance with LD₅₀ estimate greater than 3000-5000mg/kg (oral route) could be considered of low toxicity and safe.

In this study the methanolic leaf extracts of *Vitex simplicifolia* showed no significant effect on lipid

profile of the experimental animals. The methanolic extract of *Vitex simplicifolia* has no significant effect on the level of total cholesterol and low density lipoprotein. These observations may be attributed to the gut intra-luminal interactive effect of saponin. Saponins are known anti nutritional factors which reduce the uptake of certain nutrients including glucose and lipid especially cholesterol at the gut through intra-lumina physicochemical interaction. Hence saponins have been reported to have hypocholesterolemic effect.^[34] Saponin, among other secondary metabolites is found to be present in the leaves of *Vitex simplicifolia* in this study (Table 3). The low concentration of cholesterol may have contributed to the observed non-significant high serum HDL – cholesterol in the experimental animals. About 30% of blood cholesterol is carried in the form of HDL and it is hypothesized that HDL – cholesterol can remove cholesterol from antheroma within arteries and transport it back to the liver. HDL – cholesterol protect against cardiovascular disease.^[35] The observed non-significant ($P>0.05$) increase in HDL – cholesterol concentration after administration of the extracts (250, 500 and 1000 mg/kg bw) indicates that the extract does not have HDL – cholesterol boosting effect and it does not also have significant ($P>0.05$) LDL – cholesterol lowering effect at these concentrations in induced diabetic experimental animals.

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Oral administrations of methanolic extract of *Vitex simplicifolia* for 21 days significantly increase liver glycogen content. The liver glycogen content is significantly lower in the diabetic control compared to the normal control. This finding does not appear to be related to dietary conditions, since all experimental animals were feed the same type of feed. It is possible that diabetic subjects have defects in liver and muscle glycogen synthesis.^[36] The increase in liver glycogen content is by possible reversal of the earlier mentioned (defects in liver and glycogen synthesis).

Oral administration of methanolic leaf extract of *Vitex simplicifolia* in this study showed a significant decrease in vitamin A and no significant ($p< 0.05$) change in superoxide dismutase (SOD), vitamin C and E. A decrease in SOD and vitamin A is an indication of lack of antioxidant activity (Giugliano, 1995).^[37] Anti oxidant defense mechanism involves both enzymatic and non enzymatic strategies. Common antioxidants including vitamins A, C and E and superoxide dismutase etc work in synergy with each to neutralize different types of free radicals.^[5] There is paucity of data on the antioxidant activities of *Vitex simplicifolia*.

In conclusion, the methanolic leaf extract of *Vitex simplicifolia* is observed to have no hypolipidemic and antioxidant activity on alloxan induced diabetic Wistar rats.

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Phytochemical, Haematological and Histopathological Evaluation of the Ethanol Extract of *Tapinanthus globiferus* A. Rich (Loranthaceae) in Rats

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Abstract

The plant *Tapinanthus globiferus* was widely used in traditional medicine but its effect on blood indices and the spleen were not evaluated. This study is carried out to investigate the phytochemical constituents and to evaluate the haematological and histopathological effects of ethanol extract of *T. globiferus* in rats. Rats were treated daily with doses of 87.5, 175 and 350mg/kg *o.p.* extract for 28 days, after which blood samples were collected for haematological analysis and spleen for histopathological studies. The result of preliminary phytochemical screening of the extract revealed the presence of alkaloids, anthraquinones, carbohydrates, flavonoids, glycosides, saponins, steroids, tannins and terpenoids. The extract had no significant effects on the body weight and haematological parameters at doses of 87.5 and 175 mg/kg, but at 350 mg/kg the extract produced significant ($p \leq 0.05$) decrease in the serum levels of PCV, Hb and RBC compared to control treated with 1.0 ml/kg normal saline, following twenty eight (28) days daily oral treatment in rats. Histological observations showed normal spleen architecture at doses of 87.5 and 175 mg/kg, but at 350 mg/kg, the extract showed area of local degeneration and necrosis in the epithelial cells. The work revealed that, the extract contained constituents which had no toxic effects on body weight, blood indices and spleen at low doses after 28 days daily administration in rats. However, prolonged use of higher doses should be with caution to avoid the risk of toxicity.

Key words: *Tapinanthus globiferus*, Haematology, Histopathology, Phytochemical screening

Introduction

Many diseases are as a result of damage to blood cells. This can be evaluated through the haematological indices and histology profile of organs. Hence, study of these indices or profiles would help to provide valuable information for diagnosis and treatment. However, extracts are considered to be toxic when they damage blood cells or organs structure. Physical parameters observation in experimental animals is the first step in determination of safety or toxicity of chemical constituents of extract. Haematological complications consist mainly of abnormalities in the function, morphology and metabolism of erythrocytes and leukocytes [1] Spleen serves as a reservoir of erythrocytes which are transfused into

the circulation on sympathetic stimulation and change in morphology of spleen may affect the level of erythrocytes which lead to red blood cells disorders notably anaemia.[2] Some researchers have attributed the toxicity of extract and other pharmacological properties of a number of plants to their chemical constituents.[3,4,5] *Tapinanthus globiferus* belongs to family Loranthaceae. It is the most common mistletoe that grows on branches of *Vitellaria paradoxa* tree (host) in West Africa and it is a major cause of *Vitellaria paradoxa* mortality in the northern limits of the Savannah.[6] In Nigeria *T. globiferus* is referred by number of local names such as mistletoe (English), *Kauchin kadanya* (Hausa), *Eme-emi afomo* (Yoruba), and *Osisi/Okwuma osa*

(Igbo) among others.^[7] *T. globiferus* have been used in traditional medicine in the management of hypertension, epilepsy, pain relief, tinnitus and trypanosomiasis.^[8] Studies on the host plant (*Vitellaria paradoxa*) have shown that, the plant has antimycotic, antimicrobial and antifungal activities.^[9,10] Despite the use of *T. globiferus* in traditional medicine but there is little or no scientific basis on the safety of the plant as used traditionally. Therefore, there is need for safety assessment of this plant. The objective of this study is to investigate the preliminary phytochemical constituents as well as to evaluate the haematological and histopathological effects of the ethanol extract of *Tapinanthus globiferus* in rats.

Materials and Methods

Plant collection and Identification

Fresh *Tapinanthus globiferus* was collected from Huguma village of Takai local government area, Kano state. Plant was identified and authenticated in the Herbarium Unit, Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria by comparing with existing voucher specimen number 1052.

Plant Extraction

The mistletoe was air dried under shade at room temperature (27°C) until constant weight was observed. It was then grounded into fine powder using pestle and mortar. The powdered material (100 g) was subjected to Soxhlet extraction using 75% ethanol. The extract was concentrated in an automated temperature of 60°C using water bath.

Preliminary Phytochemical Screening

Preliminary screening of the ethanol extract of *T. globiferus* was conducted using standard methods.^[11,12]

Experimental Animals

Rats (weighing 150 – 180 g) were obtained from Animal House of the Department of Pharmacology, Bayero University Kano. Animals were kept in a well-ventilated room, fed with a pelletized grower mash (Vital feeds, Plc Jos) and water provided *ad-libitum*.

Animal Grouping

The method of Hodge and Sterner^[13] was used. Twenty four (24) albino rats of both sexes were used for this study; they were grouped in a simple ratio

and distributed according to their sex and weight across the groups. Rats were divided into four (4) groups of six (6) each. The first group served as control and was treated with normal saline (1 ml/kg body weight), while the second, third and fourth groups were treated with 87.5, 175 and 350 mg/kg of the ethanol extract of *T. globiferus* orally respectively on daily basis for twenty eight (28) days, after which blood samples were collected for haematological analysis and rats were sacrificed to obtain spleen for histopathology study on the 28th day.

Physical Parameters

Body weight, food, water consumption and local injury were monitored daily. Body weight was recorded on day 0 (baseline), day 7, day 14, day 21 and day 28.

Haematological Study

Blood samples were collected through cardiac puncture into a heparin containing tubes and analysed using SYSMEX SF-KX-21N Automated Haematology analyser (Sysmex Corporation, Kobe, Japan).^[14] The haematological parameters analysed include: red blood cells (RBC), white blood cells (WBC), and haemoglobin (Hb), packed cell volume (PCV) and WBC differentials such as neutrophils (Neut), monocytes (Mono), lymphocytes (Lymp), basophils (Baso) and eosinophils (Eosi).

Histopathological Study

The method of Auwioro^[15] was used. Rats were sacrificed at the end of the study period following chloroform anaesthesia. The spleens were harvested and preserved in 10% formalin solution. Spleens were fixed with 10% normal saline, dehydrated with ascending graded of alcohol, cleared with toluene, infix-treated with molten paraffin wax and embedded with paraffin wax. The microtome sections of the tissues were stained with haematoxylin and eosin staining technique. Slides were prepared and observed using a standard light microscope (H and E stain ×100) in the Histopathology Department of Aminu Kano Teaching Hospital, Kano.

Statistical Analysis

Results were expressed as the Mean ± Standard error of mean (Mean ± SEM) and the differences between means were considered significant when $p < 0.05$.

The significant differences were carried out using one - way analysis of variance (ANOVA) followed by Dunnett's post-hoc test.

Results Phytochemical Analysis

The preliminary phytochemical screening of the ethanol extract of *T. globiferus* revealed the presence of valuable chemical constituents of pharmacological importance as showed in Table 1. These were flavonoids, terpenoids, alkaloids, carbohydrates, glycosides, tannins, anthraquinones, saponins and steroids. However, coumarins and lignins were absent.

Physical Parameter

The ethanol extract of *T. globiferus* had no significant ($p \geq 0.05$) effect on weight of rats after twenty eight (28) days of daily treatment when compared to control rats treated with normal saline (Table 2).

Haematological Study

The effect of ethanol extract of *T. globiferus* on blood in rats after twenty eight (28) days daily oral treatment produced significant ($p \leq 0.05$) decrease in the serum levels of packed cell volume, haemoglobin and red blood cell at dose of 350 mg/kg and at the same dose of the extract, the serum levels of neutrophil and monocyte showed insignificantly ($p \geq 0.05$) increase compared to control group treated with 1 ml/kg normal saline (Table 3).

Histological Analysis

The histopathological evaluation of the rats spleen sections following twenty eight (28) days oral daily treatment with ethanol extract of *T. globiferus* at doses of 87.5 and 175 mg/kg revealed normal spleen architecture (Plate IB; IC) while spleen of the rat that received higher dose of the extract (350 mg/kg) showed area of local degeneration and necrosis in the epithelial cells compared to control group treated with 1 ml/kg normal saline (Plate ID).

Table 1: Preliminary phytochemical constituents of ethanol extract of *Tapinanthus globiferus*

Constituents	Inference
Alkaloids	+
Anthraquinones	+
Carbohydrates	+
Coumarins	-
Flavonoids	+
Glycosides	+
Lignins	-
Saponins	+
Steroids	+
Tannins	+
Terpenoids	+
Key: + Presence	
- Absence	

Table 2: Effect of ethanol extract of *Tapinanthus globiferus* on weight of rats following twenty eight (28) days daily oral administration

Treatment (mg/kg)	Day 0	Day 7	Day 14	Day 21	Day 28
NS 1 ml/kg	160.17 ± 7.12	176.17 ± 7.07	175.67 ± 7.00	177.33 ± 7.02	175.00 ± 6.88
TgE (87.5)	174.17 ± 5.93	174.33 ± 6.30	175.50 ± 6.02	175.50 ± 6.24	175.17 ± 5.99
TgE (175)	174.00 ± 5.14	174.67 ± 5.25	176.67 ± 5.29	177.33 ± 5.51	175.33 ± 5.44
TgE (350)	174.00 ± 6.06	174.17 ± 6.01	174.17 ± 5.56	174.33 ± 5.49	174.83 ± 5.77

No significant difference between control and treated groups, one way ANOVA followed by Dunnett's Post-hoc test. NS = Normal saline, n = 6, Data = Mean ± SEM, TgE = *Tapinanthus globiferus* extract.

Table 3: Effect of ethanol extract of *Tapinanthus globiferus* on haematological parameters in rats after twenty eight (28) days daily oral administration

Treatment (mg/kg)	PCV %	Hb g/L	RBC X 10 ⁹ /L	WBC X 10 ⁹ /L	NEUT X 10 ⁹ /L	LYMP X 10 ⁹ /L	MONO X 10 ⁹ /L	EOSI X 10 ⁹ /L
NS 1 ml/kg	40.83±1.05	13.55±0.32	6.80±0.18	7.73±0.73	9.50±3.83	85.17±4.09	0.00 ± 0.00	2.67 ± 1.02
TgE (87.5)	42.33±1.17	14.10±0.39	7.08±0.19	8.08±0.86	11.17±5.50	83.67±5.16	0.00 ± 0.00	3.00 ± 1.37
TgE (175)	41.67±1.12	13.82±0.38	6.92±0.17	9.95±1.32	7.33±1.05	90.17±1.66	0.60 ± 0.60	0.00 ± 0.00
TgE (350)	36.67±1.15 ^a	12.17±0.39 ^a	6.05±0.18 ^a	7.37±1.02	21.67±5.65	76.83±5.76	0.17 ± 0.17	1.17 ± 1.17

^aP < 0.05 compared to NS, One way ANOVA followed by Dunnett's Post-hoc test. n = 6, Data = Mean ± SEM, TgE = *Tapinanthus globiferus* extract, NS = Normal saline, PCV = Packed cell volume, Hb = Haemoglobin, RBC = Red blood cell, WBC = White blood cell, NEUT = Neutrophil, LYMP = Lymphocyte, MONO = Monocyte, EOSI = Eosinophil

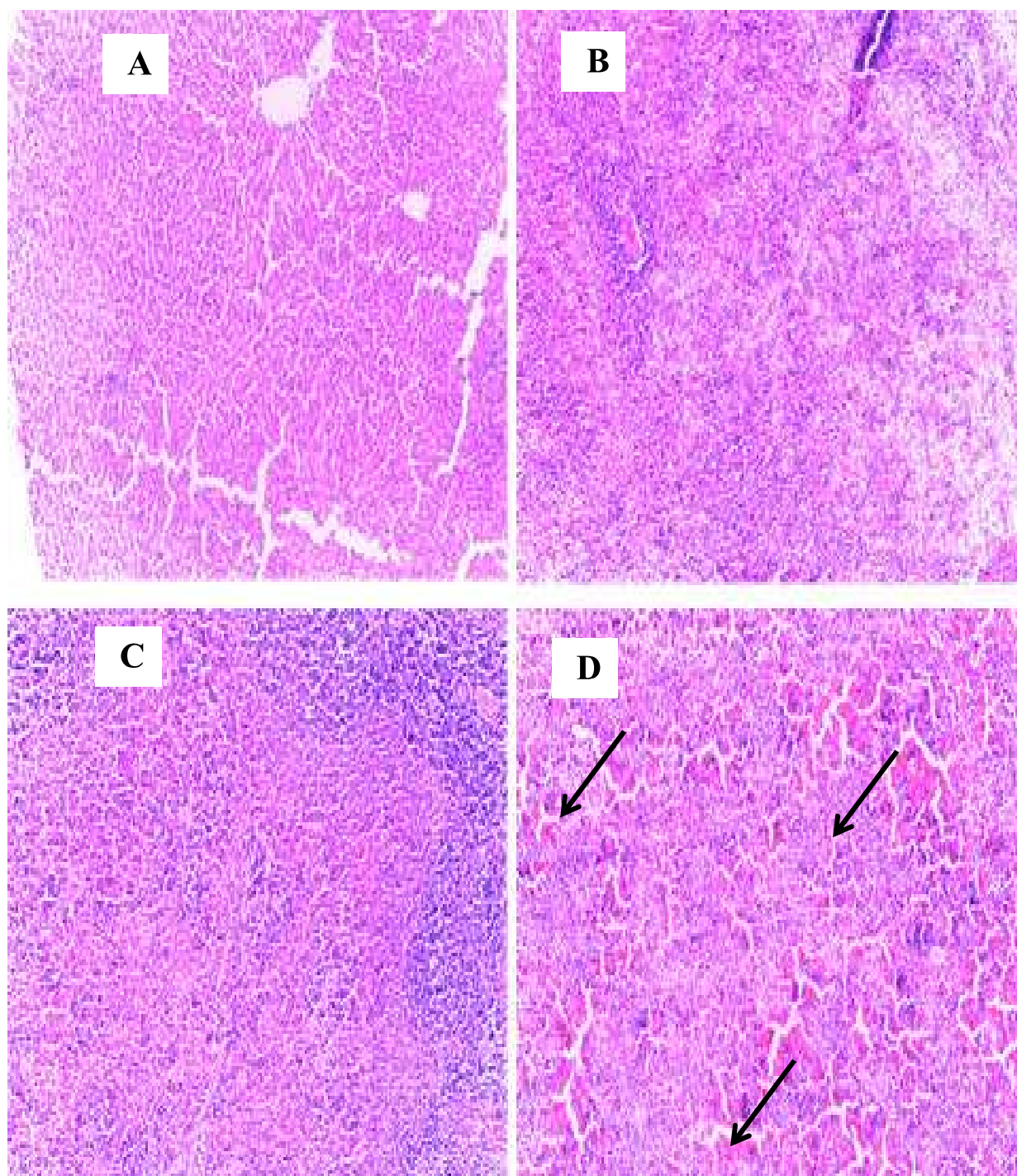


Plate I: Photomicrograph transverse section of the spleen of rat treated with the ethanol extract of *T. globiferus* following twenty eight days (28) daily oral treatment (magnification x100). (A) 1 ml/kg Normal saline; (B) 87.5 mg/kg; (C) 175 mg/kg; (D) 350 mg/kg

*(Arrow depicts degeneration in red pulp and necrosis of epithelial cell)

Discussion

The preliminary phytochemical screening conducted on the ethanol extract of *Tapinanthus globiferus* showed that the extract contained valuable constituents which might be responsible for the observed effect. Some chemical constituents such as alkaloids, flavonoids, saponins, sterols and tannins present in the extract are known to have various pharmacological activities^[16]. Some investigators have attributed the antidiarrhoeal, anti-inflammatory and other pharmacological properties of a number of plants to their alkaloids, flavonoids, saponins, sterols and tannins constituents.^[5,17,18]

The body weights observed from the rats were not affected by the ethanol extract of *T. globiferus* which probably the extract may not produce any toxic effect in the rat organs, following twenty eight (28) days daily oral treatment. A study on ethanol extract of *Cosmos caudatus* leaf showed an increase in the body weight of rats which may relatively be as a result of the toxic effect of the extract on organs which leads to alteration in the physiological functions^[19] However, chemical effect of potash on renal profile showed reduction in body weight of albino rats.^[20]

Haematological study is one of the important ways for the diagnosis of root cause of diseases.^[21] Haematological parameters such as packed cell volume (PCV), haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), neutrophil (Neut) and lymphocyte (Lymp) are used to provide useful information for diagnosis in routine clinical evaluation of the state of health of a patient.^[22] Alterations of these parameters may result in haematological disorders. Haematological disorders include a wide range of abnormal conditions indicating the profile of blood parameters, due to changes in metabolism or changes in cellular integrity, membrane permeability of cells or even due to exposure to toxic chemicals.^[21] The significant ($p < 0.05$) reduction in PCV, Hb and RBC values indicate that the ethanol extract of *T. globiferus* at 350 mg/kg may have been toxic to circulating cells and possibly interfered with RBC

production. Reduction in PCV and RBC values are indication of anaemia ^[23] and reduction in Lymp values are indication of a non-enhanced immunological status ^[24] hence, the extract insignificantly reduced Lymp values. The observed insignificant elevation of WBC values at doses of 87.5 and 175 mg/kg may be due to the stimulation of immune defence system by the extract. Stimulation of immune defence system may result in the elevation of WBC values.^[22] However, the use of *T. globiferus* in ethnomedicine should be with caution especially at higher dose, since the extract at 350 mg/kg lower the levels of PCV, Hb, RBC and Lymp in rats. Some plant materials when ingested either in the raw state or their extract have been reported to cause anaemia which may result from sequestration of red blood cell in the spleen, impaired red cell production or primary bone marrow dysfunction.^[2]

The histological observation of the spleen sections treated with low doses of extract showed no pathological effect but rat that received highest dose showed a sign of toxic effect compared to control group that received normal saline. *T. globiferus* have been reported to contain alkaloids, flavonoids, saponins, tannins and cardiac glycosides.^[25] This shows that the ethanol extract of *T. globiferus* contained chemical constituents which had no toxic effect on histopathology of spleen after low dose daily oral treatment. However, this study suggests that its prolonged usage at higher dose should be with caution.

Conclusion

The ethanol extract of *Tapinanthus globiferus* contained valuable chemical constituents which have less or no toxic effects on body weight, haematology and histopathology of spleen after short and low dose daily treatment but prolonged use of higher dose should be with caution to avoid the risk of toxicity. Therefore, *T. globiferus* at lower doses could be considered relatively safe for use in traditional medicine.

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Immunosuppressive Effects of Academic Stress among First Year Medical Students of Bayero University, Kano, Nigeria

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Abstract

Stress is an intricate series of events, consisting of a stimulus (stressor), which activates a central nervous system reaction, leading to a neuroendocrine response. Physiological studies have shown that acute physiological response to stress protects the body, while chronic stress is harmful to the body. Academic stress faced by medical students is an example of chronic psychological stressor. This study examines the effects of academic stress on serum Cortisol, CD4 cells count and some immunologic parameters among 58 randomly selected first year medical students M=41(71%) and F=17 (29%) with mean age of 22.3 ± 2.5 . The selected students were seen twice, between 12:30 pm-1:30 pm. The first interaction was four weeks earlier, while the second interaction was on the completion date before the final paper of the first semester examination respectively. Each time blood sample was taken for serum cortisol level, CD4 cells and white blood cell counts. The results showed significant increase ($p < 0.05$) in serum cortisol and Granulocytes Percentage (GRA %) and consequent significant decrease ($p < 0.05$) in CD4 cells count, total White Blood Cells count (WBC) and Lymphocyte Percentage (LYM %) during examinations compared to the before examination period. Therefore, academic stress among medical students is enormous enough to cause statistically significant decrease in immunity with elevated serum cortisol level as possible mediator. Counselling, proper time utilisation and good study skills can help reduce stress among the students and enhance their wellbeing.

Keywords: Academic Stress, Serum cortisol, Immunity, Chronic stressors, Medical students

Introduction

Hans Selye ^[1] defined stress as "the non-specific physiological response of the body to any demand placed upon it". The term "nonspecific" referred to a series of common responses which occur independently of the nature of the stressor. Stress may originate in the home, at school, at work, or within the wider community. ^[2] Stress is viewed as a negative emotional, cognitive, behavioral and physiological process that occurs as a person tries to adjust to or deal with stressors. ^[3] These stressors may be physical as in infection, chemical exposure and exhaustion; cognitive such as death of a loved one, perception of imminent danger, anxiety and depression or a combination of both physical and cognitive as in academic examination stress. ^[4]

Academic stress denotes prolonged academic pressures associated with examination periods faced

by students. ^[5] According to Wilks academic stress is the product of combination of academic-related demands that exceed the adaptive resources available to an individual. ^[6] Academic stress can be associated with psychological, physiological and physical morbidity. ^[7,8]

Physiological studies have shown that stress from any source can have influence on the endocrine, hemopoietic and immune systems. ^[9,10] The putative mechanism of cortisol release is that, the hypothalamus releases corticotrophins which act on anterior pituitary gland to release adrenocorticotrophic hormones in response to stress. The adrenocorticotrophic hormone stimulates the adrenal cortex of adrenal gland to release glucocorticoids mainly cortisol, leading to dysregulation of immunological function. ^[9,12]

The period of undergraduate education is a sensitive time in the life of an individual, and for many young adults, college life has been found to be stressful.^[13] Previous studies have indicated academics, social and family relationships, finances and daily hassles as the main stressors in college life.^[14,15]

Examination and assessments methods constitute major source of academic stress.^[15] Kumar *et al* found that academic factors were responsible for higher level of stress among undergraduate students which have negative effects on student health.^[16]

The 200 level (first year) medical students were newly exposed to professional courses (Anatomy, Physiology and Biochemistry), with a lot of new terminologies that need to be assimilated before understanding the courses. These are compounding stressors in addition to other social and institutional challenges. High level of stress results in mental and physical problems that might affect the student's academic achievement and health.^[17] Hence, this study was designed to evaluate the effects of academic stress on some immunological parameters among first year medical students of Bayero University, Kano.

Materials and Methods Study Area

The study was conducted in the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Bayero University, Kano, Nigeria.

Study Population

The study population comprised of 142 males and females first year medical students (200 level) College of Health Sciences, Bayero University, Kano, 2012/2013 session.

Sample Size Determination Sample size was determined using the formula for sample size determination in descriptive studies^[18] and $p = 0.89$ from 89% prevalence of academic stress in a previous study.^[19] With sampling interval of 2 ($142/77=1.8$), 72 subjects were systematically selected from sitting arrangement in the class. However, the study was completed with 58 subjects while 14 subjects fallout of the study.

Inclusion Criteria Level 200 medical students with willingness to fully participate in the study were included.

Exclusion Criteria

Pregnant women, those with history of Diabetes mellitus, Hypertension and those on steroid medications were excluded from the study. Ethical clearance for the study was obtained from the Scientific and Ethical Committee of Kano State Ministry of Health (Ref), before commencement of the study. An informed consent was obtained from each of the selected subjects after a detailed explanation about the study design and application of the study.

Study Design

The study was longitudinal-section design. The selected students were seen twice, between 12:30 pm-1:30 pm. First interaction was four weeks before and the second interaction was on the completion date before the final paper of the first semester examination.

Data Collection The data was collected using a structured questionnaire composed of two parts;

A. Socio-Demographic characteristics which include age and sex of the subjects B. Laboratory data, which include Serum Cortisol, CD4 Cells count, white blood cells count lymphocytes and granulocytes percentages.

B. Blood sample collection each time four milliliter of venous blood sample was collected by a trained phlebotomist under aseptic condition. Two milliliter of the blood was processed to obtain serum for cortisol analysis. The remaining 2 mls of blood was transferred into a 2 mls EDTA bottle, mixed well and transported immediately to the laboratory for full blood count and CD4 Cells count analysis.

Estimation of Serum Cortisol

Serum cortisol was estimated using Cortisol EIA Assay Kit (Monobind Inc. Lake Forest, CA USA) with REF number 3625-300A and LOT number EIA-36K2F3. The absorbance was measured spectrophotometrically at 450 nm using micro plate

reader (Model: SM600 SN:IJOTC2012 Yongchuang Medical Instrument Co. Ltd. Shanghai, China).

Determination of CD4⁺ T-Cell Count

The quantification of CD4 T lymphocytes was done with Partec CyFlow Counter (Partec GmbH, Münster, Germany) using Partec CD4 easy count kit (05-8401). Procedure as described in the product manual 2010 version.

Hematology Analysis Hematology analysis was done using Nortek automated hematology analyzer (Nortek Biomedical Co., Ltd. China) following the product's manual. The parameters analyzed include;

White Blood Cell count (WBC), Lymphocyte Percentage (LYM%) and Granulocyte Percentage (GRA%).

Data Analysis

Data obtained from the study was expressed as simple percentages, means and standard deviations. Statistical analysis was conducted using the SPSS Version 20 (SPSS Inc., Chicago, U.S.A.). The mean values of different variables both before and during examination period were compared statistically using paired 't' test. Correlation analysis was performed using Pearson's test. P value < 0.05 was considered as significance.

Results

Total of 58 medical students participated in the study, 29.3% (n=17) of which are females and

70.7% (n=41) are males. The mean age of the participants was 22.3±2.45. Table 1

Table 1: Distribution of cohort based on age and sex

	AGE (in years) Mean ± SD	Number	Percentage
Male	22.61 ± 2.48	41	70.7
Female	21.65 ± 2.29	17	29.3
Overall	22.3 ± 2.45	58	100

The result of this study showed significant increase (p<0.05) in mean serum cortisol of overall subjects during examinations period (14.47µg/dl) compared to four weeks before Examinations (5.55µg/dl). It

was also observed that the Serum Cortisol Mean difference of females (9.06µg/dl) was slightly higher than that of males (8.85µg/dl). (Table 2)

Table 2: Effect academic stress on mean serum cortisol before and during examinations

		Before Examination	During Examination	Differences	T	P
Serum cortisol in µg/dl	Males	5.44 ± 2.01	14.29 ± 3.12	-8.85 ± 3.50	-16.179	<0.0001
	Females	5.82 ± 1.55	14.88 ± 2.42	-9.06 ± 2.82	-13.26	<0.0001
	Overall	5.55 ± 1.88	14.47 ± 2.92	-8.91 ± 3.29	-20.607	<0.0001

Values expressed as (mean ± SD)

There was a significant (p<0.05) increase in Mean Serum Cortisol of the overall subjects during examination period.

Table 3: Effect of academic stress on CD4 cells count, total WBC count, lymphocytes and granulocytes percentages.

Variables		Before Examination	During Examination	T	p
CD4 Cells	Males	814.66 ± 203	756.39 ± 203.43	5.287	<0.0001
	Females	873.71 ± 248.63	790.35 ± 216.81	4.288	0.001
	Overall	831.97 ± 216.82	766.34 ± 206.11	6.782	<0.0001
WBC	Males	6.49 ± 1.67	6.20 ± 1.75	4.068	<0.0001
	Females	6.76 ± 1.89	6.12 ± 1.76	5.416	<0.0001
	Overall	6.57 ± 1.72	6.28 ± 1.63	6.036	<0.0001
Lymphocytes %	Males	37.39 ± 6.31	34.61 ± 5.21	5.449	<0.0001
	Females	39.24 ± 6.97	36.24 ± 5.45	3.00	0.008
	Overall	37.93 ± 6.51	35.09 ± 5.3	6.184	<0.0001
Granulocytes %	Males	54.78 ± 6.67	57.76 ± 5.04	-4.093	<0.0001
	Females	52.99 ± 6.97	56.88 ± 5.44	-3.57	0.003
	Overall	54.26 ± 6.75	57.52 ± 5.13	-5.383	<0.0001

Values expressed as (mean ± SD)

There was a significant reduction ($P < 0.05$) in CD4 Cells count in Cells /mm³, Mean White Blood Cell counts $\times 10^9$ cells/l and Lymphocytes Percentage while significant increase ($P < 0.05$) was observed in Granulocytes Percentage. It was observed that

females have higher mean values in CD4 cells count WBC counts and Lymphocytes Percentage and lower mean values in Granulocytes Percentage, Table 3 above.

Table 4: Pearson's correlations between Serum cortisol and CD4 count, WBC count, LYM%, GRA%.

Parameters	Pearson Correlation	p
CD4	-0.179	0.18
WBC	-0.374**	0.004
Lymphocytes %	-0.347**	0.008
Granulocytes %	0.295*	0.025

**Correlation is significant at the 0.01 level (2-tailed).

The study observed significant negative Pearson correlation between Serum Cortisol and White Blood Cell counts ($\rho = -0.374$, $p = 0.004$) and Lymphocytes percentage ($\rho = -0.347$, $p = 0.008$), and positive Pearson correlation with granulocytes percentage ($\rho = 0.295$, $p = 0.025$). (Table 4)

Discussion

The significant ($p < 0.05$) increase in serum cortisol level observed during examination was slightly more than the upper limit of normal range (3-13 µg/dl) [20] for adult during the day indicating high level of stress among these students. Our findings, support earlier studies on medical students, who documented

an increase in serum cortisol level during examination period compared to before or after the examination. [21,22,23]

The observed higher mean serum cortisol difference in females as compared to that of males, suggest that females are more prone to stress than males. Females are generally more susceptibility to stress related conditions such as high emotional overlay, anorexia and mood swings associated with menstrual cycle, although not determined by this study. Higher cortisol levels was also observed in female students during examinations period by other workers. [24] However, a study documented varying response to

stress in females corresponding to the phases of their menstrual cycle, having blunted responses in the follicular phase and increased responses in the late luteal phase.^[25] The phase of menstrual cycle was not considered in this study.

The significant ($p < 0.05$) decrease in total white blood cells, lymphocytes percentage and cd4 cells count during examination observed in this study, could be attributed to the effect of increased serum cortisol due to academic stress associated with examination period. The study also observed significant negative Pearson correlations between serum cortisol level and total white blood cell counts and lymphocytes percentage such that total white cells and lymphocytes percentage decreases with increase in serum cortisol. The relative increase granulocytes percentage could be secondary to the decrease in the lymphocyte percentage observed due to their reciprocal relationship.

Prolonged high level of Cortisol inhibits the production of new lymphocytes (T-helper cells, T-suppressor cells), pulls existing lymphocytes out of circulation, and can also cause lymphocytes to commit suicide.^[26] The significant reductions in White Blood Cells counts, Lymphocytes Percentage and CD4 Cells counts, was believed to be due to the effects of Cortisol which prevents the proliferation of T cells by rendering the interleukins-2 producer T

cells unresponsive to interleukin-1 and unable to produce the T cell growth factor.^[27] Cortisol decreases the number of lymphocytes in the blood, indeed, a finding of lymphocytopenia is an important diagnostic criterion for overproduction of cortisol by the adrenal gland.^[20] These findings are in accordance with previous studies, where they found significant decrease in the TWBC count, Lymphocyte count and CD4 cell,^[28, 29, 30] during examination in medical students.

White blood cells are the mobile units of the body's protective system.^[20] They are specifically transported to areas of serious infection and inflammation, thereby providing a rapid and potent defense against infectious agents. The lymphocytes function mainly in connection with the specific immune system.^[20] It is generally accepted that CD4 T lymphocytes plays a central role in immune response, cellular immune deficiencies of these cells may lead to increased susceptibility to infections from viruses, fungi, intracellular bacteria and protozoa.^[31]

The Limitations of our the Study

There was high attrition due to the foreign scholarships offered by then Kano State Government to its students to study abroad. The study also did not do differential white blood cells count which would have given more information.

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Toxicological Study of Combined Aqueous Leaf Extracts of *Gymnema sylvestre* and *Combretum micranthum* in Rats

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Abstract

Gymnema sylvestre R.Br. (“periploca of the woods” in English;) (Asclepiadaceae) and *Combretum micranthum*, Fam. (Combretaceae) are used in combination for the management of DM in the north-eastern part of Nigeria with little or no scientific basis for their safety. It is thus the aim of this research to determine the acute and sub-chronic toxicity risk of *G. sylvestre* (GS) and *C. micranthum* (CM) leaves, individually and in a 1:1 combined form (GSCM). The study assayed for the LD₅₀ (modified Lorke method) as well as some markers of toxicity, in the sub-chronic phase. Body and Relative Organ Weights were calculated. Qualitative/quantitative phytochemicals were also assayed for. Both plants were found to be relatively safe (LD₅₀ > 5000mg/kg) in mice. Sub-chronic studies in rats revealed that GS, CM and GSCM (1:1) showed slight increases in rats’ body weights at 600mg/kg b.w. (P< 0.05) when compared with the control after 3 weeks of administration. Relative liver weight reduced significantly only in the GS administered rats whereas GSCM reversed it; relative kidney weight, liver and kidney function parameters were not significantly affected. Both plants also showed good total flavonoids and total phenolic contents. It can be concluded that GS, CM and GSCM are safe which might be related to the flavonoids content.

Keywords: Herbalism, Combination therapy, Acute toxicity, Sub-chronic toxicity.

Introduction

Adverse effects from herbal remedies are reported, but their frequency and severity are unknown. To overcome these drawbacks scientific study of herbal remedies and their potential to cause interactions when used in combination need to be thoroughly understood and systematically studied.^[1] In sub-Saharan Africa (northern Nigeria in particular), the use of multiple herbs as decoctions or in different combinations to treat chronic non-communicable diseases like diabetes mellitus and hypertension is common. However, some of these plants, when used together, can have either positive or negative effects on the individual. It is important and necessary to provide therapy to control risk factors, including oxidative stress, dyslipidaemia, mitochondrial dysfunction and vascular complications.^[2, 3, 4] Lipid peroxidation, the oxidative degradation of polyunsaturated fatty acids, is implicated in several pathological conditions-ageing, hepatotoxicity,

haemolysis, cancer, atherosclerosis, tumour promotion, inflammation and diabetes mellitus. Selected flavonoids may exert protective effects against cell damage produced by lipid peroxidation stimulated by a variety of toxins, owing to the antioxidant properties of the compounds. Plant alkaloids usually have profound physiological actions in humans with nervous system effects being the most prominent. Because they are so reactive, even at small doses, most alkaloid rich plants are used sparingly, if at all, in Western herbalism. Indeed, use of many of the alkaloidal species is restricted by law, or listed on poison schedules. However, alkaloids - either as isolated compounds or their semi-synthetic derivatives — are widely used in pharmaceutical medicines.^[5]

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Plants used in traditional medicine are expected to have low toxicity by the consumers. The latest surveys have indicated that many medicinal plants used as traditional medicine showed undesirable effects. [6, 7, 8] Therefore emphasis should be given to elucidate the safe use of any plant for medicinal purposes. The possible toxic effects resulting from the short term and long term use of such medicinal plants have raised an alarm. To increase the confidence in their safety to the consumers especially for use in the development of pharmaceuticals, the data of the acute and sub-acute (sub-chronic) toxicity studies on medicinal plants should be obtained. Hence evaluating the efficacy and safety profile in such plants is needed. [7] Toxicity profile of *G. sylvestre*, R.Br. (“*Kafi suga*” in Hausa) and *C. micranthum*, Fam. (*Géézà* in Hausa) have earlier been reported, [8, 9, 10] however, their combined effect has not been reported so far. Thus, this study is aimed at evaluating the toxicity risks of the leaf extracts of *G. sylvestre* and *C. micranthum* used concomitantly for the management of diabetes mellitus and other ailments in the north-eastern part of Nigeria.

Materials and Methods

Materials

Plant Material Collection and Identification

Fresh samples of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) were obtained from Shira local government area (N 11° 27' 29" and E 10° 2' 48" or Latitude: 11.46 and Longitude: 10.05) of Bauchi state in Nigeria where they are used for the treatment of different ailments including diabetes mellitus and hypertension. The plants were identified at the herbarium unit of Department of Biological Sciences, Bayero University, Kano, Nigeria and voucher specimens, with number BUKHAN 0349 (for *Gymnema sylvestre*) and BUKHAN 0272 (for *Combretum micranthum*) were then deposited in the same herbarium.

Animals

Adult male wistar rats (150 – 200 g body weight) were purchased from the Department of Pharmacology and Therapeutics of Ahmadu Bello University, Zaria, Nigeria. They were kept in standard metal animal cages at room temperature in the Animal House of Biological Sciences Department, Bayero University Kano, were allowed free access to food and water and allowed to

acclimatize for a week prior to use. The protocol of the study was according to international guidelines that is the Organisation for Economic Cooperation and Development (OECD) Test Guidelines (TG407). [11]

Chemicals, Reagents and Equipment

All the chemicals and reagents used for this work were of analytical grade and purchased from reputable chemical laboratories, for example SIGMA-ALDRICH-FLUKA. The laboratory equipment was also of standard quality.

Methods Preparation of Plant Material (Extracts)

The leaves of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) used for the study were plucked from the stem, washed and then dried under shade. The dried samples were then ground into powder in a laboratory mortar using a pestle. The powdered samples were then kept in a closed plastic container for use in the experiment.

Aqueous Extraction

Two hundred grams of the air-dried samples of each plant were cold extracted with 2000 ml of distilled water – 3X.

Phytochemical Analyses

The GS and CM extracts were screened qualitatively for the phytochemical constituents utilising standard methods of analysis [12, 13, 14] to test for tannins, steroids, terpenoids, saponins, flavonoids and alkaloids. Quantitative phytochemical screening [15] was carried out to determine saponins, flavonoids, alkaloids, tannins, total phenolics and total flavonoids.

Estimation of Total Phenolic Compounds

Total soluble phenolics in the GS and CM was determined with Folin-Ciocalteu reagent according to the method of Naskar *et al.* [16] Briefly, 0.1ml of extract in distilled water (contains 1mg of each extract) was transferred into 100ml Erlenmeyer flask then final volume adjusted to 46ml by addition of distilled water. Afterwards, 1ml of Folin – Ciocalteu reagent (FCR) was added to this mixture and after 3 minutes, 3ml of Na₂CO₃ (2%) added. Subsequently, the mixture was shaken on a shaker for 2 hours at room temperature then absorbance measured at 760nm. All the tests were performed in triplicate and the results averaged. The concentration of total

phenolic compounds in extracts was determined as microgram of pyrocatechol equivalent by using Eqs. (2) and (3) that were obtained from standard pyrocatechol/gallic acid graph.

$$\text{Absorbance} = 0.001 \times \text{pyrocatechol } (\mu\text{g}) + 0.0033$$

.....

$$(2) \text{ Absorbance} = 0.0053 \times \text{gallic acid } (\mu\text{g}) - 0.0059$$

.....(3)

Estimation of Total Flavonoid Content The total flavonoid content was determined using the method as in Naskar *et. al.* ^[16] 2mL of 2 % aluminium trichloride (AlCl₃) in methanol was mixed with the same volume of the extract solution (0.1 mg/mL).

Absorbance readings at 415 nm using spectrophotometer were taken after 10 minutes against a blank sample consisting of a 2 ml extract solution with 2 mL methanol without AlCl₃. The total flavonoid content was determined using a standard curve with quercetin (25 - 200 µg/2ml methanol) as the standard. Total flavonoid content is expressed as µg of quercetin equivalents (QE)/mg of extract.

Acute Toxicity

The median lethal dose (LD₅₀) of aqueous leaf extracts of the plants was determined in mice using a modified method of Lorke ^[17] as described in the work of Adewole and Ojewole, ^[18] mice were fasted for 16 hours and randomly divided into groups of eight mice each. Stepwise, graded doses of GS and CM (separately) extracts (25, 50, 100, 200, 400, 800, 1600, 3200 and 6400 mg kg⁻¹ b.w.) were administered orally to the mice in each of the 'test' groups. Each of the mice in the 'control' group was given distilled water (3 ml kg⁻¹ b.w.) only. The mice in both the 'test' and 'control' groups were then allowed free access to food and drinking tap water, and observed over a period of 48 hours for signs of acute toxicity which include changes in feed and fluid intake, body weight, respiratory, GIT, CNS symptoms, behavioural patterns and mortality. The number of deaths (caused by the extract in each group) within this period of time was noted and recorded. 1/10th of the LD₅₀ of each extract (i.e. 600mg/kg bw) was considered as the highest dose for the sub-chronic (long term) study. ^[19]

Experimental Design (Sub-chronic studies)

According to the OECD guideline, if the acute toxicity test at one dose level of at least 5000mg/kg

b.w. produced no observable toxic effect then the full study using three dose levels is considered not necessary. As a result, total of 24 rats were used for this study and they were divided into four groups with six rats per group, using a single dose (1/10th of the LD₅₀) as outlined below:

Group 1, normal untreated rats (0.5 mL water/100 g body weight/rat/day); Group 2, normal rats treated with GS alone (600 mg/kg bw); Group 3, normal rats treated with CM alone (600 mg/kg bw); Group 4, normal rats treated with 1:1 GSCM (600 mg/kg bw); All experiments were carried out in overnight fasted rats. The aqueous plant extracts were given orally daily using an intragastric tube for 21 days.

Body Weight

The body weight of each rat was assessed during the acclimatization period, once before commencement of dosing, once every 7 days during the dosing period and once on the day of sacrifice. The relative body weight (RBW) of each animal was then calculated using the formula RBW=[absolute body weight of one time interval (g) / body weight of rat on commencement of dosing day(g)] × 100. ^[20]

Biochemical Assays and Relative Organ Weight

On 22nd day of the experiment the animals were humanely sacrificed under light anaesthesia, followed by decapitation after recording the final body weight. Blood was collected from the dorsal aorta by a syringe and the serum separated by centrifugation at 2800g for 5 minutes for the estimation of biochemical parameters and toxicity markers as specified below.

After taking the blood, the abdominal cavity of each animal was opened and the liver and kidney quickly removed, cleaned with ice-cold saline and weighed. The relative organ weight (ROW) of both organs for each animal was then calculated using the formula below. ROW = [absolute organ weight (g)/body weight of rat on day of sacrifice (g)] × 100. ^[20]

Liver Marker Enzymes

Serum Aspartate Aminotransferase (AST) and Serum Alanine Aminotransferase (ALT) were evaluated using the method of Reitman and Frankel; ^[21] serum alkaline phosphatase (ALP) using DGKC method; ^[22] total protein (TP) using direct biuret method ^[23] while total bilirubin (TB) according to the method of

Jendrasik and Grof; [24] all as described by Randox laboratories, using Randox kits (United Kingdom).

Kidney Function Tests

Estimation of serum urea: This was done following the method of Bauer *et. al.* [25]

Estimation of serum creatinine

Creatinine concentration was determined following the method of Butler. [26]

Results

Phytochemical

Aqueous extracts of *G. sylvestre* (GS) and *C. micranthum* (CM) leaves gave respective yields of 17.07 ± 0.07 % w/w and 18.38 ± 0.04 % w/w. All phytochemicals assayed for were found to be present in the aqueous GS and CM extracts except steroids, saponins and soluble starch (Table 1). Alkaloids and monosaccharides were not detected in the GS extract (Table 1). Aqueous extract of GS had significantly higher total flavonoids and total phenolics contents than that of CM ($P < 0.05$) (Table 2).

Acute Toxicity

G. sylvestre and *C. micranthum* aqueous leaf extracts (100-6400 mg kg⁻¹ b.w.) were found to be safe in mice. Thus, LD₅₀ values were found to be > 5000 mg kg⁻¹ i. p. in mice, which make the plants to be relatively safe in mice.

Sub Chronic Toxicity

The animals generally did not show much physically observable behavioural toxicity.

Body Weight Changes

When the rats were treated with 600mg/kg b.w. doses of GS and CM aqueous leaf extracts and their 1:1 combination (GSCM), the GSCM recorded the least percentage increase in weight compared to the separate administration of each plant (Table 3).

Relative Organ Weights

Relative liver weights reduced significantly ($p < 0.05$) in the groups normal + 600mg/kg b.w. GS, (Figure 1); normal + 600CM, (Figure 1); and normal + 600GSCM (Figure 1). The relative kidney weights displayed no significant change (Figures 1).

Liver Function Tests

Except for the 600mg/kg b.w. GSCM treated group which showed significant increase ($P < 0.05$) in serum AST compared with normal, no significant change was observed in levels of AST, ALT, ALP, TP and Total Bilirubin in all the treated groups (Table 4).

Renal Function Tests

Serum urea and creatinine levels in the treated rats did not show any significant change (Table 5).

Table 1: Qualitative phytochemical composition of aqueous extract of *G. sylvestre* and *C. micranthum* leaves

Phytochemical	Sample	
	<i>Gymnema sylvestre</i>	<i>Combretum micranthum</i>
Tannins	+	+
Anthraquinones	+	+
Steroids	-	-
Terpenoids	+	+
Saponins	-	-
Flavonoids	+	+
Alkaloids	-	+

Key: (+) means present; (-) means not detected.

Table 2: Quantitative phytochemical composition of aqueous extracts of *G. sylvestre* (GSAq) and *C. micranthum* (CMAq) leaves

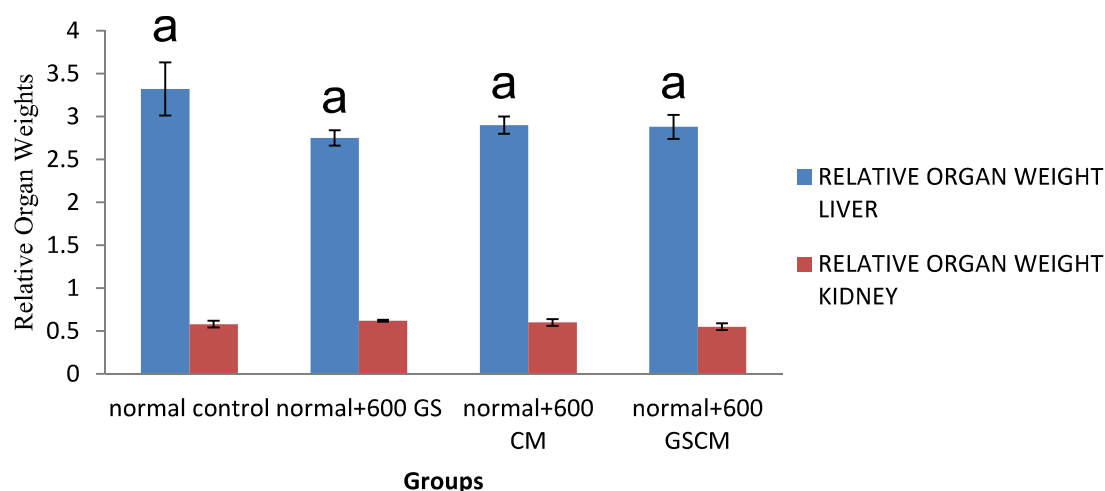
Phytochemical	Sample	
	GSAq	CMAq
Tannins (g/100g dry weight)	1.48±0.015	1.06±0.067
Saponins (g/100g dry weight)	ND	ND
Flavonoids (g/100g dry weight)	10.69±0.012	3.143±0.033
Alkaloids (g/100g dry weight)	ND	10.03±0.03
Total Flavonoids (mg/g QE)	53.45±7.93*	19.66±1.03*
Total Phenolics (mg/g GAE)	53.69±0.36**	48.98±0.09**

Values expressed as Mean ± SEM of triplicate measures. * and ** = values respectively significant (p<0.05) with each other. ND = not detected, QE = Quercetin equivalent, GAE = Gallic acid equivalent.

Table 3: Percentage (%) change in body weight of rats administered *G. sylvestre*, *C. micranthum* and 1:1 combination of their aqueous leaf extracts

Group/Treatment	Percentage (%) Change in body weight		
	Week 1	Week 2	Week 3
normal control	6.00±0.90	7.29±1.24 ^a	10.69±3.06
normal 600 GS	9.29±2.02	14.49±2.54 ^a	13.49±3.38
normal 600 CM	3.22±4.85	10.05±6.95	11.30±6.61
normal 600 GSCM	5.66±0.79	6.88±2.58	5.98±4.36

Values are mean ± standard error of mean with those bearing same superscripts under the same column being significantly (p< 0.05) different. N = 6.

**Figure 1:** Relative organ weights of rats administered *G. sylvestre*, *C. micranthum* and 1:1 combination of their aqueous leaf extracts

Values are mean ± standard error of mean (bars) with those bearing same superscripts being significantly (p< 0.05) different with respective control.

N = 6.

Table 4: Liver function indices of rats administered *G. sylvestre*, *C. micranthum* and 1:1 combination of their aqueous leaf extracts for 21 days.

Groups	AST(U/l)	ALT(U/l)	ALP(U/l)	TP(g/dl)	TBIL(mg/dl)
normal control	56.56±10.55 ^a	23.33±0.88	158.15±70.28	2.97±9.48	1.78±0.12
Normal+ 600 GS	73.71±8.26	21±0.01	363.91±87.91	4.67±2.85	1.68±0.08
Normal+ 600 CM	81.21±6.73	16.5±4.50	305.33±62.17	2.61±1.10	1.64±0.15
Normal+ 600 GSCM	91.33±8.2 ^a	31.5±14.50	256.82±13.8	4.81±1.52	1.45±0.12

Values are Mean ± standard error of mean with those bearing same superscripts within the same column being significantly (p< 0.05) different. N = 6.

Table 5: Renal function indices of rats administered *G. sylvestre*, *C. micranthum* and 1:1 combination of their aqueous leaf extracts for 21 days.

Group	Serum Urea (mg/dl)	Serum Creatinine (mg/dl)
normal control	42.16±4.21	0.63±0.11
normal 600 GS	37.08±1.57	0.75±0.06
normal 600 CM	41.39±14.98	0.75±0.04
normal 600 GSCM	52.3±1.88	1.67±0.55

Values are mean ± standard error of mean with those bearing same superscripts within the same column being significantly (p< 0.05) different. N = 6.

Discussion

Safety profile on medicinal plants is important as safety of herbal medicine use has recently been questioned due to reports of illnesses and fatalities.^[27] General behaviour and body weights are one of the critical parameters for the evaluation of first signs of toxicity.^[28] In the acute study, and in compliance with Organisation of Economic Cooperation and Development (OECD) guidelines 423^[29] and OECD Test Guidelines TG407,^[11] there were no observable signs of morbidity or mortality throughout the duration of the experiment at all the doses used, which is an indication that the extracts were well tolerated by the mice. The LD₅₀, being greater than 5000 mg/kg b.w., is thought to be safe as suggested by Lorke.^[17] The low acute toxicity observed might be associated with the low amount/concentration of some toxic phytochemicals like alkaloids.^[5, 20, 30]

This concurs with toxicity studies of *Gymnema sylvestre* extract which had shown its safety when taken in recommended doses.^[31] Moreso, in an acute toxicity assay, it was demonstrated that treatment with *Combretum lanceolatum* ethanol extract and *Combretum micranthum* aqueous extract in limit dose of 5000 mg/kg orally did not promote any behavioural changes nor mortality in animals.^[9, 32] However, acute toxicity alone does not suffice to qualify a drug

as being safe. As a result, long-term (sub-chronic) toxicity was carried out to further ascertain the toxicity risk of GS and CM. There are a number of markers of toxicity, for instance, changes in body weight, food and water intake, urine output, and organ weight may all be sensitive indicators of either general or specific toxicity. Thus, animals often consume less food and lose weight after exposure to toxic compounds, and increased organ weight may be due to a tumor, fluid, or triglyceride accumulation, hypertrophy, or enzyme induction.

The present study revealed that GS, CM and GSCM extracts may help regain body weight losses normally associated with some chronic diseases as seen in body weight increases observed. However, the combined form of the extracts (GSCM) could be good in weight control especially in obesity-related ailments as it recorded lower percentage increases in body weight compared to the plant extracts used separately as well as with the normal control. Weight reduction property of the plants agrees with the work of Preuss *et al.*^[33]

Moreover, relative organ weight is an important index of swelling, atrophy, or hypertrophy. An increase in this parameter indicates inflammation while a decrease may be associated with cellular constriction.

[34, 35] Thus, the extracts and their 1:1 combination might be good in reversing hepatomegaly associated with chronic ingestion of some chemicals leading to liver diseases. The extracts did not show any adverse effect on the kidney weight too.

Aminotransferases are localised in periportal hepatocytes where they are involved in amino acid metabolism; transamination reactions and their serum activities presumably increase as a result of cellular membrane damage and leakage. [7, 36] Increases in serum ALT and AST indicate hepatic damage which may be associated with altered membrane permeability. Alkaline phosphatase (ALP) is a marker enzyme for the integrity of the hepatobiliary system and the flow of bile into the small intestine. Increase in serum ALP activity indicates obstructive event or cholestatic effect following chronic ingestion of a toxin. Thus, the insignificant change of aforementioned parameters observed in the rats by administration of GS, CM and GSCM at varying dose levels indicates that the plants are less toxic to the liver.

Furthermore, lack of significant change in total bilirubin levels observed in the present study, is an indication that the plants could not cause post-hepatic toxicity by an interruption to the drainage of bile in the biliary system. Evaluation of serum total protein

level is a good criterion for assessing the secretory ability of the liver. [7, 36] The slightly increased effect on the serum total protein levels observed in the experimental animals in the present study suggests that the plants, GS and CM, have the potential of improving secretory function of the liver since the bulk of total protein in the plasma is contributed by albumin.

Kidney damage is associated with decline in renal function which could lead to renal failure. However, there was no change of the kidney function indices in the extract-treated rats which is an indication of non-interference in reabsorption at the renal epithelium and a reflection of lack of impairment in the kidneys, particularly for glomerular filtration rate.

In conclusion, the plants GS, CM and their 1:1 combination are relatively safe and have the potential of being medicinal plants but should be used with caution, especially at 600mg/kg b.w.

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Comparative *In Vitro* Antioxidant Activity of Methanolic and Ethanolic Leaf Extracts of Drum Stick Leaves (*Moringa oleifera*)

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Abstract

Moringa oleifera (Moringa or drum stick tree) is highly nutritious and potential source of bioactive compounds with antioxidant activity. This study was aimed at comparing the ethanol and methanol extract of *Moringa oleifera* leaves (MOL) antioxidant activity and total phenolic content present in *Moringa oleifera* leaves. Total phenolic content (TPC), *in vitro* total antioxidant activity (TAA) and ferric ions (Fe³⁺) reducing anti-oxidant power (FRAP) estimations were determined in methanol and ethanol extracts of MOL at 100µg/cm³, 50µg/cm³ and 25µg/cm³ each, respectively. The result for all the parameters measured showed their properties are dependent on the type solvent used during extract and at the extract concentrations in which they estimated. The results showed that methanol is more efficient than ethanol in the extraction of phenolic compounds and antioxidant activity of MOL. Both parameters however depends significantly ($P < 0.05$) on extract concentration of both solvents extracts. On the over hand, ethanol extract had better reducing power and less dependent on the extract concentration of both solvent extracts. These findings are useful in exploring the applications of *Moringa oleifera* as a natural source of antioxidants and methanol appears to be the best solvent for this purpose.

Keywords: *Moringa oleifera*, Antioxidant capacity, Total phenolics, Methanol extract, Ethanol extract.

Introduction

Nowadays, there is an upsurge of interest in antioxidants. Many of the potential antioxidants have been detected in surprisingly high amounts in natural products. ^[1] Natural antioxidants are less toxic and more easily degradable by the body system than artificial on antioxidant. ^[2] Natural antioxidants comprises of, mostly, phenolic compounds. These compounds are normally found in significant quantities in plant leaves, seeds, fruits, stem, flower and roots. ^[3] When substantial amount of reactive oxygen species (ROS) and free radicals accumulated in cells, oxidative stress may occur. ^[4] Antioxidants are responsible for preventing or delaying the oxidation of products by free radicals through scavenging ^[5], and hence reduce, prevent, repair or even reverse oxidative stress. ^[6] If not dealt with, oxidative stress can cause macromolecules, enzymes,

protein and DNA to be oxidized and may cause significant damage to the intermediate metabolites producing diseases. ^[7] Appearance of antioxidants in food improves desirable properties. ^[8] Plants have been used for several centuries worldwide for traditional treatment of many diseases. ^[9] This demonstrates the potential of plants as sources of bioactive compounds; including potential antitumors, antioxidants, antiobesity and antimicrobial molecules. ^[10]

Moringa oleifera (moringa or drum stick tree); a rapidly growing perennial tree was used by the ancient Egyptians and then the Romans and Greeks not only as source of nutrients ^[11] but also as medicines, perfumes, lotions, astringent liquids and wine making. ^[12] *Moringa oleifera* has naturalized

from the tropics to the sub-Himalayan regions, Oceania, Latin America, Africa and Tropical Asia.^[13] The plant is significant source of fats, protein, beta-carotene, vitamin c, iron, potassium, and other nutrients.^[13] In addition, some parts of the plant have drawn much attention and have been studied for its various biological attributes, including anti-atherosclerotic^[14], immune-boosting^[15] antiviral^[16], antimicrobial^[17], anti-inflammatory^[18] properties and tumor suppressive effects in skin papellomagnesis^[19], hepato-carcinoma cancer^[20], colon cancer, and myeloma.^[21] Hence it has been called a “Miracle Plant”.^[22]

Solvent extraction is the most frequently used technique for the isolation of bioactive compounds from plants.^[23] The recovery of bioactive compounds from desired source is typically accomplished using various solvents, such as methanol and ethanol, as well as hot water and buffers.^[21] In majority of studies, organic solvent extracts has been shown to be more efficient than simple water extractions.^[18] Organic solvents can dissolve the many useful organic molecules found in plants, such as phenolic compounds.^[23] As such solvent extract is the first step in exploring the usefulness of the plant with desirable properties of interest. For this reason, the current research compared the efficiency of methanol and ethanol in the extraction of *Moringa oleifera* leaves’ TPC, TAA and FRAP.

Materials and Methods

Sample Collection

Fresh leaves of *M. oleifera* were obtained from Yankaba market, Nassarawa local government Kano State, Nigeria and authenticated in the Herbarium Unit, Department of Botany, Bayero University, Kano, with the accession number (BUKHAN0011).

Sample Preparation

Raw *M. oleifera* leaves were washed with distilled water, shade dried and grounded into powder. The powdered leaves were stored in clean air-tight container at 25°C for further analysis.

Extract Preparation

To 50g of MOL powder, 500 cm³ of methanol was added and allowed to stand for 48 hours at 25°C. The

soaked sample was shaken during the extraction period and subsequently filtered using Whatman No. 1 filter paper. The resulting extracts were concentrated under vacuum in a rotary evaporator at 45°C for complete solvent removal. The extract was reconstituted at 100µg/cm³, 50µg/cm³ and 25µg/cm³ with distilled water. The procedure was repeated but now with ethanol as extracting solvent.

Estimation of TPC, TAA and FRAP

In vitro total phenolic content (TPC) were determine using the Folin Ciocalteu method described by Singleton *et al.*^[24]. Ferric ions (Fe³⁺) reducing antioxidant power (FRAP) was estimated using the method described by Oyaizu^[25]. Total antioxidant activity (TAA) was evaluated using the phosphomolybdenum method described by Preito *et al.*^[26]. All the estimations were carried out at different concentrations of 100µg/cm³, 50µg/cm³ and 25µg/cm³ of both leaf extracts, respectively. Percentage differences of methanol extracts over ethanol extracts at a particular extract concentration of all parameters were calculated as:

(1) Percentage Difference(%)

(2).
$$\frac{(\text{ethanol extract value}-\text{methanol external value})}{\text{Methanol extract value}} \times 100$$

Statistical Analysis

Results are in triplicate and each were presented as mean ± standard deviation. Data were statistically analysed by one-way ANOVA with Tukey multiple comparison post-test using GraphPad InStat[®] software (2000), version 3.05 (32 bit for Win 95/NT) from GraphPad Incorporated[™] and statistical limit of significance was set at $P < 0.05$.

Results and Discussion

Results

Total phenolic content (TPC), total antioxidant activity (TAA) and ferric ions (F³⁺) reducing antioxidant power (FRAP) of methanol and ethanol leaf extracts of *M. oleifera* are presented in the Figure 1, Figure 2 and Figure 3, respectively.

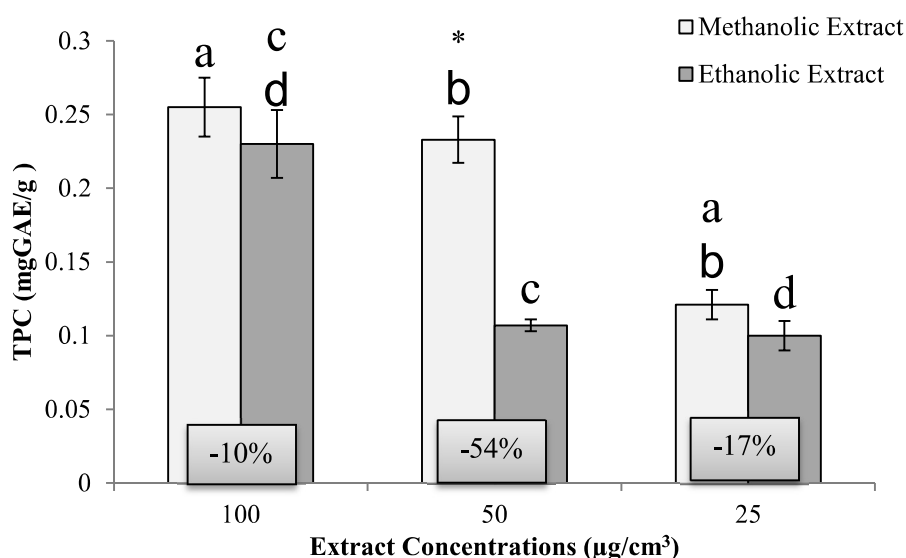


Figure 1: Total Phenolic Content (TPC) of methanol and ethanol leaf extracts of *Moringa oleifera*. Readings with the same letter differs significantly ($P < 0.05$) with each other and methanol extracts readings bearing “*” differs significantly ($P < 0.05$) with their ethanol extract counterpart at the same extract concentration.

The TPC results showed percentage differences of -10%, -54% (significant at $p < 0.05$) and -17% for methanol extract over ethanol extract at 100 µg/cm³, 50 µg/cm³ and 25 µg/cm³ of the extracts, respectively. A significant decrease ($p < 0.05$) was observed

between 25 µg/cm³ methanol extract with both 100 µg/cm³ and 50 µg/cm³ of the same solvent, respectively. For ethanol extract, the 100 µg/cm³ extract showed significant increases ($p < 0.05$) over both 50 µg/cm³ and 25 µg/cm³, respectively.

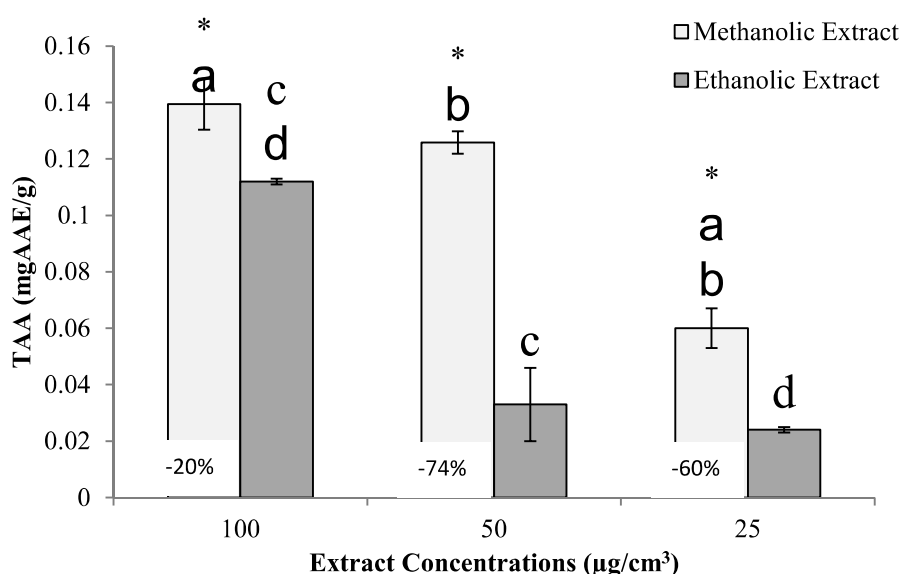


Figure 2: Total antioxidant activity (TAA) of methanol and ethanol leaf extracts of *Moringa oleifera*. Readings with the same letter differs significantly ($P < 0.05$) with each other and methanol extracts readings bearing “*” differs significantly ($P < 0.05$) with their ethanol extract counterpart at the same extract concentration.

The TAA result showed significant percentage differences ($P < 0.05$) of -20%, -74% and -60% for methanol extract over ethanol extract at $100\mu\text{g}/\text{cm}^3$, $50\mu\text{g}/\text{cm}^3$ and $25\mu\text{g}/\text{cm}^3$ of the extracts, respectively. A significant decrease ($P < 0.05$) was observed

between $25\mu\text{g}/\text{cm}^3$ methanol extract with both $100\mu\text{g}/\text{cm}^3$ and $50\mu\text{g}/\text{cm}^3$ of the same solvent, respectively. For ethanol extract, the $100\mu\text{g}/\text{cm}^3$ extract showed significant increases ($P < 0.05$) over both $50\mu\text{g}/\text{cm}^3$ and $25\mu\text{g}/\text{cm}^3$, respectively.

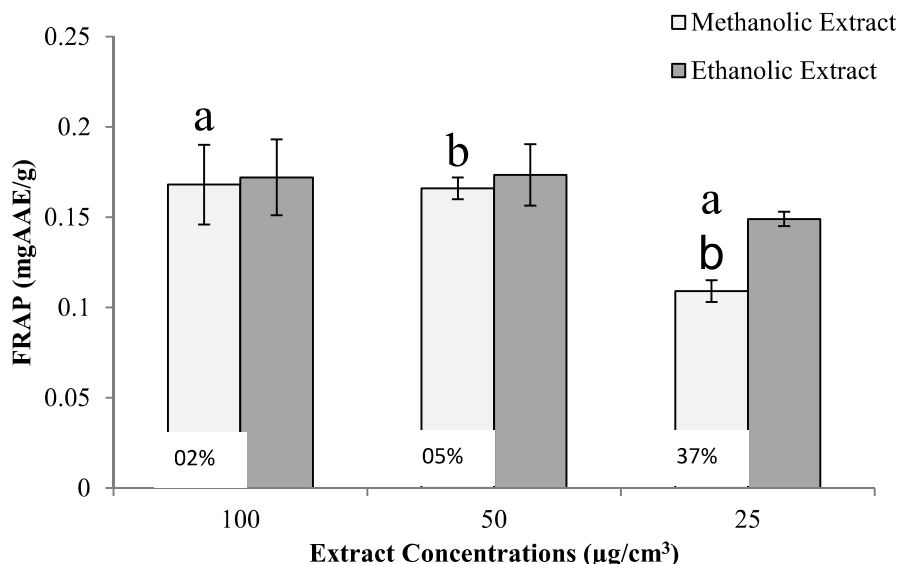


Figure 3: Ferric Ions (F^{3+}) Reducing antioxidant power (FRAP) assay of methanol and ethanol leaf extracts of *Moringa oleifera*

Readings with the same letter differs significantly ($p < 0.05$) with each other and methanol extracts readings bearing ‘*’ differs significantly ($p < 0.05$) with their ethanol extract counterpart at the same extract concentration.

The FRAP results showed non-significant ($p \geq 0.05$) percentage differences of 02%, 05% and 37% for methanol extract over ethanol extract at $100\mu\text{g}/\text{cm}^3$, $50\mu\text{g}/\text{cm}^3$ and $25\mu\text{g}/\text{cm}^3$ of the extracts, respectively. The methanol's $100\mu\text{g}/\text{cm}^3$ and $50\mu\text{g}/\text{cm}^3$ extract concentrations appear to be at the same level of reducing capability while the $25\mu\text{g}/\text{cm}^3$ extract is significantly lower ($p < 0.05$) than both the other two extract concentrations, respectively. The ethanol extracts showed similar pattern but with non-significant lowering ($p \geq 0.05$) of $25\mu\text{g}/\text{cm}^3$ extract reducing capacity.

Discussion

TPC in MOL appears to be extract concentration dependent. Methanol extracts higher TPC content compared to ethanol extracts (Figure 1) is probably due to the higher polarity of methanol [27] that is capable of solubilizing much of the polar phenolic phytochemicals in the plants leaves over ethanol. [28] This solvent polarity-dependency of methanol on

TPC is seen in green walnut fruits [29], *Zingiber officinalis* leaves [30], *Tamarix aphyllia* leaves [31], *M. vulgare* [32] and *Artemisia annua* leaves. [9] TAA showed a similar pattern of higher methanol extract activity over ethanol extract in a concentration-dependent manner (Figure 2). This could suggest that phenolics are the major contributors of antioxidant activity in *M. oleifera* leaves owing to the similar patterns of TPC and TAA observed in the current research. However, both methanol and ethanol have relatively similar polarities as such it did not really explain the noticeable differences observed between the two solvents extracts in both TPC and TAA results. The reason could, however, be due to low solvation provided by ethanol, probably because of the presence of the ethyl radical that is longer than the methyl radical present in methanol resulting in a lower solvation of antioxidant molecules in *M. oleifera* leaves. [33] The result for FRAP, however showed an opposite pattern with ethanol extracts

presenting a higher reducing powers than methanol extracts with lesser dependency on concentration (Figure 3). This higher ethanol extract FRAP was also observed in green walnut husk^[34], blueberry leaf^[35] and *B. Retusa*.^[36] Phenolic compounds are believed to exerts their antioxidant activity by reducing free radicals to stable compounds via electron transfer while they themselves (the phenols) becomes stable radicals.^[37] This electron transfer is what the reducing power assays are attempting to quantify.^[38] However the FRAP readings in the current research appears to contradict the results observed in TCA and TAA of methanol extract having higher TPC and consequently higher TAA. This could imply that phenolic compounds extracted from *M. oleifera* leaves suggested to be responsible for TAA observed do so via a different electron transfer process not necessary the one modelled under the FRAP procedure of singlet electron reduction of ferrous to

ferric ion^[38] used in the current research. Furthermore, it insinuates that ethanol dissolves a bit more antioxidants that follow the singlet electron transfer mechanism that have lesser contribution to TAA of the plant.

Conclusion

The results of this study clearly indicate that all investigated *Moringa oleifera* leaf extracts have antioxidant activity which is influence by extract concentration. It also indicated that the methanol extract of *Moringa oleifera* leaf showed a better phenolic contents and potential antioxidant activity over that of ethanol leaf extract while ethanol extract showed a better reducing power capability over the methanol extract. These findings are useful in investigating the applications of *Moringa oleifera* as a natural source of antioxidants.

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Characterisation and Nutritional Quality of Bambaranut (*Vigna subterranea* (L) Verdc.) Protein Grown in Madobi LGA, Kano State, Nigeria

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Abstract

Bambaranut (*Vigna subterranea* (L) Verdc.) is one of the neglected leguminous crops in Kano, State, Nigeria, despite its potential nutritional contents. This study was aimed at evaluating the amino acids content and nutritional quality of bambaranut protein grown in Madobi local government of Kano state, Nigeria. The amino acids content was determined using Technicon sequential Multi-sample Amino acid Analyzer. The total amino acid content of bambaranut was 94.76g/100g protein, with total essential and nonessential amino acids of 43.83g/100g protein (46.25%) and 50.93g/100g protein (53.75%) respectively. The total aromatic and sulphur containing amino acids were 11.69g/100g protein (12.34%) and 2.87g/100g protein (3.02%) respectively. All the essential amino acids, aromatic and sulphur containing amino acids are present in good proportion and exceed the WHO/FAO/UNICEF standard values except leucine, which is below the standard value for children. The result indicates that bambaranut has an excellent amino acid profile and could be used as an ingredient in high nutrient processed foods and amino acids supplements.

Keywords: Bambaranut, Protein, Amino acids profiling, Nutrition

Introduction

World demand for proteins is increasing from both conventional and new sources of protein. Although all proteins have nutritional value, food industry requires that the protein should have good functional properties to be acceptable as a food ingredient. ^[1] Protein plays an important role in biochemical, biophysical and physiological processes. Proteins in the body come from both plant and animal sources. ^[2] Presently there is growing evidence that cereals and legumes play important roles in the prevention of chronic diseases. ^[3] Grain legumes serve as cheap sources of protein to a large proportion of the population in poor countries of the tropics. Bambaranut (*Vigna subterranea* (L) Verdc.), an indigenous African legume, plays an important socio-economic role in the semi-arid regions of Africa. It is a rich source of protein and along with other local sources of protein could help to alleviate nutritional

problems in these areas. As an underutilised crop in the past, bambaranut has not received sustained research input largely because most funding agencies are not willing to support research on crops of unproved potential and unknown commercial value. ^[4] Nevertheless, despite the uncertainties in research sponsors, in recent years there has been a growing awareness of its potential to contribute to food production in Africa and the need to improve the crop. ^[4]

One of the most disturbing and prevalent nutritional problems in developing countries is protein-energy malnutrition. ^[5] The cause has been attributed to many factors including high cost of first class proteins. The use of cereal-legume based food is therefore advocated as alternative protein and energy source for infant and adult food products. Bambaranut could be

a good source of protein, lipid, carbohydrate and mineral elements thus has nutritional potentials that can alleviate nutritional problems when properly explored. ^[6] The oil of bambaranut contains both saturated and unsaturated fatty acids and also contains 1-(+)-Ascorbic acid 2, 6-dihexadecanoate which is vitamin C fatty acid conjugate, indicating the presence of vitamin C. The fatty acids present include arachidic acid (eicosanoic acid). ^[7] The presence of unsaturated fatty acid in bambaranut indicates that it could play a significant role in cardiovascular fitness while that of saturated fatty acids implies that it could be a non-carbohydrate source of energy. This study is aimed at evaluating the amino acids content and nutritional quality of bambaranut proteins grown in Madobi local government of Kano state, Nigeria.

Materials and Methods

Sample Collection

The sample (bambaranut pods) used in this research was collected from farmers in Madobi. The sample was dried and the seeds were removed from the hulls and brought to the laboratory, ground using a mortar and the flower obtained stored in a dry place.

Amino Acid Analysis

The amino acid profile in the sample was determined using the method described by Benitez, 1989. ^[8] The sample was dried to constant weight, defatted, hydrolysed, evaporated in a rotary evaporator and loaded into the Technicon Sequential Multi-Sample Amino Acid Analyzer (TSM). Crude protein was determined by the micro-Kjeldahl method. ^[9]

Hydrolysis of Sample and Amino Acid Profile

A portion of the defatted sample was weighed into glass ampoule. A quantity (7cm³) of 6N HCl was

added and oxygen was expelled by passing nitrogen into the ampoule (to avoid possible oxidation of some amino acids like methionine and cystine during hydrolysis). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105°C ± 5°C for 22 hrs. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humus. It should be noted that tryptophan is destroyed by 6N HCL during hydrolysis. The filtrate was then evaporated to dryness at 40°C under vacuum in a rotary evaporator. The residue was dissolved with 5cm³ of acetate buffer (pH 2.0) and stored in plastic bottles and kept in the freezer.

Determination of Protein Quality

The essential amino acid score was calculated using the formula: amino acid score = amount of amino acid per test protein (g/100g)/amount of amino acid per protein in reference pattern (g/100g). ^[10] Amino acid score for essential and non-essential amino acid was calculated based on whole hen's egg. ^[11] Percentage total essential amino acid (TEAA) in total amino acid (TAA) i.e. (%TEAA/TAA), percentage total non-essential amino acid (TNAA) in TAA that is (%TNAA/TAA), total sulphur amino acid (TSAA), percentage cystine in TSAA (%Cys/TSAA), total aromatic amino acid (TArAA), and the Leucine/Isoleucine ratios were calculated. The predicted protein efficiency ratio (P-PER) was determined using the equation: ^[12] P-PER= - 0.468 + 0.454 (Leu) – 0.105 (Tyr).

Results and Discussion

The amino acid concentration, amino acid score and other parameters in respect of amino acid contents of bambaranut are presented in Table 1 through 3.

Table 1: Amino acid concentrations of bambaranut (*Vigna subterranea*) grown in Madobi local government, Kano state, Nigeria

Amino acid	g/100g Protein
Lysine (Lys)*	6.92
Histidine (His)*	3.06
Arginine (Arg)*	6.73
Aspartic acid (Asp)	11.35
Threonine (Thr)*	3.68
Serine (Ser)	5.21
Glutamic acid (Glu)	16.66
Proline (Pro)	4.27
Glycine (Gly)	4.01
Alanine (Ala)	4.48
Cystine (Cys)	1.46
Valine (Val)*	4.75
Methionine (Met)*	1.41
Isoleucine (Ile)*	4.06
Leucine (Leu)*	8.08
Tyrosine (Tyr)	3.49
Phenylalanine (Phe)*	5.14

*essential amino acids

Table 2: Amino acid score for bambaranut (*Vigna subterranea*) grown in Madobi local government, Kano State, Nigeria

Amino acid	Amino acid (g/100g protein)	WHO Ideal Protein (g/100g protein)		[(%Amino acid/ideal) x 100]	
		Children	Adult	Children	Adult
Isoleucine	4.06	2.8	1.3	145.00	312.31
Leucine	8.08	8.3	6.6	97.35	122.42
Lysine	6.92	4.2	5.8	164.76	119.31
Histidine	3.06	1.9	1.6	161.05	191.25
Valine	4.75	4.4	3.5	107.95	135.71
Threonine	3.68	3.0	3.4	122.67	108.24
Total Sulphur	2.87	1.6	2.5	179.38	114.80
Total Aromatic	11.69	7.4	6.3	157.97	185.56

Table 3: Parameters of bambaranut (*Vigna subterranea*) protein grown in Madobi local government, Kano State, Nigeria.

Amino acid	g/100g protein	Percentage (%)
Total amino acid (TAA)	94.76	
Total non-essential amino acid (TNEAA)	50.93	53.75
Total essential amino acid (TEAA)	43.83	43.02
No Histidine	40.77	
Total neutral amino acids (TNAA)	11.76	12.41
Total acidic amino acid (TAAA)	28.01	29.56
Total basic amino acid (TBAA)	16.71	17.63
Total sulphur amino acid (TSAA)	2.87	3.02
% Cys in TSAA		50.87
Total aromatic amino acid (TArAA)	11.69	12.34
Predicted protein efficiency ratio (P-PER)	2.83	
Leucine/Isoleucine ratio	2.00	
Leucine – Isoleucine (difference)	4.02	4.24

The concentrations of amino acids in the studied bambaranut are presented in Table 1. Only seventeen amino acids were detected in the current study; this might be due to a complete destruction of tryptophan during acid hydrolysis and the conversion of amide glutamine and asparagine to their corresponding amino acids. [13] The percentage of the essential amino acids, total sulphur and aromatic amino acids were compared with the reference standard value established by WHO [14] for both adult and school children (Table 2). The values obtained in the current study for essential amino acids, sulphur amino acids and aromatic amino acids were higher than the reference value for both adults and children except that of leucine in children (Table 2). The total non-essential amino acids (50.93g/100g protein) were higher than essential amino acids (43.83g/100g protein) (Table 3). The findings of the present study supports the report that bambaranut could be an excellent source of protein. [6]

The total amino acid (TAA) found in this study (94.76g/100g protein) was higher than that of degutted white grubs (82.89g/100g protein), [15] African yam bean (91.70g/100g protein), [16] cowpea (64.0g/100g protein), [17] *A. occidentale* and *C. acumina* (65.90g/100g protein and 35.60g/100g protein respectively) [18] and that of *Monodora myristica* (65.60g/100g protein). [2] The result of the current study indicated that bambaranut has a commendable essential amino acid composition. Dietary essential amino acids deficiency was reported to remarkably decrease food intake. [19, 20] Depletion of a single essential amino acid leads to quick deacylation of its (essential amino acid) tRNA and the levels of charged tRNA fall, leading to disruption of global protein synthesis. [21]

The total essential amino acid (TEAA) 43.83g/100g protein found in this study was comparable to that of soya bean (44.40g/100g protein), [22] pigeon pea (45.20g/100g protein), [23] cowpea (42.60g/100g protein) [24] and *Cajanus cajan* (43.60g/100g protein). [25] The TEAA of 43.83g/100g protein was higher than that of *M. myristica* (31.25g/100g protein), [2] Kersting's groundnut (*Macrotyloma geocarpum*, 32.70g/100g protein) [17] and degutted white grubs (31.48g/100g protein). [15] This finding indicates that bambaranut can be used in malnourished children

especially in kwashiorkor where there is decrease in essential amino acids, usually the branched chain amino acids leucine, isoleucine and valine. Looking at the amino acid profiles (Table 2), bambaranut can be used to replenish these amino acids. The result also indicated that bambaranut used in the study are rich in lysine (Table 2) an amino acid that enables the synthesis of carnitine, which converts fatty acids into energy and also plays an important role in the production of hormones, antibodies and enzymes. Having a deficiency in lysine can lead to niacin deficiency and causes pellagra characterised by dermatitis, diarrhoea, and dementia.

Valine is necessary for muscle metabolism and the repair of tissues and can be useful in the treatment of liver and gallbladder disorders. Methionine aids in the production of sulphur, which is necessary for normal metabolism and it is also essential for the synthesis of haemoglobin and glutathione that fights against free radicals while isoleucine is important in the regulation of blood sugar. Leucine increases muscle mass, helps muscle recover after exercise and regulates blood sugar. Clinically leucine helps the body heals, affects brain function and can be used in place of glucose in fasting states. Phenylalanine is a precursor to catecholamines that regulate the central and peripheral nervous system, while threonine is important for antibody production. [26] Significant decrease in leptin and insulin is related with the consumption of essential amino acid deficient diet. [21] The hepatic triglyceride levels tends to be elevated upon consumption of diet deficient in lysine whereas consumption of diet deficient in methionine, tryptophan, valine and threonine tend to decrease the hepatic triglycerides. Feeding of essential amino acid-deficient diet was also reported to disturb estrous cycles in female rats. [21]

The total non-essential amino acid of 50.93g/100g protein found in the current study was comparable to that of degutted white grubs (51.41g/100g protein) [15] and higher than that of kersting's groundnut and cowpea (41.40g/100g protein and 36.10g/100g protein respectively), [17] *A. occidentale* (32.30g/100g protein), African yam bean (45.4g/100g protein) [18] and *Monodora myristica* (34.35g/100g protein). [2] In kwashiorkor, the non essential amino acids tyrosine and arginine are very low; bambaranut could

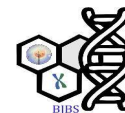
therefore be used to correct the deficiency. The total sulphur containing amino acids (TSAA) 2.87g/100g protein was lower than that of degutted white grubs (3.35g/100g protein) ^[15] and higher than that of *Monodoramyristica* (1.19g/100g protein), ^[2] cowpea and kersting's groundnut (2.10g/100g protein and 2.60g/100g protein respectively). ^[17] In terms of total aromatic amino acid (TArAA) the value of 11.69g/100g protein found in this study was higher than that of degutted white grubs (8.02g/100g

protein), ^[15] cowpea and kersting's groundnut (3.90g/100g protein and 4.40g/100g protein respectively) ^[17] and *Monodoramyristica* (3.98g/100g protein). ^[2] Based on the findings of this study it could be inferred that Bambaranut has a commendable amino acid profile that could be used to support the nutritional need of the society and can also be used as amino acid supplement in baby foods and in pharmaceutical industries.

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Growth Kinetics Modelling of *Klebsiella* spp. FIRD 2 on TBT-Resistant Containing Zinc

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Abstract

Tributyltin (TBT) is an organotin that is widely used as a component of antifouling paints, wood preservative, pesticide, fungicide and bactericide. Kinetic equations, which describe the activity of microorganism or an enzyme on a particular substrate, are crucial in understanding many phenomena in biotechnological processes. They allow the mathematical prediction of growth parameters important for identifying key parameters for controlling growth. This study modeled growth kinetics of TBT-resistant *Klebsiella* sp. FIRD 2 containing various concentrations of zinc using different kinetic models such as Haldane, Teissier, Monod, Aiba, Luong and Webb and then evaluated the accuracy of the fitted models using statistical analysis such as coefficient of determination, adjusted coefficient of determination (R^2) and root mean square (RMSE). The study indicated that Luong model was fitted to the experimental growth kinetics data and gave a very good fit with an R^2 of 0.9921 and RMSE of 0.00169 respectively. The calculated value for the Luong constants such as maximal growth rate, half saturation constant and half inhibition constant rate symbolised by μ_{max} , K_S , and K_i were 0.03493 hr⁻¹, 0.338 mg/dm³ and 0 mg/dm³ respectively. Luong model also predicted the significant substrate concentration (S_m) value at which specific substrate degradation rate falls to zero (102.2 mg/dm³). The growth kinetic parameters calculated using these models showed better tolerance to various zinc concentrations.

Keywords: Growth, Kinetics models, *Klebsiella* sp. FIRD 2, TBT-resistance, Zinc.

Introduction

Heavy metals are group of metals with density greater than 5 g/dm³. They persist in nature and consequently tend to accumulate in food chains.^[1] Although relatively high levels of these elements occur in natural environment, their presence as a contaminant in ecosystems result mainly from anthropogenic activities.^[2] Some heavy metals such as cadmium, silver, and mercury have no biological role and are harmful to the organisms, even at very low concentrations.^[3] Others like zinc, copper, iron, and nickel are essential to metabolic reactions and

are required as trace elements by the organisms. Tributyltin (TBT) is an organotin that is widely used as a component of antifouling paints, wood preservative, pesticide, fungicide, and bactericide. Even though in developed countries, the manufacture, use and export of TBT have been in existence since the 1990s by the International Maritime Organisation (IMO), some countries have continued to utilise this agent until its complete prohibition, which was scheduled to go into effect in 2008.^[4] Consequently, TBT pollution has been

recognised as a serious problem around the world. More so, there are high concentrations in sediments from fresh and marine waters in many places across the world including Strait of Johore, Malaysia, South Africa, north-west coast of Portugal, Australia among others.^[5,6] Organotin compounds are highly toxic to many prokaryotic and eukaryotic organisms and have also been identified as immune system inhibitors and endocrine disruptors in humans.^[7,8] In many marine organisms, its toxic effect is also recognised; it causes immunosuppression, growth suppression and imposex in higher animals.^[9]

Mendo *et al.*,^[10] reported that organotin compounds are toxic to both Gram positive and Gram negative bacteria collected from sediment; nevertheless, the former showed increased sensitivity to triorganotin compounds. It has been reported that trisubstituted organotin compounds (TBT) are more toxic than di-substituted (DBT) and monosubstituted (MBT) compounds, and seem to exert toxicity through their interaction with membrane lipids.^[5]

Previously, TBT-resistant bacterium was isolated from contaminated surface sediment along Strait of Johor, Malaysia. The bacterium was identified as *Klebsiella* sp. FIRD2.^[11] Several works were reported on TBT-resistance, growth and degradation processes. Astonishingly, no work has so far been published on growth kinetics of TBT-resistant bacterium containing zinc based on specific substrate consumption. This study therefore presents the growth ability of *Klebsiella* spp. FIRD2 containing various zinc concentrations and the effect of initial substrate (TBT) concentration on its degradation capacity.

Materials and Method

Chemicals and Media

The chemicals (Tributyltin chloride (TBTCl) and 96% Methanol HPLC grade) used were purchased from Sigma-Aldrich Co. USA. All kinetic experiments were conducted with the Minimal Salt Media containing (g/L): 5 NaCl, 5 NH₄Cl, 0.01 CaCl₂, 0.2 MgSO₄·7H₂O, 0.01 FeSO₄·7H₂O, 1.0 KH₂PO₄, 5-yeast extract. The media contain 1000 µg/L of TBT and various concentrations of zinc in addition to the above compositions. TBT stock was prepared from 96% TBTCl, 21,000 µg/L (1 µL in 20

mL methanol) and kept in the dark at 4°C.^[12] All gradient concentrations were prepared using the dilution formulae $M_1V_1=M_2V_2$ from the stock.

Kinetic Experiments

Shake flask studies were carried out in 250 cm³ Erlenmeyer flask containing 50 cm³ of the TBT medium and incubated on a rotary shaker at 150 rpm for 48 hrs at room temperature (27°C). Samples were drawn after every 6 hrs and TBT growth-containing zinc was measured. The initial temperature and pH of the medium were adjusted to room temperature and neutral pH. The seed culture was transferred to 25cm³ of TBT liquid media containing various initial zinc concentrations ranging from 1 to 100 mg/dm³ in the 250 cm³ Erlenmeyer flask. Samples were collected at different time intervals and measured for cell growth.^[13-16]

Mathematical Model

Agarwal *et al.* ^[16] reported that inhibition of substrate occurs at high substrate concentrations. Primarily, it is caused by more than one substrate molecule binding to the active site, and/or often by different parts of the substrate molecules binding to different sub-sites within the substrate-binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction.^[16] At various initial zinc concentrations, the specific growth rates (μ) have been calculated and data were tested in different deterministic models as per Table 1, which shows different substrate inhibition models that have been used to explain cell growth kinetics of copper. All the kinetic models were fitted to the experimental data by using a curve fitting toolbox available from MATLAB R2012a based on Windows vista.^[17]

The bacterial growth rate can be represented as cell production rate. The formula for various kinetics models is as shown in Table 1, where S , S_m , μ , μ_{max} , K_s , K_i and n are specific substrate concentrations (mg/dm³) above which cell growth of TBT-resistant bacterium containing zinc completely stops (mg/dm³), cell growth rate (hr⁻¹), maximum cell growth rate (hr⁻¹), saturation constant or half velocity constant (mg/dm³), inhibition constant (mg/dm³) and the exponent representing the impact of the substrate to μ_{max} , respectively. For each

initial concentration of zinc, specific growth rate was calculated based on the linear portion of the growth against time in an exponential phase. The specific growth rate (μ) in exponential phase was calculated by the following equation:

Where, X_1 and X_2 are the cell dry weight obtained at time t_1 and t_2 , respectively. All experiments were conducted in triplicates under identical conditions and all results had a mean standard deviation.^[13]

Table 1: Various Kinetic Models for Effect of Substrate on TBT Cell Growth Containing Various Zinc Concentrations

Author	μ (Growth rate)	References
Monod		[18]
Haldane		[19]
Luong		[20]
Aiba		[21]
Teissier		[22]
Webb		[23]

Statistical Analysis

To decide whether there is a statistically substantial difference between models with different number of parameters, in terms of the quality of fit to the same experimental data was statistically evaluated through various methods such as the root-mean-square error (RMSE), coefficient of determination (R^2) and the adjusted coefficient of determination (R^2).^[24]

Result and Discussion

Roane *et al.*^[25] and Okpokwasili and Nweke^[26] reported that in sites environmental contamination with organic compounds and heavy metals inhibits the activity of organic degrading microorganisms, impacting both their physiology and ecology, thus reducing the growth and biodegradation rate of the organic compounds. Heavy metals toxicity to microorganisms is dependent on its bioavailability. Quantification of bioavailable metal concentration is an important step in the process of standardising experiments to determine the impact of metals on organic pollutant growth and biodegradation.^[26] The

effect of various zinc concentrations on TBT-resistant growth kinetics was determined by measuring the cell growth rate at different time intervals for 48 hrs with different initial concentrations of the heavy metals.

Effect of Initial Zinc Concentration on Growth Curve

Figure 1 shows the time course profile of the TBT-resistant growth curve of *Klebsiella* sp. FIRD 2 by the shake flask culture. The culture is able to resist up to 40 mg/dm³ zinc in almost 48 hrs. It was observed that the time taken for the culture to grow in the presence of zinc is dependent on the initial concentration of the zinc. It was found that as the zinc concentration increased, the bacterial growth decreased signifying that the high zinc concentration has effect on the bacterial growth. At a concentration of 1 mg/dm³ the bacterial isolate grows optimally with an OD₆₀₀ of 1.652 while at a concentration of 100 mg/dm³, it inhibits the growth of *Klebsiella* sp. FIRD 2 as shown in Figure 1.

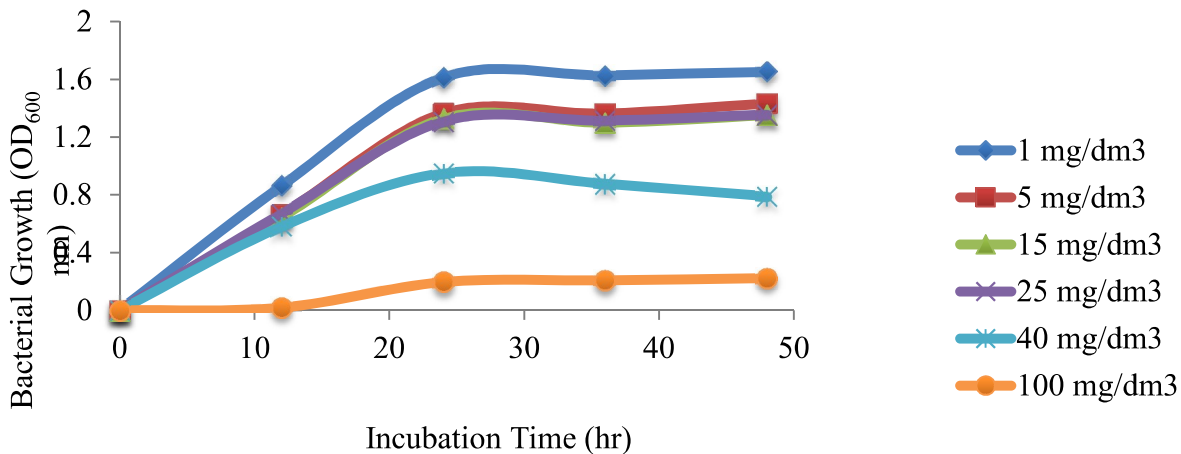


Figure 1: Effect of Various Zinc Concentrations on *Klebsiella* spp. FIRD 2 Growth Containing TBT. Data Represent Mean $\pm$ STDEV, n=3.

Effect of Initial Substrate Concentration on Specific Growth Rate

Based on the growth curves of *Klebsiella* sp, FIRD 2, the specific growth rate (μ) at each initial zinc concentration (S) was calculated. The plot shows a definite increase in cell growth rate with increase in zinc concentration until 1 mg/dm³, beyond which there was a decrease in cell growth rate as zinc concentration increased, signifying zinc inhibition kinetics. A plot of specific growth rate vs. specific substrate consumption rate containing zinc (Figure 2) according to Equation (1) was used to evaluate the growth of the isolate, which is a valuable tool in biotechnology. This relationship is represented by a set of empirically derived rate laws referred to as

theoretical models. These models are nothing but mathematical expressions generated to describe the behaviour of a given system.^[14,26] The classical models, which have been applied to microbial population growth, include the Gompertz and Verhulst function.^[26,27] The Gompertz function was originally formulated for actuarial science for fitting human mortality data but it has also been applied deterministically to organ growth.^[28] The Gompertz function is based on an exponential relationship between specific growth rate and population density.^[26]

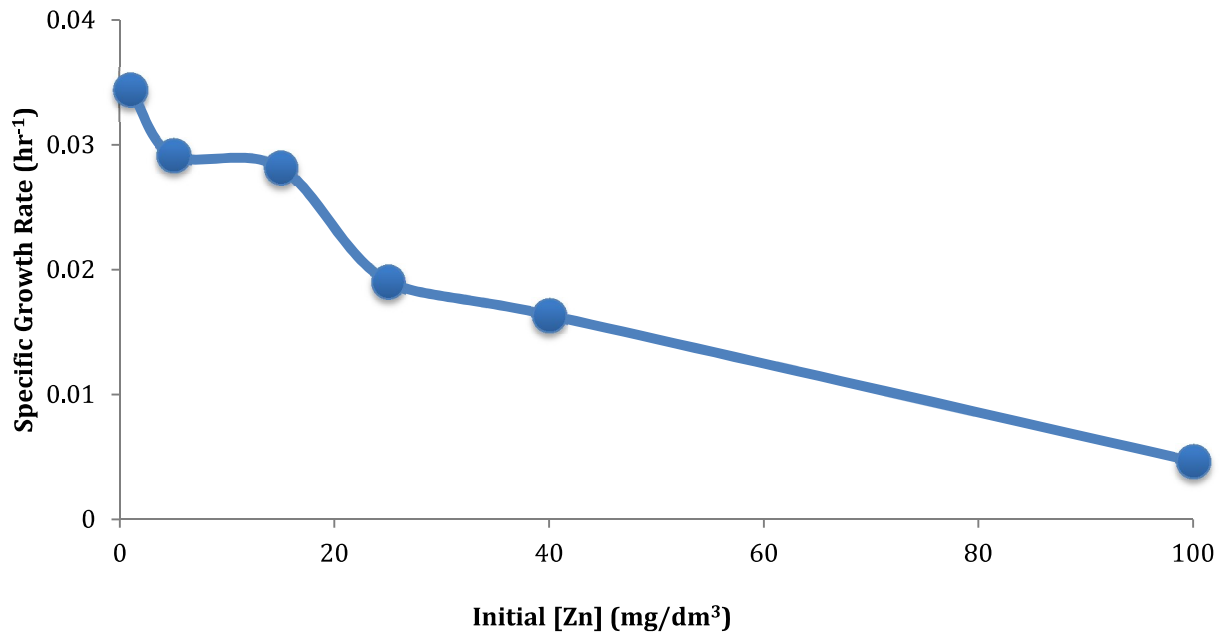


Figure 1: Replotted Data of the Specific Growth Rate against the Initial Substrate (TBT) Concentration Containing Various Concentrations of Zinc

Microbial Growth Kinetics

The results of the curve fitting are shown in Figure 3 as the data from the experimental values in batch studies were fitted to kinetic models. Models such as Teissier and Monod failed to fit the experimental data as their correlation coefficient R^2 was very low. All of the other models tested gave reasonably good fitting based on software output and by visual observation. The accuracy and statistical analysis of the six kinetic models used shows that Luong was

the best model with the highest value for adjusted R^2 and the lowest value for RMSE. The R^2 and adjusted R^2 values were also excellent for Luong with their values being close to 1.0 as shown in Table 2, which could be attributed based on the models themselves, which are considered more refined from the standpoint of development of these models.^[29]

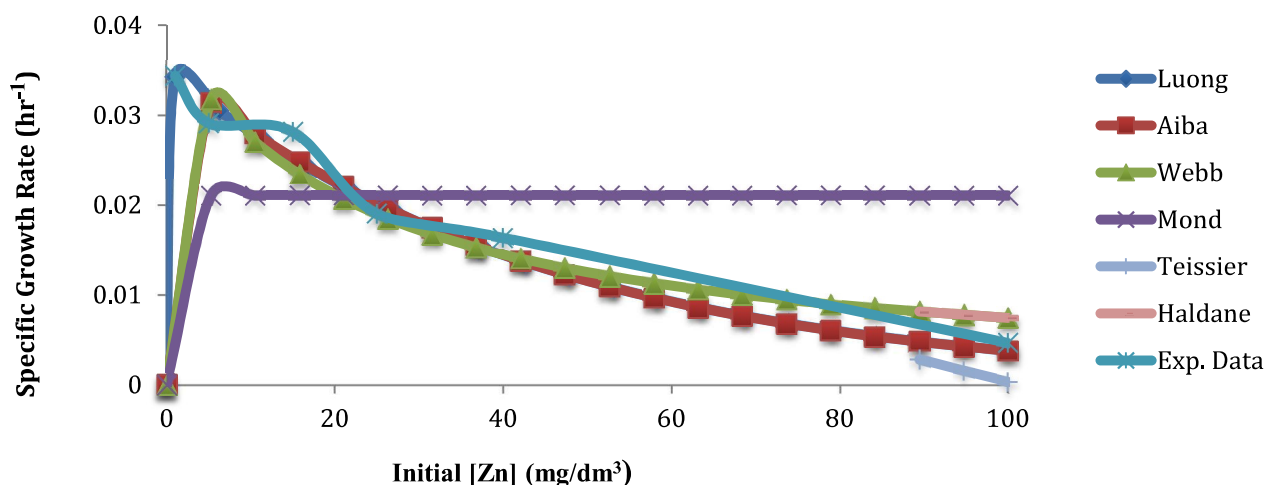


Figure 3: Zinc Growth Kinetics Resistance Experimental Values with Six Different Kinetic Models.

The growth kinetic constant of the batch culture as shown in Table 2 reported the value μ_{max} and K_s by nonlinear regression method as per Monod. The calculated value for the Luong constants in this work such as maximal growth rate, the Monod half saturation constant, the half inhibition constant, the maximum substrate inhibitory concentration rate and n symbolised by μ_{max} , K_s , and S_m were 0.03493hr^{-1} , 0.338mg/dm^3 , and 102.2 mg/dm^3 respectively. The value of μ_{max} estimated by Luong model (0.03493hr^{-1}) was closer to the experimental value of 0.03442hr^{-1} obtained at 1 mg/dm^3 . In addition, Luong model predicted maximum substrate inhibitory

concentration rate (S_m) of 102.2 mg/dm^3 beyond this concentration, there will be no growth on TBT containing zinc will be observed which was closer to the experimental value of 100 mg/dm^3 . The exponents represent the impact of the substrate to μ_{max} (n). The constant ' n ' was found to be 1.7324 , indicating a non-linear correlation between specific growth and the initial substrate concentration. This work is in accordance with the result obtained by Othman *et al.*,^[30] where it was reported that Luong model is the best model that fitted the experimental data for the reduction of Molybdenum-to-Molybdenum blue by *Bacillus* spp. strain A.rzi with an R^2 of 0.99 .

Model	$\mu_{max}(\text{hr}^{-1})$	K_s (mg/dm^3)	$K_i(\text{mg/dm}^3)$	$K(\text{mg/dm}^3)$	$S_m(\text{mg/dm}^3)$	R^2	Adjusted R^2	RMSE	n
Haldane	0.03935	0.119	26.07			0.9199	0.8799	0.004649	
Teissier	1.015	13.1	14.09			0.5675	0.3513	0.0108	
Monod	0.01878	0.4649				0.5371	0.4446	0.009998	
Luong	0.03493	0.383			102.2	0.9921	0.9841	0.00169	0.7324
Aiba	0.03587	0.3667	48.15			0.9699	0.9549	0.002848	
Webb	0.03456	0.1977	36.93	5.162		0.9098	0.8198	0.005698	

Table 2: Statistical Analysis of Kinetic Models.

In most of the studies concerning substrate inhibition on microbial growth are carried out using toxic substrate such as aromatic hydrocarbons and hence it can be concluded that at high concentration growth rate will be severely affected and the normal use of the Monod model will fail.^[24] There were other discontinuous models for describing substrate inhibition kinetics developed.^[31] These discontinuous model was developed because the previous models developed such as Webb, Andrews And Noack, and Haldane describe inhibitory effect on microbial growth but could not explain or adequately model for certain situations where the growth rate completely become zero at very high substrate concentration. However, the discontinuous model of the Wayman and Tseng is a major drawback.^[32] Luong^[20] developed a continuous type of the model above that has popular support due to its close agreement to experimental data in some cases.^[24,30,33,34] A central attraction of the Luong

model is its ability to successfully predicts the value of S_m , the maximum substrate concentration above which growth is completely inhibited.

Conclusion

Growth and degradation kinetics of bacteria can be modeled using different models to ascertain the most effective one. This study reveals that Luong model was fitted to the experimental growth kinetics data and gave a very good fit with an R^2 of 0.9921 and RMSE of 0.00169 respectively with regard to *Klebsiella* spp, FIRD 2 growth in various zinc concentrations. The calculated value for the Luong constants such as maximal growth rate, half saturation constant and half inhibition constant rate symbolised by μ_{max} , K_S , and K_i , were 0.03493 hr⁻¹, 0.338 mg/dm³, and 0 mg/dm³ respectively. Amongst all the kinetic models, Teissier gave a poor R^2 of 0.3515.

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Effect of Angiotensin Converting Enzyme Inhibitor (Captopril) on Liver and Kidney Function Parameters of *Plasmodium berghei*- Infected Mice

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Abstract

Recent discoveries indicate the potential role of the renin-angiotensin system in malaria infection, and the type of antihypertensive medication taken has been shown to influence the development of malaria in susceptible individuals. In the present study, the effect of captopril on serum liver and kidney function parameters was assessed in *Plasmodium berghei*-infected mice. A total of 40 apparently healthy mice were randomly divided into five groups of 8 mice each; normal control group (not infected and untreated), malaria control (malaria infected, untreated), standard control (malaria infected, treated with 0.03mg/kg of standard drug; Artemeter 20mg + Lumefantrine 120mg), group 4 (malaria infected and treated with 0.03mg/kg of captopril) and group 5 (malaria infected and treated with 0.09mg/kg of captopril). The mice were treated for 14 days and parasitemia monitored every other day. On the 15th day, mice were sacrificed and blood obtained to determine the level of serum liver enzymes (aspartate transaminase, alanine transaminase, and alkaline phosphatase), total protein, serum electrolyte concentration (HCO_3^- , Cl^- , Na^+ , and K^+), urea and creatinine. From the results, there was a significant increase ($P < 0.05$) in parasitemia, level of serum liver enzymes, urea and creatinine after induction of malaria. This decreased significantly ($P < 0.05$) when mice were treated with 0.03mg/kg of standard drug (Artemeter 20mg + Lumefantrine 120mg) and 0.09 mg/kg of captopril. Also, serum total protein, Na^+ , HCO_3^- , and Cl^- decreased significantly ($p < 0.05$) in the malaria control group but increased significantly when treated with 0.03mg/kg of standard drug (Artemeter 20mg + Lumefantrine 120mg) and 0.09 mg/kg of captopril. There were no significant differences in parasitemia and levels of creatinine, urea and electrolytes in mice treated with 0.09 mg/kg captopril and control mice. This study has shown that captopril, may be safe as an antihypertensive in patients with plasmodium infection and may confer some form of protection against liver and renal disorders in infected individuals.

Keywords: *Plasmodium berghei*, Malaria, Captopril, Liver, Kidney

Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoan belonging to the *Plasmodium* family.^[1] The disease is wide spread in tropical and sub-tropical regions,^[2] including much of sub-Saharan Africa, Asia and Latin America. In 2015, there were 214 million cases of malaria worldwide.^[3] This resulted in an estimated 438,000 deaths, 90% of which occurred in Africa.^[4] The rates of the disease decreased from 2000 to 2015 by 37%.^[3] Five species of *plasmodium* infect humans,^[2] but most deaths are

caused by *Plasmodium falciparum*. *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* generally cause a milder form of malaria.^[1,2] The species *Plasmodium knowlesi* rarely causes disease in humans.^[1]

The malaria parasites usually affect the kidney, liver and brain,^[5] and severity of the infection can be determined by both renal and hepatic malfunction. The clinical manifestations of renal involvement associated with several infectious diseases including

malaria are electrolyte imbalance and mineral disturbances.^[6] Serious cases of renal problems are usually associated with malaria, and without treatment, can lead to renal failure.^[7] Common electrolytes that are measured for clinical routine investigation in the blood include sodium, potassium, chloride, and bicarbonate. Sodium (Na^+) is the major cation of extracellular fluid that regulates the distribution of water and osmotic pressure in various body fluids;^[8] while Potassium is a crucial electrolyte for accurate functioning of all body cells, tissues and organs. It is particularly important in skeletal and smooth muscle contraction.^[9] These electrolytes play important roles in maintenance of pH, regulation of heart and muscle function, electron transfer reactions as well as serving as cofactors for enzymes. Disorders of water balance include hyponatremia, hypernatremia, hypokalaemia and hyperkalaemia, which also occur in conditions of electrolyte disturbance, such as severe malaria infection.^[10,9] With respect to the liver, exoerythrocytic forms of the malaria parasite invade the liver and so, cause the activities of liver enzymes in serum to increase, an evidence of liver dysfunction and compromise in membrane integrity.^[11]

The renin angiotensin-aldosterone system is one of the most important regulatory systems of blood volume, arterial blood pressure and cardiovascular homeostasis. Angiotensin II is the principal effector hormone of renin angiotensin-aldosterone system in vascular biology mediating effects through two receptors: Angiotensin type I and II receptors.^[12] New findings highlight the importance of host specific signaling pathways that can control parasite invasion and development.^[13] Some reports have shown that Angiotensin peptides can induce impairment of erythrocytic cycle of plasmodium, reducing parasite growth *in vitro*.^[14,13] It has been reported that treatment of *plasmodium* infected mice with captopril, known to reduce angiotensin II level decreases parasitemia and protect against experimental cerebral malaria.^[15] Other studies have reported the protective effect of angiotensin II and other peptides of renin angiotensin aldosterone system (RAAS) in malaria outcome.^[16,17] However, there is paucity of information on the effect of these treatments on kidney and liver function, and ultimately, malaria outcome.

Materials and Methods

Chemicals and Reagents Reagent kits for alkaline phosphatase, aspartate amino transferase, alanine amino transferase, urea and creatinine were obtained from Randox laboratories (Antrim, United Kingdom). Kits for serum chloride, potassium, sodium, and bicarbonate assays were from Teco diagnostic laboratories (Antrim, United Kingdom). All other reagents and chemicals used were of analytical grade.

Experimental Animals

A total of forty (40) apparently healthy mice weighing between 24g to 30g were obtained from the Animal Breeding Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The animals were maintained 8 per cage under standard conditions and fed with standard animal feeds and water *ad libitum*. They were allowed to acclimatize for two weeks before commencement of experiment.

Experimental Design

The mice were randomly divided into five groups of 8 mice each as follows; Group I: Normal control (not infected, not treated) Group II: Malaria control (infected with *P. berghei*, not treated) Group III: Malarial, and treated with 0.03mg/kg of standard drug (Arthemeter 20mg + Lumefantrine 120mg) Group IV: Malarial, and treated with Captopril (0.03mg/kg) Group V: Malarial, and treated with Captopril (0.09 mg/kg).

Doses were chosen after a pilot study was conducted to confirm the anti-plasmodium effect of captopril and determine the dose that would significantly reduce parasitemia and decrease mortality. In addition, the newly inoculated animals were monitored daily to determine the blood level of parasites. Treatment began a day after the parasites were first detected in the blood stream and lasted for fourteen days. Parasitemia was monitored every other day, and on the 15th day, mice were sacrificed and blood obtained for biochemical analysis. All experimental protocols were approved and conducted with strict adherence to guidelines of the Institutional Animal Care and Use Committee of Bayero University, Kano State, Nigeria.

Parasite Inoculation

Anka strain of *Plasmodium berghei* was obtained from Department of Parasitology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Blood from a highly parasitized mouse was obtained by cardiac puncture, collected into an EDTA-coated sample bottle, and diluted appropriately with physiological saline to serve as inoculum. Mice from groups II, III, IV and V were infected intraperitoneally with 0.2 mL of the inoculum containing about 1×10^7 *P. berghei* parasitized red blood cells/mL.

Estimation of Parasitaemia

Malaria parasite was estimated by microscopic test using thick and thin blood smears stained with Giemsa and Leishman stains as described by^[18]. Blood was collected from the tail of the mice. The relative malaria parasite count in each blood sample was determined from Giemsa stained thick blood film and Leishman stained thin blood film. Malaria parasite was confirmed by microscopic examination using x100 objective lens (oil immersion lens). Percentage parasitaemia was calculated after staining for 30 minutes and viewed under the microscope:

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized red blood cell}}{\text{Total number of normal red blood cells counted}} \times 100$$

Total number of normal red blood cells counted

Biochemical Analysis

Serum AST, ALT and ALP were determined using Randox kits (Antrim BT29 4QY, United Kingdom).

Total protein was assayed by Biuret method,^[19] and creatinine was determined by colorimetric method^[20]. Serum urea was determined by the urease-Berthelot's method,^[21] chloride was assayed as described by Skeggs and Hochstrasser.^[22] Sodium was determined based on modifications of Soundararajan et al.^[23] and Trinder^[24] methods, while bicarbonate was determined colorimetrically.

Statistical Analysis

The results were presented as Mean $\pm$ Standard deviation. One way ANOVA was performed to depict statistical differences using Graphpad instat3 software (version 3.05) and a p-value < 0.05 was considered significant.

Results

Figure 1 shows the percentage (%) parasitemia of the treated and control mice before and after treatment. After induction with *Plasmodium berghei*, there was a significant increase in parasitemia level of all groups when compared to that of the normal control group. However, after 14 days of treatment, the parasitemia decreased significantly ($p < 0.05$) in mice treated with 0.09 mg/kg of captopril ($0.03 \pm 0.01\%$) and 0.03 mg/kg of standard drug ($0.02 \pm 0.01\%$) when compared to that of the negative control. The % parasitemia level of the group treated with 0.03mg/kg of captopril ($4.26 \pm 0.11\%$) slightly reduced when compared to that of the negative control ($6.17 \pm 0.06\%$) but was not as effective as those of the group treated with 0.03 mg/kg of standard drug and 0.09 mg/kg of captopril, showing a dose dependent effect.

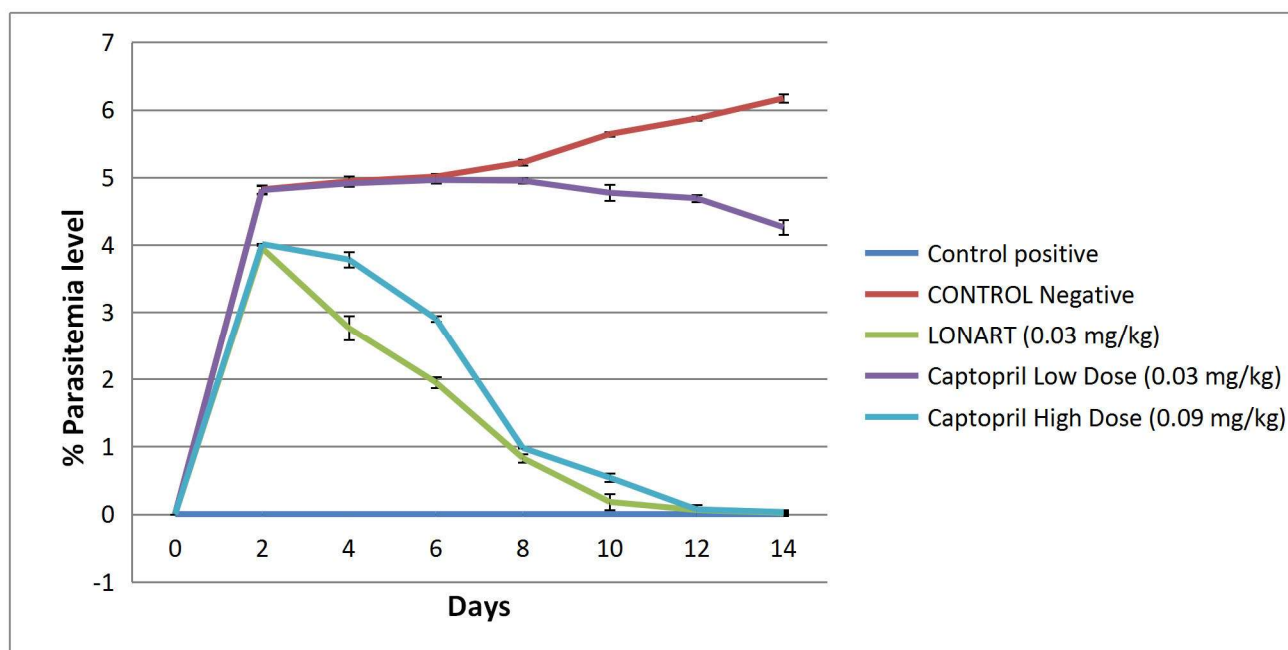


Figure 1: Percentage (%) parasitemia before and after administration of drugs in treated and control mice.

The serum levels of ALP, AST and ALT in malaria control mice were significantly ($p < 0.05$) higher than those of mice in all groups. Treatment with 0.03 mg/kg of standard drug and 0.09 mg/kg captopril significantly ($p < 0.05$) decreased the level of these enzymes. Captopril at 0.03 mg/kg also decreased the

liver enzymes, but significantly ($p < 0.05$) lower than mice given 0.09 mg/kg captopril. The serum protein level of malaria control mice was not significantly ($p > 0.05$) different from normal control group, but was significantly ($p < 0.05$) lower than those of mice treated with 0.03 mg/kg standard drug and those treated with 0.09 mg/kg of captopril (Table 1).

Table 1: Effect of captopril on some parameters of liver function in *Plasmodium berghei*-infected mice

Parameters	Normal Control	Malaria Control	Standard Control	Captopril (0.03 mg/kg)	Captopril (0.09 mg/kg)
ALP (U/L)	35.3 ± 1.52 ^a	80.33 ± 2.51 ^b	35.00 ± 2.00 ^a	51.00 ± 4.00 ^c	39.33 ± 2.51 ^a
AST (U/L)	87.33 ± 4.04 ^a	200.0 ± 11.13 ^b	106.67 ± 10.40 ^c	132.33 ± 4.16 ^d	104.33 ± 4.04 ^c
ALT (U/L)	29.00 ± 3.00 ^a	51.60 ± 2.51 ^b	31.00 ± 3.00 ^a	39.66 ± 1.52 ^c	33.00 ± 4.35 ^c
Total protein (mg/dl)	5.60 ± 0.40 ^a	5.40 ± 0.25 ^a	6.50 ± 0.30 ^b	6.10 ± 0.20 ^b	6.30 ± 0.55 ^b

Values are Mean ± standard deviation

Values with different superscript along a row are significantly different at $P < 0.05$.

Table 2 shows the results of serum creatinine and urea concentration in both treated and control mice. From the results, the creatinine level in the malaria control mice (7.14 ± 3.56 mg/dL) was significantly ($p < 0.05$) higher than the normal control and all treated groups. The level of urea increased significantly ($p < 0.05$) in the malaria control ($88.14 \pm$

7.36 mg/dL) compared to the normal control (21.25 ± 7.62 mg/dL) mice. However, treatment with the standard drug (28.58 ± 13.50 mg/dL), 0.03 mg/kg captopril (50.05 ± 8.24 mg/dL) and 0.09 mg/kg captopril (29.26 ± 18.9) decreased it significantly ($p < 0.05$).

Table 2: Serum concentration of creatinine and urea in treated and untreated *Plasmodium berghei*-infected mice.

Parameters (mg/dl)	Normal Control	Malaria Control	Standard Control	Captopril (0.03 mg/kg)	Captopril (0.09 mg/kg)
Creatinine	0.70 ± 0.1 ^a	7.14 ± 3.56 ^c	0.70 ± 0.10 ^a	3.86 ± 1.05 ^b	0.73 ± 0.10 ^a
Urea	21.25 ± 7.62 ^a	88.14 ± 7.36 ^c	28.58 ± 13.50 ^a	50.05 ± 8.24 ^b	29.26 ± 18.9 ^a

Values are Mean ± standard deviation

Values with different superscript along a row are significantly different at p<0.05.

The serum level of Na⁺ and Cl⁻ in the normal control, standard control and group treated with 0.09 mg/kg captopril were not significantly (P>0.05) different. In 0.03 mg/kg captopril treated mice, Na⁺, HCO₃⁻ and Cl⁻ levels were significantly (P<0.05) lower than all other groups, except the malaria control group,

which decreased significantly (P<0.05) upon induction of malaria. With respect to K⁺, the mean value increased significantly (P<0.05) in the malaria control group when compared to that of the normal control, standard control, and groups treated with 0.03 and 0.09 mg/kg captopril (Table 3).

Table 3: Serum electrolyte concentration in treated and untreated *Plasmodium berghei*-infected mice.

Parameters (mg/dl)	Normal Control	Malaria Control	Standard Control	Captopril (0.03 mg/kg)	Captopril (0.09 mg/kg)
Sodium (Na ⁺)	151.68 ± 17.78 ^a	97.34 ± 8.57 ^c	149.41 ± 11.40 ^a	121.30 ± 14.24 ^b	148.52 ± 6.19 ^a
Potassium (K ⁺)	5.11 ± 0.03 ^a	15.50 ± 0.51 ^c	5.12 ± 0.13 ^a	10.37 ± 0.52 ^b	5.39 ± 2.26 ^a
Chloride (Cl ⁻)	103.24 ± 11.31 ^a	31.75 ± 18.72 ^c	101.23 ± 22.32 ^a	67.49 ± 5.15 ^b	100.67 ± 22.27 ^a
Bicarbonate (HCO ₃ ⁻)	100.67 ± 22.27 ^a	8.51 ± 1.36 ^c	17.37 ± 1.35 ^a	14.04 ± 0.87 ^b	17.32 ± 1.41 ^a

Values are Mean ± standard deviation

Values with different superscript along a row are significantly different at p<0.05.

Discussion

In the current study, the reduction in parasitemia after treatment with captopril was dose-dependent, as parasite level of mice treated with 0.09 mg/kg captopril decreased significantly (p<0.05) back to normal after 14 days of treatment, unlike the 0.03 mg/kg captopril treated group. This result correlates with studies by Silva-Filho *et al.* [15] who showed that losartan and captopril reduced parasitemia, promoted survival benefits and attenuated signals involved in the development of cerebral malaria; and attributed this to the inhibition of ACE by captopril, resulting in low levels of Ang II, and accumulated Ang I being converted to Ang (1-7), which binds specifically to the Mas receptors decreasing the T-cell response and PKA activity and consequently reducing the parasite invasion. However, this result contradicts reports by Maciel *et al.* [14] who first demonstrated that angiotensin II

(Ang II) and related peptides impair parasite development in the sexual cycle of *P. galinacium*, an avian malaria parasite. They postulated that Ang II reduces the accumulation of sporozoites in the salivary gland of the mosquito vector by directly disturbing the parasite membrane. Also, Maciel *et al.* [16] demonstrated a significant reduction in blood parasitemia in mice infected with *Plasmodium berghei* ANKA treated with a supraphysiological concentration of Ang II.

The high levels of ALP, AST and ALT in untreated *P. berghei*-infected mice agrees with the report of Onyeson *et al.* [25] that demonstrated malaria infection significantly increased the serum activities of AST, ALT, and ALP in a manner that positively correlates with the parasites density and concluded that the infection confers a measure of hepatic compromise.

Among complications that are associated with malaria, renal dysfunctions are common in malaria endemic regions.^[26,27] This study showed a significant increase in the level of urea and creatinine in the malaria control mice when compared to that of the normal control mice (Table 2). The observed increase could be as a result of sequestration of the parasite into the renal microvasculature bed, which may lead to ischemia.^[28] In this study, there was significant decrease in the level of Na^+ , Cl and HCO_3^- , while K^+ increased in the malaria control group compared to that of the normal control. This result concurs with previous report of hyponatraemia and hyperkalemia during malaria infection.^[29,30] It has been reported that impaired glomerular filtration as a result of malaria infection could be responsible for the reduction in the Na^+ available in the renal tubule for K^+ exchange. This may lead to the increase in the

serum K^+ level^[31,29] as a result of imbalance in hydrogen ions, causing retention of K^+ in the serum. However, the significant decrease in K^+ levels on administration of captopril, conforms with reports that angiotensin converting enzyme inhibitors, such as captopril can be used for the treatment of renal (kidney) related disorders.^[32]

Conclusion

This study has shown that angiotensin converting enzyme inhibitors at high concentration (0.09mg/kg) has antiplasmodial activity, reduces kidney-related disorders associated with malaria and possesses hepatoprotective effect, hence, maybe safe as antihypertensive treatment in malaria endemic regions. However, there is need for further research to establish the possible mechanism of action through which captopril exerts its antiplasmodial activity.

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Glycaemic Index of Bambara Groundnut (*Vigna subterranea* L. Verdc.) – Based Meals Commonly Consumed in Northern Nigeria

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Abstract

Bambara groundnut (*Vigna subterranea* L. Verdc.) was a crop that is indigenous to the African continent. It is grown mainly for its edible and nutritionally rich seeds. In this study the glycaemic index of Bambara groundnut-based meals commonly consumed in northern Nigeria is determined. Two landraces (Yobe and Kano) of Bambara groundnut were used to prepare the test meals; roasted snack (*pashe*) and steamed cake (*alale*). *Pashe* and *alale* were separately fed to 10 apparently healthy participants after an overnight fast. Glycaemic responses were calculated at 0, 30, 60, 90 and 120 minutes post meal consumption. The Kano Bambara groundnut landrace meals exhibit lower glycaemic indices, with *pashe* having the lowest glycaemic index value of $16 \pm 9\%$ compared to *pashe* (Yobe) with $44 \pm 25\%$. Similarly glycaemic index values of $24 \pm 6\%$ and $62 \pm 23\%$ were observed in the *alale* (Kano) and *alale* (Yobe) respectively; the differences are statistically significant ($p < 0.05$). These suggest that *alale* and *pashe* can be a excellent diets for diabetics.

Keywords: Bambara groundnut, Roasting, Steaming, Domestic processing, Glycaemic index.

Introduction

Legumes are important source of plant proteins in many parts of the world. Grain legumes are widely consumed in Nigeria. ^[1] Different processing methods (boiling, steaming, frying, dehulling and grinding) are often combined to produce different products that are eaten as snack or main meal. ^[2] Bambara groundnut is an underutilised legume and is readily available in Nigeria. Bambara groundnut can serve as a cheap source of protein and other nutrients.

Glycaemic index is a quantitative way of assessing foods based on their postprandial blood glucose response. ^[3] It is determined by measuring the effect of 50 g available carbohydrate of a test food on blood glucose and comparing it with that of a control food, usually glucose or white bread. ^[4] The concept of glycaemic index was developed to complement information available on chemical composition of foods given in FAO/WHO food table, which has been approved for inclusion in guide to food choices. ^[5]

Generally, Nigerian diabetics consume other legumes because of their low glycaemic index. Low glycaemic index diets are desirable in the management of diabetes, as they reduce postprandial blood glucose level in both diabetic and healthy individuals. ^[6] Low postprandial blood glucose concentration and diets with low glycaemic index are associated with reduced risk of degenerative diseases such as diabetes mellitus, obesity and cardiovascular diseases. ^[7, 8, 9] Foods that causes slow rise in blood sugar and steady release of continuous energy are low in glycaemic index, while high glycaemic index food induce a sharp rise in blood glucose, which declines within a short time. ^[10]

Although the glycaemic indices of some processed grain legumes have been reported by Oboh *et al.*, ^[6] there is little information on the glycaemic index of Bambara groundnut. Onimawo *et al.* ^[11] reported that *okpa* (steamed cake prepared from Bambara groundnut) is a better diet for diabetics than *moi moi* (steamed cake prepared from cowpea) in terms of glycaemic index and postprandial blood glucose

response. The availability of different landraces of Bambara groundnut in northern Nigeria makes the choice of this underutilised legume. In Nigeria Bambara groundnut is processed through various techniques like boiling, roasting, and frying. The aim of this study was to determine the glycaemic index of Bambara groundnut-based meals that are commonly consumed in northern Nigeria.

Materials and Methods

Bambara Groundnut Sample Collection

Seeds of two different landraces of Bambara groundnut were used in the study. The Kano landrace (mottled, brown in colour) was purchased from Dawanau grain market in Kano state, while the Yobe landrace (cream in colour) was obtained from Potiskum grain market in Yobe state. For glycaemic index determination, white bread was used as control.

Preparation of Test Meals

i) Steamed cake (*alale*)

The Bambara groundnut seeds were sorted and washed in cold water twice its volume to remove foreign materials. Seeds were then peeled to remove the testa and subsequently wet-milled into a paste. Two medium size onions, three large pepper, salt to taste, four serving spoon of palm oil, and seasoning to taste were added and thoroughly mixed. The mixtures were then put into plastic containers and steam cooked for 1 hr.

ii) Roasted snack (*pashe*)

The Bambara groundnut seeds were soaked in water overnight at room temperature with a seed to water ratio of 1:5 (w/v). Thereafter, the soaked seeds were washed twice and rinsed with distilled water and allowed to drain for 1hr. Subsequently, salt to taste was added and the seeds roasted in a frying pan for 20 minutes.

Proximate Analysis

The processed Bambara groundnuts were analysed for moisture content, ash, crude protein, and crude fiber by the AOAC method. ^[12] Carbohydrate was determined by difference.

Study Participants

Ten apparently healthy human volunteers of both sexes between the ages of 25-35 years were recruited for the study based on standard guidelines for glycaemic index determination. ^[13] Participants were excluded if they had diabetes, vascular diseases,

hypertension or other chronic diseases and if they are pregnant or lactating. The study was approved by the Faculty of Biomedical Science of Bayero University, Kano, participants gave their consent after being fully briefed about the experimental nature of the study. The study subjects had mean body mass index of $21.09 \pm 1.25 \text{ kg/m}^2$, mean fasting blood glucose of $4.57 \pm 0.04 \text{ mmol/L}$ and mean systolic /diastolic blood pressure of $116 \pm 5.16/74.00 \pm 9.60 \text{ mmHg}$. On each test day, between 7-9 am after an overnight fasting (10-12 hrs), participants were randomly assigned to consume either of the two test meals or the control meal. After one week interval, participants were then given a different test meal/control. On each test day, the test/control diet was given to the subjects and their blood glucose determined at 30 minutes interval for a period of 2 hrs.

Determination of Blood Glucose and Glycaemic Index

On the day of each test, the participants were asked to restrict intake of caffeine containing drinks and alcohol and to refrain from strenuous physical activity. Capillary blood samples (drops) were collected by finger prick and gentle pressure on the index finger. Blood glucose concentration was determined before feeding (fasting) and at 30, 60, 90 and 120 minutes after feeding the test meals using Accu-chek Blood Monitoring System (Roche Diagnostics GmbH, Mannheim, Germany). The incremental Area Under the Curve (iAUC) of the test and control meals were calculated by the trapezoidal rule. ^[14] The area above (or below, as sometimes occurs) the curve of the test meal was calculated and expressed as a percentage of the area obtained after the ingestion of the standard meal (control); the higher the area under the curve the higher the glycaemic index of the food. The glycaemic index (GI) value was calculated by the method of Jenkins *et al.* ^[15] and is given by:

$$GI = \frac{\text{IAUC of the test meal}}{\text{IAUC of the reference meal (control)}} \times 100$$

Statistical Analysis

Experimental results were expressed as mean $\pm$ SD. Results were analysed using one way analysis of variance (ANOVA) with Tukey multiple comparison post-hoc test, significant difference was established at ($p < 0.05$). The analyses were done using SPSS version 16.0.

Results

The proximate analysis of processed and unprocessed Bambara groundnut indicated that Kano landrace has higher crude fibre and protein content but lower content of carbohydrate, lipid, ash and moisture compared to the Yobe landrace. Among the samples analysed, it was observed that *alale* had significantly ($p<0.05$) higher fat, ash and moisture content in both

Bambara groundnut landraces. Conversely, the fat and moisture contents were significantly ($p<0.05$) lower in *pashe*.

Figures 1 and 2 present the blood glucose levels in the test participants in response to the intake of the test meals against the control meal.

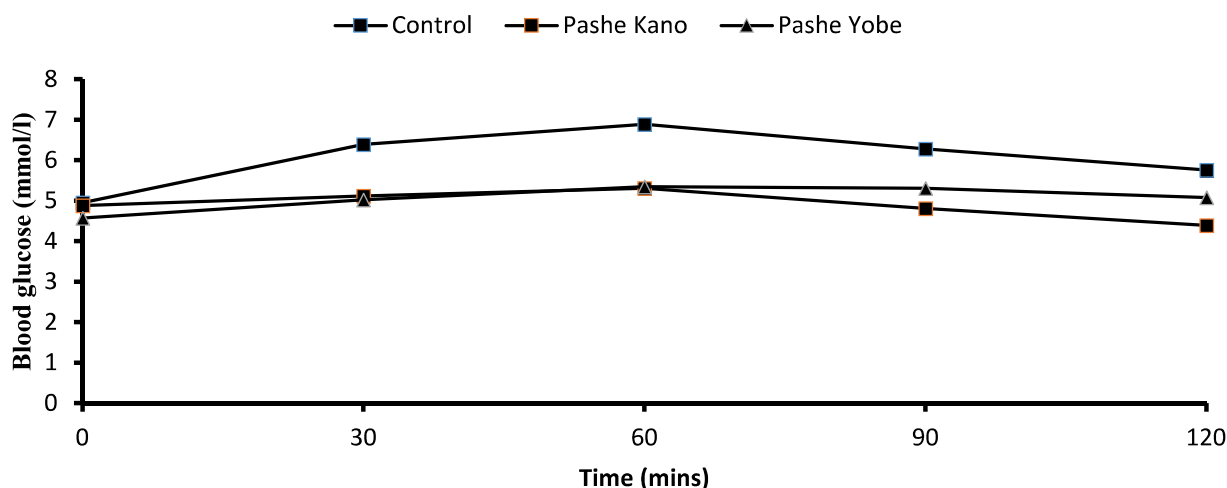


Figure 1: Mean blood glucose response curve (mmol/L) of control meal compared to *pashe* intake

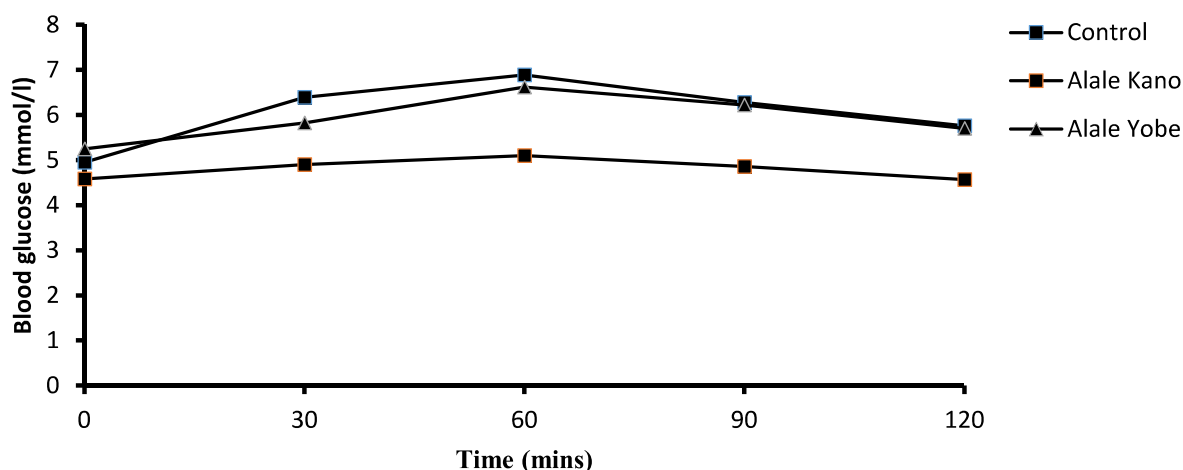


Figure 2: Mean blood glucose response curve (mmol/L) of control meal compared to *alale* intake

The results revealed that meals prepared with Kano Bambara groundnut landrace have significantly ($p<0.05$) lower glycaemic index (GI) value compared to the Yobe Bambara groundnut landrace. The GI of the analysed meals are shown in Figure 3. The mean GI values ranged from 16 to 62%. The GI's of the test meals are significantly ($p<0.05$) lower compared to

the GI of white bread (control). In addition, GI of *alale* is significantly ($p<0.05$) higher than that of *pashe*. Generally, both *pashe* and *alale* prepared from the Kano Bambara groundnut landrace had significantly ($p<0.05$) lower GI value compared to the meals prepared from the Yobe landrace.

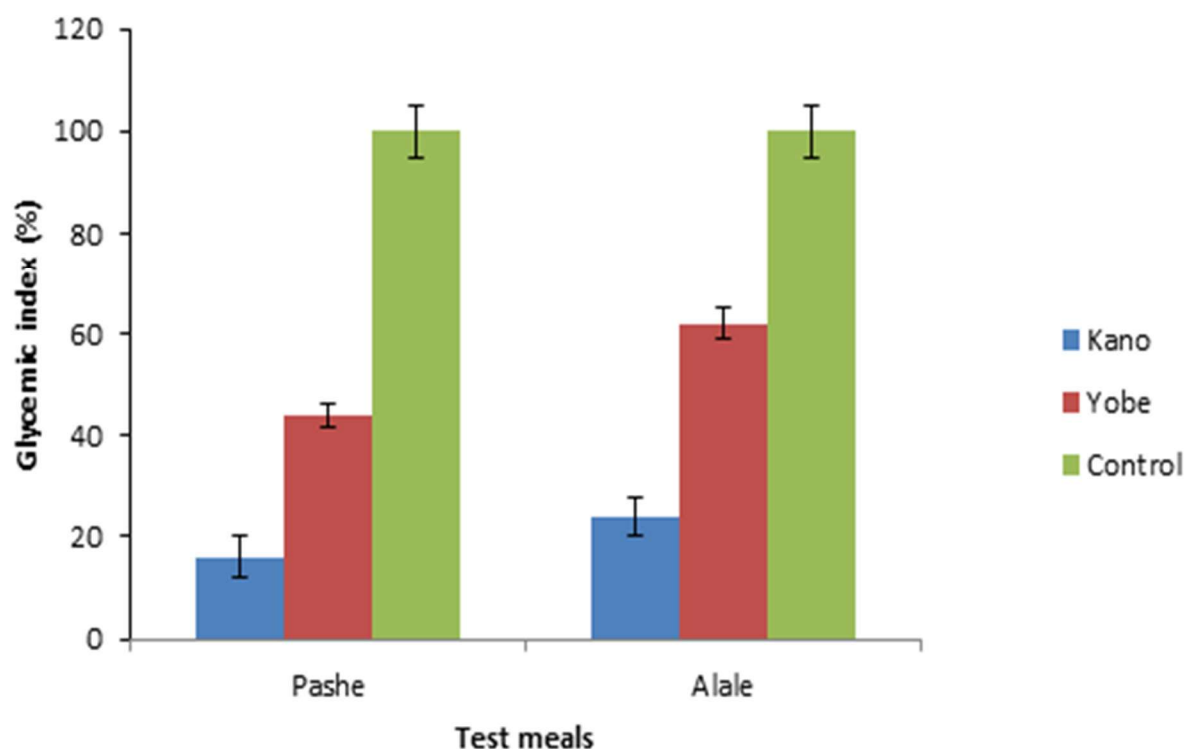


Figure 3: Glycaemic index (GI) of the participants to different processed types of Bambara groundnut.

Results are presented as mean $\pm$ SD; bars with the same letters within same meals and/ or between meals are significantly different ($p < 0.05$).

Discussion

The crude fibre content of raw Bambara groundnut was higher ($p < 0.05$) than that in the processed meals. Corresponding findings were reported by many others researchers. [16, 17] The moisture content of both landraces was found to increase in steamed cooked meal (*alale*). The increase in the moisture content is due to the change resulting from the uptake of water by the seed during cooking. However there is insignificant reduction in moisture content in the roasted meal (*pashe*). Similar trend was observed by Ndidi *et al.* [18] and Okafor *et al.* [19] The application of moisture and heat affects starch granules causing the disorganisation of the crystalline structure of starch thereby making it available for hydrolysis. [20] The protein content in both landraces significantly ($p < 0.05$) decreased after cooking but insignificantly after roasting; similar trend was observed by Mazahib *et al.* [17] and Ndidi *et al.* [21] The observed decrease in protein content after cooking and roasting could be a consequence of the solubilisation of the proteins by heating that leads to loss of protein in the final product. [22] The carbohydrate content of the

processed meals were lower ($p < 0.05$) than the raw Bambara groundnut. Similar results were reported by Ahima *et al.* [16] and Ramadan. [23] Starch can form insoluble complexes with protein (Maillard reaction) making it unavailable for digestion and absorption. [24]

Glycaemic index determination is a method of classifying carbohydrate based on their effect on blood glucose; it replaces the older method of classifying carbohydrates according to their chemical and physical structure i.e complex and simple carbohydrate. [16, 25] GI measures how rapid a food can raise blood sugar; high GI foods cause a rapid and large release of glucose and are associated with increased risk of diabetes, whereas foods with low GI contain slowly digested carbohydrate and cause slower and lower release of the blood glucose level. In this present study, the GI values of different Bambara groundnut-based meals varied across a considerable range with Kano Bambara groundnut landrace having significantly lower GI values compared to Yobe Bambara groundnut landrace.

The glycaemic index according to Brand-Miller [26] categorically classifies foods into three general categories: High GI food (GI 70 and above) which causes rapid rise in blood glucose levels, intermediate GI food (GI 55-69) causing a medium rise in blood glucose and lastly low GI food (GI 54 or less) causing a slower rise in blood glucose. The variation in the GI observed in this study may be attributed to the quality of starch in each of the landrace of Bambara groundnut and processing method. It has been reported by Amelsvoort and Weststrate [27] that GI is affected by the proportion of amylose to amylopectin in legumes. Amylose is a linear molecule with D-glucose unit linked by α (1-4) linkages and amylopectin is a branched chain molecule linked by both α (1-4) and α (1-6) linkages. [24] Amylopectin which is made up of branched starch molecules is more readily hydrolyzed in the gut compared to the linear chain amylose. [19] These suggest that Yobe landrace may possibly contain higher proportion of amylopectin that may be responsible for the observed higher GI. Glycaemic index does not assess the quantity of carbohydrate, but rather the quality. [28] The result is consistent with the findings of Onimawo *et al.* [11] that legumes produce relatively low glycaemic responses. The results of the present work suggest meals prepared from Kano Bambara groundnut landrace is more suitable for use in controlling postprandial rise of blood glucose levels than Yobe landrace.

It has been shown that the fibre content of Bambara groundnut could be responsible for decreasing postprandial blood glucose by increasing the viscosity of the digest and reducing the gastric emptying time. [29] The extent to which the fibre in a particular food is responsible for its GI is a subject of much debate. Wolever [30] reported that total dietary fibre is weakly related to the GI while other studies in which fibre was added to a carbohydrate meal suggested that only soluble fibre has an effect on the postprandial blood glucose concentration and not the insoluble component. [31] Other factors that could contribute to the low GI content could be the raffinose oligosaccharide components which are not digested in

the human gut. [29] The structure of the starch and fiber present in the seeds and the interactions of other constituents that make up the Bambara groundnut legume which can bind starch may affect the blood glucose levels. [6]

It has been reported by Glen *et al.* [32] and Kouassi *et al.* [33] that methods used to cook, store as well as cooking time changes the structure of starch thereby affecting its GI. The GI of *alale* tends to be higher than the GI of *pashe*. It has been reported by Pi-Sunyer [20] that grinding, rolling, pressing or even chewing of starch containing food can disrupt the granules and increase the GI. The observed higher GI of *alale* may also be explained by the effect of high temperature and humidity in modifying the physical and chemical properties of starch. Soh and Brand-Miller [34] and Englyst and Cummings [35] have reported that during cooking starch granules takes up water and gelatinize which irreversibly disrupts the crystalline structure of the starch, making it to be readily hydrolyzed by amylase. Conversely the low GI of roasted Bambara groundnut *pashe* could be attributed to the weak gelatinization of the starch because of the fact that no external water was added during processing. Therefore the starch structure becomes more resistant and slowly digestible by amylase. This study shows that roasting and cooking of Bambara groundnut leads to low glycaemic index (GI) products.

Conclusion

The results obtained from this study demonstrated that the GI of Bambara groundnut-based diet is low and varies based on the processing method. This finding is significant as low glycaemic index diets are important in the management of hyperglycaemia and hyperinsulinaemia. Roasted Bambara groundnut meal (*pashe*) may be better for glycaemic control compared to steamed Bambara groundnut meal (*alale*). In addition the Kano Bambara groundnut landrace has significantly lower GI compared to the Yobe Bambara groundnut landrace.

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