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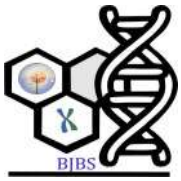

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Guide to Authors

Aim and Scope

Bayero Journal of Biomedical Sciences (BJBS) is a journal that publishes original research and critical reviews in the fields of Anatomy, Biochemistry, Microbiology, Nutrition and Dietetics, Pharmacology and Physiology. It is a biannual publication (June & December) with intent to publish quarterly in the future depending on availability of accepted manuscripts.

Submission

Articles for publication should be sent to the Editor-in-Chief, BJBS, Faculty of Basic Medical Sciences, Bayero University, PMB. 3011, Kano or through the Journal email address: bjbs.fbms@buk.edu.ng

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Charges

Manuscripts submitted from Nigeria must be submitted along with a processing fee of ₦5, 000.00 or $20, while articles submitted from other countries are not accompanied with processing charges. Articles submitted within Nigeria and not accompanied by the assessment fee will not be processed. Upon acceptance of a paper for publication, the corresponding author must submit the original paper (reviewed), corrected paper, CD version of the paper (CD-R) in word document or through an email and a publication fee of ₦13,000.00 (within Nigeria) or $120 (outside Nigeria).

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A manuscript will be reviewed for possible publication with the understanding that it is being submitted to Bayero Journal of Biomedical Sciences alone at that point in time and has not been published anywhere, simultaneously submitted, or already accepted for publication elsewhere. One of the authors should correspond with the Journal for all matters related to the manuscript. All manuscripts received are duly acknowledged. On submission, the editorial board reviews all submitted manuscripts for suitability before the formal review. Sub standard manuscripts or those with technical flaws are rejected before proceeding for formal peer-review. Articles for publication will be peer reviewed by a minimum of 2 reviewers to ensure accuracy. The Board's decision on publishing a paper will be guided strictly by the reviewer's comments.

Authorship Criteria

Participation based on data collection, or fund acquisition does not justify authorship, so also supervision of the research group is not sufficient for authorship. Authors should contributor sufficiently in the appropriate portions of the content of the manuscript. The order of naming the contributors should be based on the relative contribution of the author towards the study and writing the manuscript.

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Upon acceptance of a paper by the Journal, the author(s) have automatically transferred copyright of the paper to BJBS. The transfer will ensure widest possible dissemination of information.

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Authors are to submit three (3) copies of the manuscript. Submitted papers should contain results from original researches that are not being considered for publication elsewhere or previously

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Text
The text of the manuscript should appear in the following order. Title, authors name(s) and address (es), abstract, introduction, materials and methods, results, tables, figures, and plates, discussions, conclusion, recommendation(s), acknowledgement, references, (authors should please comply strictly to this format).

Title page
The title of the paper should clearly reflect the content and the main theme of the paper. The full names and addresses of authors (including names of institutions, country, email address and telephone numbers) should be stated. In the case of joint authorship or multiple authorship, the name and mailing address of the author to whom further correspondence would be referred should be indicated; otherwise, the name of the first author on the list is assumed to be the correspondence author, not corresponding author. Authors' names should start with surnames then initials of other names.

Introduction
This should contain mainly essential background information and important relevant references. The objectives of the work and its relevance or contribution must be clearly stated. The literature cited must be discussed to show the relationships between the published and the research study.

Materials and Methods
New methods and modifications of established ones should be described in detail to enable duplication by other workers. For the established and published methods, references should be made to the relevant publications. Due attention should be given to experimental design and statistical analysis of data where applicable.

Ethics
When reporting studies on human beings, indicate whether the procedures followed were in accordance with the ethical standards of the authorized committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000 (available at http://www.wma.net/e/policy/17-c_e.html). For studies involving human participants, authors are expected to mention about approval of (regional/ national/ institutional or independent Ethics Committee or Review Board, and should also obtain informed consent from adult research participants or assent from guardian for children participating in the trial. Authors should ensure confidentiality on all matters relating to subjects by avoiding mentioning of participants' names,

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Individuals who have done substantial work on the subject/field are expected to write these articles. A short summary of the work done by the contributor(s) in the field of review together with appropriate references should accompany the manuscript. The manuscript must have at least 50 references. The format of writing the review article should be similar to the standard articles presentation in BJBS.

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Note
Results and discussion could be presented together. In such case, each result should be presented immediately by discussion specific to the result and this should continue until all the results have been presented and discussed

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Recommendations
Should be optional and must be drawn from the study.

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

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The reference style should be the Vancouver format i.e. numbering format, number is used instead of name(s) of the authors in the text. References should b*e* numbered *c*onsecutively in the order in which they are first mentioned in the text (not in alphabetic order). Identify references in text, tables, and legends by Arabic numerals in superscript with square bracket after the punctuation marks. References cited only in tables or figure legends should be numbered in accordance with the sequence established by the first identification in the text of the particular table or figure. The titles of journals should be abbreviated according to the style used in Index Medicus. Use complete name of the journal for non-indexed journals. Avoid using abstracts as references. Information from manuscripts submitted but not accepted should be cited in the text as "unpublished observations" with written permission from the source. Avoid citing a "personal communication" unless it provides essential information not available from a public source, in which case the name of the person and date of communication should be cited in parentheses in the text.

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Contents

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 3, Issue 1, 2018 to the teeming scholars and young scientists across the globe, who like to be abreast with current research findings in the field 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 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.

Dr. Salisu AI ***PhD***
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Right and Left Ear Asymmetry among Hausa Adolescents in Zaria, Northwestern Nigeria

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Abstract

Some degree of asymmetry of auricles is present in most normal human beings and is acceptable. Ear prints of the right and left auricles in different sizes from crime scenes do not exclude that they may belong to the same offender. Several studies exist on ear asymmetry in various populations. However, studies on Nigerians are sparse and none of these studies have explored the right and left ear discrimination potential of ear morphometric variables. The present study included apparently healthy 400 subjects (200 males and 200 females) in the age range of 10-19 years of Hausa ethnic group resident in Zaria, North Western Nigeria. A digital vernier calliper was used to measure the total ear height (TEH), ear width (EW), lobular height (LH) and lobular width (LW). Auricular index (AI) and lobular index (LI) were computed and the right and left discrimination potential of these variables evaluated. Results of the study showed significant asymmetry in the right and left ear measurements irrespective of the sexes ($p < 0.05$). The parameters when analysed within the sexes, females had larger dimensions of right TEH, right and left LH and LW when compared to their male counterpart. On the other hand, males had larger values for left TEH, right and left EW, left AI and right and left LI. In addition, the ear parameters gave a moderate to the good, right and left ear identification accuracy of 58.5% using discriminant function analysis. Hence, emphasis on its utility as an adjunct for right and left ear determination in Hausa adolescents resident in Zaria should be made.

Keywords: Asymmetry, External Ear, Forensic Identification, Discriminant Function Analysis.

Introduction

The auricle is important for the maintenance of a harmonious and pleasing appearance of the face [1, 2] as it can convey subtle signs of age and sex although not easily defined. Knowledge of the morphometry of auricle is relevant in the diagnosis of congenital malformations and syndromes, in planning plastic surgeries of the external ear and designing hearing aids [1, 2, 3, 4]. Also, it is important in personal identification in forensic sciences i.e.,ear prints of the right and left auricles in different dimensions from crime scenes does not exclude that they may belong to the same offender [5, 6, 7].

Fine and extensive studies on the morphometry of the auricle have been reported in non-Nigerians [1-4, 8, 9, 10]. Reports on the Nigerian population are scarce [11, 12, 13] and since reference databases on every ethnic group in Nigeria are needed in forensic anthropology, then it is important to develop one for the Hausa ethnic group resident in Zaria. Also, reports have shown that studies investigating the asymmetry of the auricle are scanty [14, 15, 16, 17] and even where reported, none of them have explored the right and left ear discrimination potential of these parameters.

The present study is intended to evaluate the extent of asymmetry of right and left morphometric ear variables and their efficacy in right and left ear discrimination among Hausa population resident in Zaria belonging to the age group of 10–19 years using a digital vernier calliper method.

Materials and Methods

Subjects

The study was carried out on 400 adolescent Hausas resident in Zaria with no evidence of congenital ear anomalies, history of craniofacial trauma, ear disease or previous ear surgeries. The study cohort consisted of 200 females and 200 males aged 10-19 years randomly selected from four secondary schools. The purpose of the study was explained to the subjects and permission was obtained from the school management.

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Anthropometric Measurements

The auricle was measured according to the standard landmark points defined by De Carlo et al., [18] and the methodology was adopted from McKinney et al., [19] and Brucker et al., [1]. The Parameters measured were total ear height (TEH), ear width (EW), lobular height (LH) and the lobular width (LW) for each right and left ear of the subject. Figure 1 shows the measurement of reference points used for anthropometric measurements. The TEH was measured as the distance between the inferior projection of ear lobule and the most superior projection of helix (A-B). The EW was measured as the distance between the most anterior and the most posterior points of the auricle (C-D). The LH was taken as the distance between the most inferior ends of lobule to the base of the tragal notch (G-B). The LW was measured as the horizontal width of the lobule (E-F). Figure 2 shows the measurement of the ear by vernier calliper.

In addition, indices defining the proportions of the ear such as auricular index (EW*100/TEH) and lobular index (LW*100/LH) were calculated. All the measurements were taken by a single investigator in order to minimize inter-observer error. A digital vernier calliper with measuring accuracy to the nearest 0.1mm was used. For each subject, the measurement was taken in duplicate and the mean of the two measurements was taken in order to minimize intra-observer error.

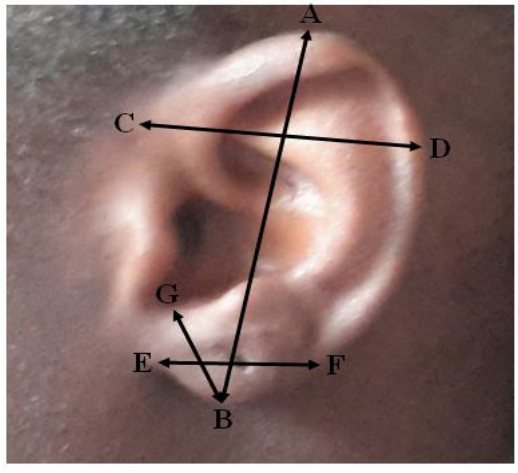

Figure 1: Landmarks used for anthropometric measurement of ear (total ear height=A-B, ear width=C-D, lobular height=G-B, lobular width=E-F

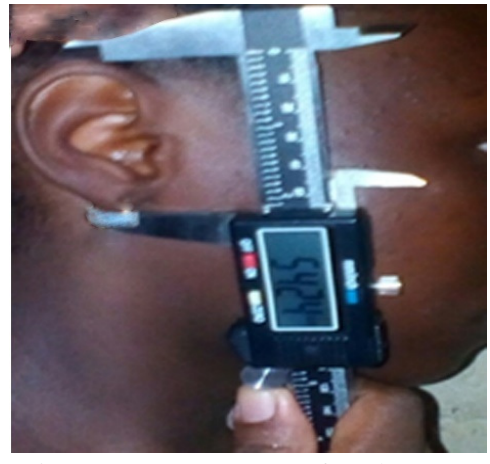

Figure 2: Measurement of ear by vernier calliper (total ear height)

Statistical Analysis

The symmetry of the right and left ears, the difference in the ear symmetry between sexes were evaluated using paired t-test. The comparison according to the auricular side of the measured parameters was evaluated using discriminant function analysis. All the statistical analyses were done on an SPSS 20.0 software package (SPSS Inc., Chicago, Illinois, USA) and $p \leq 0.05$ was set as the level of statistical significance.

Result

Table 1 shows the degree of symmetry of ear measurements analysed using paired t-test. The right total ear height (54.86 mm) was significantly larger than the left total ear height (54.12 mm). The left ear width (27.85 mm) was significantly wider in comparison to the right ear width (27.62 mm). The lobular height (right: 14.75 mm vs left: 14.33 mm) and lobular width (right: 18.73 mm vs left: 17.85 mm) were statistically significantly larger on the right ear compared with the left ear. The auricular and lobular indices on the left ear were significantly larger than the right ear ($p < 0.05$)

Table 2 depicts the symmetry of the ear within the sexes. Right total ear height, right and left lobular

height, right and left lobular width were significantly larger in females than in males; while left total ear height, right and left ear width, and right and left auricular index, in contrast, were significantly larger in males than in females ($p< 0.05$). The lobular indices did not show statistically significant differences in both sexes for right and left ear ($p> 0.05$). Though, values for the right and left ear were higher than the ones in the female.

A forward stepwise discriminant analysis for right and left ear prediction using external ear features is presented in Table 3. Two variables entered into the stepwise discriminant function analysis, i.e., lobular width (LW) and auricular index (AI). The Wilks' Lambda decreases as more variables were added to the equation. Also, the p-value of <0.0001 indicated that all the steps can discriminate right and left ear more than by chance. For the overall equation below the table, it can be inferred that a value of 0.170 or greater obtained using the equations, indicates right ear, whereas a value less than 0.170 (including negative value) indicates left ear.

Table 4 is a classification summary of right and left ear discrimination using external ear features. This analysis proved effective in the identification of right and left ear, with overall accuracy being 58.5%, in which 61.3% of the right ear and 56.8% of left ear were correctly classified. The left ear had the highest level of misclassification (44.3%) compared to the right ear (38.8%).

Table 1: Symmetry of ear measurements analysed using paired t-test (n= 400)

Parameters		**Mean ± SD**	**t- value**	**p- value**
Total Ear Height (mm)	Right	54.86 ± 3.94	6.19	0.001
	Left	54.12 ± 4.40		
Ear Width (mm)	Right	27.62 ± 3.99	-2.03	0.043
	Left	27.85 ± 3.84		
Lobular Height (mm)	Right	14.75 ± 3.02	6.24	0.001
	Left	14.33 ± 2.92		
Lobular Width (mm)	Right	18.73 ± 3.55	8.86	0.001
	Left	17.85 ± 3.41		
Auricular Index	Right	50.31 ± 6.06	-5.94	0.001
	Left	51.62 ± 7.90		
Lobular Index	Right	130.38 ± 28.49	2.88	0.040
	Left	127.83 ± 28.04		

SD= Standard deviation

Table 2: Symmetry of ear measurements within the sexes using paired sample t-test.

		Males	**(n=200)**	**Females**	**(n=200)**
Parameters		**Mean ± SD**	**t- value**	**Mean ± SD**	**t- value**
Total Ear Height (mm)	Right	54.83 ± 4.12	6.31*	54.90 ± 0.27	4.03*
	Left	54.24 ± 4.10		54.01 ± 0.33	
Ear Width (mm)	Right	28.00 ± 4.11	-1.49	27.25 ± 0.27	-1.49
	Left	28.17 ± 4.25		27.52 ± 0.24	
Lobular Height (mm)	Right	14.22 ± 3.14	4.09*	15.28 ± 0.20	4.71*
	Left	13.84 ± 3.15		14.82 ± 0.18	
Lobular Width (mm)	Right	18.69 ± 3.81	7.04*	18.77 ± 0.23	5.65*
	Left	17.8 ± 3.63		17.91 ± 0.22	
Auricular Index	Right	51.04 ± 6.15	-3.73*	49.59 ± 0.42	-4.72*
	Left	51.88 ± 6.39		51.36 ± 0.65	
Lobular Index	Right	135.80 ± 32.89	1.56	124.97 ± 1.56	2.55
	Left	133.77 ± 34.18		121.90 ± 1.30	

*$p \leq 0.05$; SD= Standard deviation

Table 3: Stepwise discriminant analysis for right and left ear prediction using external ear features

Steps	Selected Variables	Wilks' Lambda	Exact F	P value
1	LW	0.98	12.65	<0.0001
2	LW+ AI	0.97	11.53	<0.0001

LW: Lobular width; AI: Auricular index

Auricular side= *0.47 + LW (0.244) + AI (-0.970) [Right ≥ 0.170 < left]*

Table 4: Classification summary of right and left ear discrimination using external ear features

		Predicted Group Membership		
		Rightear	Left-ear	Overall accuracy
Percentage	Right ear	61.3	38.8	58.5%
	Left ear	44.3	56.8	

Discussion

According to Ferrarioet al., [20] asymmetry between right and left halves of the face, especially in paired structures, are well known in human beings. In the present study significant asymmetry was noted in all the ear measurements in both sexes i.e., the right total ear height and right lobular height were larger than the ones in the left ears while, contrastingly, the left ear width was larger than the right ear width. Our findings are in agreement with the works of Farkaset al., [4], Barut and Aktunc [9], Azaria et al.,[21] and Sharma et al., [22]. However, only a few previous studies have reported ear symmetry with contrasting findings, for example, a study by Alexander et al., [23] whose data were from a mainly adult sample, showed generally good symmetry and also Ekanemet al., [12] who worked with adult Nigerians residents in Maiduguri Metropolis. The differences seen could be governed by factors such as age, body constitution, environmental and nutritional factors which are known to affect the structure of the human ear.

The parameters when analysed within the sexes, females had larger dimensions of right TEH, left EW, right and left LH and LW when compared to their male counterpart. And with regards to auricular and lobular indices of both ears, Ferrarioet al., [8] and Barut and Aktunc [9], observed that values in males were significantly higher than those in females. Their findings are in accordance with the present study in which female lobular parameters were found to be larger than the ones in the male. These discrepancies in our findings could be the result of factors such as weight bearing effects of earring worn by females, race, body constitution, environment, age and nutritional factors which can affect the structure of the human ear.It can also be empirically said that there is no normal appearance of the ears, even among people of the same ethnic origin, which makes them as unique as fingerprints and can aid in identification [16].

Although several external ear parameters have been used in sex determination [16], the right and the left ear assessment potential of ear variables is largely unexplored. In the present study, on using all the variables; the right and left ear classification accuracy was 58.5%, with around 61.3% of the right ear and 56.8% of left ear being correctly classified. This accuracy is almost similar to external ear parameters used in the comparison of genders [16]. No other study related to ear parameters exists for us to compare our findings, and we believe that ear parameters may have the potential to serve as an adjunct for right and left ear identification in forensic scenarios along with other routinely used methods. Nevertheless, it is well-known fact that anthropological attributes vary among different populations, thus necessitating specific standards of assessment for each population.

Conclusion

This present study revealed significant asymmetry in the external ears and has also shown that some morphometric variables gave a moderate to good (58.5%) right and left ear assessment accuracy, which emphasizes its utility as an adjunct for right and left ear estimation in Hausa ethnic group resident in Zaria. Nevertheless, we believe that larger samples should be examined in detail to further validate the findings of this study and come to definitive conclusions.

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Detection of *Mycobacterium tuberculosis* by Fluorescence Microscopy and its Susceptibility to Isoniazid and Rifampicin by Line Probe Assay in Kano, North Western, Nigeria.

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Abstract

Smear microscopy for Acid Fast Bacilli (AFB) in the diagnosis of pulmonary tuberculosis has played a crucial role particularly in Sub-Saharan Africa where facilities for sputum culture for Mycobacterium tuberculosis (MTB) are unavailable or are very expensive. This study was carried out to detect Mycobacterium tuberculosis and its susceptibility profile to rifampicin and isoniazid in Kano, Nigeria between January and August, 2016 with the aim of determining the incidence rate of the infection in the study population. A total of 400 sputum samples were collected, from the subjects studied comprising 203 (51%) males and 197 (49%) females, using a sterile wide mouthed translucent container, processed in a biosafety cabinet II and stained by Auramin O staining method (fluorescent). Fresh samples were recollected for the smear positive samples and were processed using N-acetyl-L-cysteine (NALC) and cultured on Lowenstein Jensen (LJ) medium for 8 weeks in an incubator and monitored weekly. The growth cultures were harvested in a biosafety cabinet II and DNA extracted using Hains Genolyse reagent according to standard methods. The extracted DNA was used to determine the susceptibility pattern of Mycobacterium tuberculosis to Isoniazid and Rifampicin by Hain's Line Probe Assay (reverse hybridization method) using Geno-type MTBDR plus VER 2.0 reagent. An overall positivity rate of 62 (15.5%) was found with incidence rate higher in males (51.9%) than females (48.1%) and age groups 16-30 years had the highest with 43.5% followed by 31-45years with 33.9%. Out of the 62 cultured on Lowenstein Jensen (LJ) medium, 53 grew up, no growth in 7 and 2 were contaminated. Two (2) samples were multi-drug resistant (male and female), one (1) was mono resistant-rifampicin resistance and isoniazid sensitive (male); forty seven (47) samples were susceptible to both isoniazid and rifampicin (24 males, 23 females), and three (3) Non Mycobacterium Tuberculosis (NTM). Sputum (AFB) for Mycobacterium remains a cheap and common method of diagnosing TB in our environment. Significant numbers of the patients are missed using this method, therefore effort should be made to increase the diagnostic yield by providing facilities such as culture and newer molecular methods, e.g., Gene-Xpert.

Keywords: Fluorescence Microscopy, *Mycobacterium tuberculosis*, Multi Drug Resistance, Isoniazid, Rifampicin, Kano

Introduction

Tuberculosis (TB) is a major public health concern worldwide: despite a regular, although slow, decline in incidence over the last decade, as many as 8.6 million new cases and 1.3 million deaths were estimated to have occurred in 2012. TB is by all means a poverty-related disease, mainly affecting the most vulnerable populations in the poorest countries.[1] It remains a global emergency health problem in 21st century,[2, 3] mainly affecting people in sub-Saharan Africa.[4] It is an infectious and transmissible disease caused by *Mycobacterium tuberculosis* comple.[5] In 2013, there were an estimated 9.0 million incident cases of TB (range, 8.6 million–9.4 million) globally, equivalent to 126 cases per 100 000 population .[6]

To reduce this burden of TB, detection and treatment gaps must be addressed, funding gaps closed and new tools developed. In 2014, 6 million new cases of TB were reported to WHO, fewer than two-thirds

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(63%) of the 9.6 million people estimated to have fallen sick with the disease. This means that worldwide, 37% of new cases went undiagnosed or were not reported. The quality of care for people in the latter category is unknown. Of the 480 000 cases of multidrug-resistant TB (MDR-TB) estimated to have occurred in 2014, only about a quarter of these – 123 000 – were detected and reported.[7] In 2014, an estimated 190, 000 people died of MDR-TB. More TB patients were tested for drug resistance in 2014 than ever before. Worldwide, 58% of previously treated patients and 12% of new cases were tested, up from 17% and 8.5% respectively in 2013. This improvement is partly due to the adoption of rapid molecular tests.[7]

Improved tuberculosis case detection and enlarged capacity for the detection of drug resistance are global priorities for tuberculosis control in resource limited settings. The development and implementation of fluoresce staining method could be a useful strategy and alternative to culture methods for TB treatment in resource limited settings. Without treatment, TB mortality rates are high. In studies of the natural history of the disease among sputum smear positive/HIV-negative cases of pulmonary TB, around 70% died within 10 years; among culture-positive (but smear negative) cases, 20% died within 10 years.[1, 6] Effective drug treatments were first developed in 1940s. The most effective first-line anti TB drug, rifampicin became available in the 1960s. The currently recommended treatment for new cases of drug-susceptible TB is a regimen of four-line drugs: isoniazid (INH), rifampicin (RMP), ethambutol (ETM) and pyrazinamide. Treatment for multidrug- resistant TB (MDRTB), defined as resistant to Isoniazid (INH) and rifampicin (RMP) (the two most powerful anti-TB drugs) is longer, and requires more expensive and more toxic drugs. For the first time in four decades new TB drugs are starting to emerge from the pipeline and combination regiment that include new compounds are being tested in clinical trials. There are several TB vaccines in Phase I or Phase II trial. For the time been however, a vaccine that is effective in preventing TB in adults remains elusive.[1]

Multidrug-Resistant TB (MDR-TB) must be addressed as a public health crisis. In 2012 the large number of new TB cases occurred in Asia accounting for 60% of new cases globally. However, sub-Saharan Africa carries the greatest proportion of new cases per population with over 255 cases per 100, 000 population.[1] In North -West Nigeria, with Kano having the largest population in the region and second to the largest population in the country, indeed there is need to establish the current trend of the disease and the use of rifampicin and isoniazid in the therapy of the disease due to increasing MDR cases in the study area and nation at large. At present, there is no much available data on that, and such data will inform the Programme managers/researchers on the current status and the appropriate course of action to improve the diagnostic performance of the programme. Hence the need for this study that was aimed to assess the current incidence rate of *Mycobacterium tuberculosis* diagnosed using fluorescent microscopy and susceptibility to Isoniazid (INH) and Rifampicin (RIF) in Kano, Nigeria.

Materials and Methods

Study Area

The study was conducted after an ethical approval was obtained at North West Zonal Tuberculosis Reference Laboratory, Aminu Kano Teaching Hospital Kano, AKTH, Nigeria. AKTH is a tertiary referral centre for diagnosis of both normal and drug resistant tuberculosis established in 1988 and is located in North Western Geopolitical Zone of Nigeria, with over 400 beds capacity.

Sample size

Four hundred (400) samples were collected which was determined using the standard formula [8] based on local prevalence study done in Zamfara (North-West) Nigeria with 40% .[9]

$$N=\frac{Z^2\times P(1-P)}{d^2}$$

$$\frac{1.96^2\times 0.4(1-0.4)}{0.05^2}$$

Where N= sample size-?
Z= standard normal variate (95% confidence level) =1.96
P= incidence rate from past studies-40%
d= absolute error – 5%

Sample Collection

Sputum samples from presumptive TB patients who reported to the laboratory on first visit for diagnosis were used. Two sputum samples were collected on 2 consecutive days from each patient (One spot and one early morning sample) in clean, sterile, leak proof, wide mouth containers. A structured questionnaire was administered. The processing of the sample was carried out in a biosafety cabinet. Each sample was subjected to fluorescent Auramine-O (AO) staining.

Smear_Preparation

Smears was selected from fine, pale -white moist (cheesy), yellowish purulent or blood tinged particles of the sputum specimen and spread over a 2x1 cm area in a clean, grease-free slide with a wooden applicator stick and allowed to air-dry and heat-fixed with Bunsen burner flame. Positive and negative control smears were used.[10]

Staining Procedure

The smears were arranged in serial order on staining bridge, with smear side up and flooded with filtered 0.1% Auramine-O and allowed to stain for 15 minutes after which they were rinsed with water and drained. Decolonization was done with 0.5% acid alcohol for 2 minutes. The smears was rinsed with water, drained and counter stained with 0.1% Potassium permanganate solution for 2 minutes, and rinsed with water, drained and allowed to air-dry. The smears were examined microscopically using the dry (40x) objective.[11, 12]

Reading of Fluorochrome Smear

Auramin-O stained smears were viewed using 40 x objectives under ILED fluorescence microscope (Zeiss Primo Star).[13] The results were graded in a defined manner as per the guidelines of International Union against Tuberculosis. The bacteria appeared brilliant greenish yellow against dark background under the microscope. Samples were viewed at the Reference laboratory using the World Health Organization (WHO) grading system. For positive results, the patients/clients were contacted through phone calls; a sterile container was given for them to produce a sterile sample for culture. Once the sample was produced, it was immediately processed for culture using Lowenstein Jensen medium and inoculated for 8 weeks for identification of growth. Negative samples were not use for further assay.

Susceptibility Testing -Line Probe Assay (LPA)

The reagents were procured from Hain's Life Science Company (UK, Ltd). Method adopted by CDC/ Institute of Human Virology, Nigeria. North West Zonal Reference Laboratory, AKTH, Kano version 2016, and manufacturer's instruction was followed strictly. Only positive samples were subjected to this assay to determine its susceptibility to Rifampicin and Isoniazid.

Sputum Inoculation into Lowenstein Jensen Medium Slants

Positive samples were decontaminated and inoculated into Lowenstein Jensen (LJ) Medium. On the LJ slants, inside a Biosafety Cabinet (BSC) level 2, the cap was carefully opened and the water of condensation was discarded to guard against contamination. Using a sterile transfer pipette, 2 drops of the decontaminated sample were dispensed into each tube and the cap loosen, the slopes were then placed at lying position for 24h before raising them up. After two days the culture was observed for a possible growth which could be cause by contaminants (as *Mycobacterium tuberculosis* are slow growers which start at 4th week of inoculation) were monitored weekly for up to 8weeks before they were concluded to be negative. Only the bacterium that grew up on the medium were considered *Mycobacterium tuberculosis*

Standard Diagnostics SD Bioline TB Ag MPT64 and Ziehl-Neelsen confirmatory test for *Mycobacterium tuberculosis* complex

All culture positive samples were confirmed using SD bioline and Ziehl-Neelsen (ZN) according to the National Committee for Clinical Laboratory Standards; NCCLS (2000). Organisms that were SD bioline positive and ZN positive DNA was extracted for Line Probe Assay (LPA) while for SD bioline negative, ZN positive, LPA was not done.

Results

The current work recorded an incident rate of 15.52 % of *Mycobacterium tuberculosis* (MTB) among the 400 sputum samples examined. Out of all the *Mycobacterium tuberculosis* (MTB) cultured samples, growth was observed in 53 samples, two (2) contaminated and no growth in seven. Line probe assay were done for the 53 samples, 2(3.8%) were multi-drug resistant tuberculosis (1 male and 1 female), 1 mono-resistance tuberculosis, 47 were susceptible to both rifampicin and isoniazid (24 males and 23 females) and 3 were Non Tuberculosis Mycobacterium (NTM).

The incidence of tuberculosis in relation to sex and age shows males had the highest rate (53.2%) against females (46.8%); Age group 16-30 has the highest rate 27(43.5%) followed by 31-45 , which had 21(33.9%), next is 46-60, which has 8(12.9%); 1-15 and 61-75 had the same incidence rate 3(4.8%) while 76-90 had zero (0%) as shown in Table 1, and in relation to HIV status, highest incidence was recorded in HIV negative 45(72.6%), followed by HIV positive 15(24.2%) and lastly those with unknown HIV status 2(3.2%) as presented in Table 2. The incidence of tuberculosis in relation to marital status and educational status indicated that the married had the highest rate of 56.5%.

The least was recorded in widows with 1.61%. For educational status, the non-educated has the highest incidence rate of 53.2%, the least was recorded in those that attended tertiary education with 6.5% as shown in tables 3 and 4. The incidence of tuberculosis in relation to occupation, the unemployed had the highest incidence of 27.4% and civil servants had less 1.6% (Table 5).

Susceptibility of tuberculosis to isoniazid and rifampicin in the study population indicates two samples were resistant to isoniazid and rifampicin (3.8%); one was resistant to rifampicin and susceptible to isoniazid (1.9%). Forty seven were susceptible to rifampicin and isoniazid 47(88.7%). Three (5.7%) were suggestive of non tuberculosis mycobacterium (NTM). The resistance was due to presence of mutation on the rPOB-MUT gene, KatG-MUT and inhA-MUT genes; absence of rPOB-WT, KatG-WT and inhA-WT genes on the probes while susceptibility was due to presence of wild type (WT) genes and absence of mutant (MUT) genes on the probes (Tables 6 and 7).

Table 1: Incidence of *Mycobacterium tuberculosis* in relation to age and sex to the studied population at Aminu Kano Teaching Hospital

Age Group	M	F	No. Pos. (%)	M	F	Total
1-15	36	26	3(4.8)	1	2	62
16-30	53	68	27(43.5)	14	13	121
31-45	69	64	21(33.9)	12	9	133
46-60	23	26	8(12.9)	4	4	49
61-75	20	12	3(4.8)	2	1	32
76-90	2	1	0(0)	0	0	3
Total	203	197	62(15.5)	33(53.2%)	29(46.8%)	400

P value < 0.00001; P < 0.05, df =5, X^2=59.8, there is a significant difference.

Table 2: Incidence of *Mycobacterium tuberculosis* in relation to HIV status of the studied population at Aminu Kano Teaching Hospital

TB /HIV Status	No. Pos.(%)	M	F	Total
Unknown	2(3.2)	1	1	20
Pos.(co-infection)	15(24.2)	6	9	131
Neg.	45(72.6)	24	21	249
Total	62	31	31	400

P value = 0.1402, P>0.05; df =2, X^2=3.93; there is no significant difference.

Table 3: Incidence of *Mycobacterium tuberculosis* in relation to marital status in studied population

Marital Status	No. Pos.(%)	Total
Single	24(38.7)	158
Married	35(56.5)	220
Widow	1(1.61)	9
Divorce	2(3.23)	13
Total	62	400

P value = 0.9826, P>0.05, df =3, X^2=0.168; there is no significant difference.

Table 4: Incidence of *Mycobacterium tuberculosis* in relation to educational status of the studied population

Educational Status	No. Pos. (%)	No. Neg.	Total
None	33(53.2)	155	188
Primary	15(24.2)	79	94
Secondary	10(16.1)	51	61
Tertiary	4(6.5)	53	57
Total	62 338	400	

P value = 0.2887; P> 0.05; df =3, X^2=3.759; there is no significant difference.

Table 5: Incidence of *Mycobacterium Tuberculosis* in relation to occupation of the studied population

Occupation	No. Pos.(%)	No. Neg.	Total
Civil servant	1(1.6)	20	21
Housewife	7(11.3)	43	50
Student	9(14.5)	58	67
Business	14(22.6)	78	92
Unemployed	17(27.4)	104	121
Farmer	6(9.7)	10	16
Artisan	8(12.9)	25	33
Total	62	338	400

P value = 0.1173; P>0.05; df =6, X^2=10.18; there is no significant difference.

Table 6: Susceptibility Pattern of *Mycobacterium tuberculosis* to isoniazid and rifampicin using line probe assay

Drugs	Resistant (%)	M	F	Susceptible (%)	NTM	Total
Isoniazid	2(3.8)	1	1	48(90.6)	3(5.7)	53
Rifampicin	3(5.7)	2	1	47(88.7)	3(5.7)	53
Total	5	3	2	95	6	106

Table 7: Resistance and susceptible gene pattern in the isolates.

Drugs Patterns Genes	TUB	rPOB-WT	rPOB-MUT	KatG-WT	KatG-MUT	inhA-WT	inhA-MUT
1. rRIF and rINH	+	+	+	+	+	+	+
2. rRIF and rINH	+	---	+	---	+	+	--
3. rRIF and sINH	+	---	+	+	---	+	---
4. sRIF and sINH	+	+	---	+	---	+	---

r = resistance, s = susceptible, TUB = tuberculosis control band, rPOB-WT = rifampicin wild type gene, rPOB-MUT= rifampicin mutant gene, KatG-WT = isoniazid Wild type gene, KatG-MUT= Isoniazid mutant gene, inhA-WT= isoniazid wild type gene, inhA-MUT= isoniazid mutant type gene**.**

Discussion

According to 2014 Nigeria Tuberculosis Fact Sheet, Nigeria ranks third among the 22 high burden TB countries in the world, and ranks 1st in Africa. In this study a smear positivity rate was found to be 15.5%, slightly higher than that reported which documented a smear positivity rate of 13.9% in Ile-Ife.[14] The difference may be due to the high sample size in the study area. Study conducted in South-East Nigeria however, indicated a high incidence rate of 51.5%.[15] In North-West Nigeria, 40.3% was reported as positivity rate.[9] In North- East Nigeria a positivity rate of 14.4% using fluorescent microscopy was documented, similar rate to this study.[16]

The incidence rate in this study was higher in males (53.2%) than Females (46.8%). This is not surprising since in India showed a similar incidence rate of 54.6% in males against females 47.2%.[17] The age distribution reveals highest incidence rate to be in age group 16-30 (43.5%) followed by 31-45 (33.9%). A study also showed highest prevalence rate in age groups 21 – 30yrs (28.2%) followed by 31-40yrs (21.6%).[15] This group represents the most productive age groups economically and correlates with work done in North Eastern Nigeria.[16]

HIV status indicates that HIV positive clients with TB have 24.2%, while HIV negative with TB have 72.6%. The co-infection rate in this study is higher than 13.9% recorded in Ile Ife, South West, Nigeria. [14] This might be due to depressed immune status which paves way for the TB to set in. Other reason why the HIV negative is higher than the positive is because the survey was not done in Anti-retroviral therapy clinic. In Uganda, survey conducted showed that the prevalence of HIV infection in TB is 41.4% higher than the one reported in this study.[18]

Marital status shows that the incidence was higher in married than others with incidence rate of 56.5%. This could be due to the fact that the married engage in many activities for livelihood which exposes them to the infection. Educational status reveals a high incidence rate in non-educated of 53.2%. Reason might be that there is little or no knowledge about the infection and its preventive measures. Occupation also shows a highest incidence rate in the unemployed (27.4%) followed by business men/women. This could be due to the fact that the unemployed could be left to the mercy of hunger which could lead to malnutrition and immune suppressed which could trigger latent TB to active form. For the business class, this might be due to exposure to carriers during business activities. A study in Ile Ife, South West, Nigeria recorded a high incidence rate in artisans.[14]

This study recorded an MDR rate of 3.8% from new patients which was higher than that found in National drug resistance survey conducted in Uganda in 2010(1.4%) and that found in Kampala drug resistance survey conducted in 2008 (1.1%) . [19, 20] In Nigeria, the 2012 National drug resistance survey is documented at 2.9 %.[21] In Rivers state, a 5.2% Multi-drug Resistant rate was also reported.[22]

There was high incidence rate of 15.5% using fluorescent microscopy in the study population. This rate is similar to that conducted in Abia, Nigeria in 2006 with an incidence rate of 15.9%.[23] The co-infected rate of TB-HIV was 24.2% which is high. HIV increases the rate of TB spread could be due to suppressed immunity. This was contrary to a study which documented 32.2% co-infection rate.[5]

Conclusion

Fluorescent microscopy has precisely updated an incident of tuberculosis as 12.52% in the population of Kano. Poor knowledge of tuberculosis could have contributed to the spread of the infection in the study area. Multi-drug resistant tuberculosis strain is present at 3.8% of intensity in the study population and if quick intervention is not done, the strain could spread in the city to lead to epidemic. Advanced molecular technique like GeneXpert could be employed which will increase the detection of more cases in Kano.

Recommendations

Incidence of tuberculosis in the study population can be reduced to undetectable level through the following recommendation.

1. The rate of resistant strain in the study is scary and more active case finding from community to community needs to be intensified to reduce the burden of tuberculosis in Nigeria.
2. Molecular method such as Genexpert should be utilized for all first line diagnostic method to increase the case detection
3. Regional laboratories should be designated as reference laboratories and equipped to perform basic tests and possibly carry out surveillance studies on MDR- TB strains and gene responsible for resistance.
4. Active case finding should be extended to communities both urban and rural to reduce the burden of the disease.
5. Awareness creation about the disease should be carried out to mosques, churches, schools and markets/ intensifying public awareness campaign of this infection and how to recognize early pulmonary TB is very important especially in the paediatric and the elderly age groups need to be achieved.
6. Laboratory workers (Clinical microbiologists, technologists, technicians and researchers) should be sent for refresher courses to update their knowledge and skills. These are surmountable tasks, which require the efforts of international donor agencies and private sector participation and with International collaborations substantial gaps that exists in TB research between poor resource countries and the developed world can be bridged.
7. It is also recommended here that infected patients should be closely monitored early enough to put in place appropriate therapy to promote the patients' health, reduce drug resistance problem and finally prolong life. Treatment of TB should be by directly observed Therapy (DOT).

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A Survey on Usage of Codeine Containing Cough Syrups in Urban Areas of Kano State, Nigeria

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Abstract

Narcotic and non-narcotic antitussive preparations are available as over the counter and prescription-only medications in Nigeria. The narcotic antitussives usually contain codeine with or without other agents (antihistamines, expectorants, decongestants and soothing agents). It is reported that the use of codeine containing cough syrups (CCCS) in Kano and most cities of the Northern Nigeria is widespread. This is accompanied by its attendants socio-political and health consequences. The present study investigated the usage of CCCS in Kano Urban areas. Structured questionnaire were administered to two hundred and twenty users of CCCS recruited within the study area. Data obtained were analysed using descriptive and inferential statistics. Youths in their twenties constituted the majority (69.0%) of the users of CCCS and they were mainly senior secondary school leavers (58.5%). Students formed a large proportion (40.5%) of users while others include drivers (9.5%) and labourers (5.5%). Male users constituted 70.5% while female were 29.5%. Volunteers described various rewards associated with use of CCCS ranging from 'feeling of comfort' (33.0%), increased concentration (15.0%), forgetting worries (10.0%) and feeling high (11.0%). About 34.0% admitted that they cannot go through a day without taking CCCS. Planned and unplanned withdrawal of CCCS was greeted by unpleasant effects like 'sickness' (22.5%), 'unhappiness' (14.0%), 'weakness/sleepy' (11.0%) and 'aggressive behaviour' (2.5%). Perceived problems associated with CCCS consumptions were: 'excessive sleep' (8.0%), 'excessive laughter' (3.5%), 'forgetfulness' (2.5%), and missing of lectures (2.0%). The users of CCCS were mainly youths in their twenties with educational attainment up to the level of senior secondary school certificate and mainly self employed. There was a surge in the proportion of female users of CCCS and some of their personal expressions suggest high level of addiction.

Keywords: Codeine, Cough-syrups, Drug abuse, Kano

Introduction

Drug abuse has been described as the use of illicit drugs, *or the misuse of prescription or over-the-counter drugs with negative consequences.*[1] Some of the drugs most often associated with this term include alcohol, amphetamines, barbiturates, benzodiazepines, cocaine, methaqualone and opioid, and they have varying degrees of physical and psychological dependence when taken for prolonged periods of time and usually at increasing dosages.[2]

There are growing health concerns on the rate at which codeine containing cough syrups (CCCS) are abused and cases of mental disorder have been linked to CCCS abuse.[3,4] The illegal use of CCCS and the ease of obtaining them is increasing at an alarming rate. Users on National Health Insurance Scheme have learned which symptoms to describe to get a prescription for the cough syrups.[5] In Denmark a new trend concerning the abuse of codeine has been reported. Danish drug abusers have discovered that codeine is easily separated from a number of medicine mixtures which is then used either orally or intravenously.[6]

A survey of primary abuse of CCCS in Nagaland (in India) indicated that most addicts were male in their early twenties who were unmarried and educated up to matriculation.[7] Most addicts in Assam (also in India) were Hindus (85.4%) and in Nagaland, Christians (81.6%).[7] Unemployment was predominant in both the groups. Mild forms of physical and psychiatric disorders amongst users

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were reported. Easy over-the-counter availability, lesser expenditure, milder withdrawals and ease of consumption without secrecy were some of the reasons observed by the study group for the emergence of this new form of addiction in Assam and Nagaland.[7]

In Nigeria, CCCS were transferred from the 'Over the Counter' (OTC) drug-category to the 'prescription only' (scheduled drugs or PoM) category. Such drugs can only be obtained from pharmacy shops after presenting drug prescriptions from physicians. Against this background, health care providers were called upon to understand that some drug users have figured out ways to manipulate the system to get CCCS.[8] The Directorate of Narcotic and Controlled substances of the National Agency for Food and Drug Administration and Control (NAFDAC) disclosed that a large number of Nigerians are abusing the prescription of codeine.[9]

The present survey characterised the users and usage of CCCS in Kano urban area of Northern Nigeria.

Methods Study Area

Kano city is the capital of Kano state, Nigeria. The state is situated in the Sahelian geographic region south of the Sahara. Kano is the commercial centre of Northern Nigeria and is the second largest city in Nigeria after Lagos. According to the 2006 census, Kano was the most populous state in Nigeria, with about 9,383,682 million people. The Kano urban area covers 137 km^2 and comprises six local government areas (LGAs) - Kano Municipal, Fagge, Dala, Gwale, Tarauni and Nasara wa - with a population of 2,163,225 at the 2006 Nigerian census. The principal inhabitants of the city are majority Muslims. As in most parts of Northern Nigeria, Hausa language is widely spoken in Kano.[10]

Selection of Study Centres

The survey was conducted in the Kano Urban area where pharmaceutical premises and patent medicine shops were identified as data collection centres. Willingness to participate in the study by pharmacy directors and patent medicine shop owners was the sole criterion of selecting study centres.

Study Population

A total of two hundred and twenty (220) volunteers recruited from local clubs (Daba) and thirty-two (32) premises (pharmacies and patent medicine shops) distributed across the study area were used for the survey. Respondents for this study gave informed consent to participate in the study.

Ethical Clearance and Study Period

The work was approved and given ethical clearance by the Research Committee of the University of Maiduguri in 2012. Pilot study was conducted in 2013 following grant offer from National Institute of Pharmaceutical Research and Development. It was carried out between March 2014 and March 2015.

Study Design

An observational descriptive design was adopted in the study.

Questionnaire and Data Collection

Structured mixed and non-disguised questionnaire were administered to 220 recruited volunteers out of whom 11 were invalidated prior to data analysis. The first part of the questionnaire addressed the demographic and simple characteristics of the respondents while the second part, habits and attitudes surrounding the use of CCCS.

Limitations of the Study

Inability to implement a scientifically acceptable method of sampling in choosing study centres for fear of legal litigation by owners of medicine stores. Selection was limited to consented shops and volunteers.

Data Presentation/Analysis

Data were grouped and presented as frequency and percentage tables and was further analysed by use of Statistical Package for Social Sciences (SPSS) version 16.0®. Chi-square analysis was used to test for relationship or association between categorical variables. A P-value of ≤ 0.05 was considered statistically significant. Descriptive analysis was used to discuss the outcome of the survey.

Results

The demographic and basic characteristics of users of CCCS in Kano Urban Area are described in table 1, while attitudes and trends of usage are presented in tables 2 and 3.

Table 1: Demographic and basic characteristics of respondents using CCCS in Kano Urban area

Demography	Variables	Number (Percentage)
Sex	Male	141(70.5)
	Female	59 (29.5)
	Total	200 (100)
Age group (Years)	7 – 12	1(0.5)
	13 – 19	16 (8.0)
	20 – 30	138 (69.0)
	31 – 49	44 (22.0)
	> 50	--
	No response	1(0.5)
Educational background	Non-formal education	--
	Primary (Pri.Sch.Cert)	5 (2.5)
	Secondary (Sec Sch. Cert.)	117(58.5)
	Diploma	6 (3.0)
	Degree	5 (2.5)
	Post Degree	--
	None	29 (14.5)
	No response	38 (19.0)
Occupation	Civil servant	15 (7.5)
	Student	81(40.5)
	House wife	9 (4.5)
	Self-employed/Business	54 (27.0)
	Driving	19 (9.5)
	Drug vendors	--
	Labourers	11(5.5)
	Applicant	5 (2.2)
	Unemployed	--
	No response	6 (3.0)

Male users constituted 70.5% while female were 29.5%.Youths in the age group of 20-30 years constituted the majority (69.0%) of the users (respondents) of CCCS and were mainly senior secondary school leavers (58.5%). Students formed a large proportion of users while others included drivers and labourers (Table 1).

Table 2: Characteristics of usage of CCCS in Kano urban area

Variables	Options	Frequency (Percentage)
Perceived associated feelings after taking CCCS	Feel high	22 (11.0)
	For comfort	67 (33.0)
	Strong/active	6 (3.0)
	Sleep/forgetting worries	20 (10.0)
	To concentrate	30 (15.0)
	Feel tipsy	6 (3.0)
	Allow to respect others	12 (6.0)
	Gain respect from others	19 (9.5)
	Decreasing hyperactivity	3 (1.5)
	Decrease fighting urge	3 (1.5)
	No response	12 (6.0)
Ability to go through a day without taking CCCS	Able	128 (64.0)
	Not able	68 (34.0)
	No response	4 (2.0)
Feeling when CCCS is not taken	Normal	96 (48.0)
	Sick	45 (22.5)
	Not happy	28 (14.0)
	Sleepy/week	22 (11.0)
	Aggressive	5 (2.5)
	No response	4 (2.0)

Data in Table 2 describes the feelings associated with taking CCCS, the respondents used variable rewards ranging from 'feeling of comfort' (33.0%), increased concentration (15.0%), forgetting worries (10.0%) and feeling high (11.0%). About 34.0% admitted that they cannot go through a day without taking CCCS. Planned and unplanned withdrawal of CCCS was greeted by unpleasant effects like 'sickness' (22.5%), 'unhappiness' (14.0%), 'weakness/sleepy' (11.0%) and 'aggressive behaviour' (2.5%). However 48% admitted feeling normal when CCCS is not taken.

Table 3: Challenges and financial involvements among the respondents using CCCS in Kano urban area

Variables	Options	Frequency (Percentage)
Perceived problems associated with taking CCCS	Excessive sleep	16 (8.0)
	Quiet all day	3 (1.5)
	I missed some lectures	4 (2.0)
	Laugh too much	7 (3.5)
	No problems	19 (9.5)
	Fighting	3 (1.5)
	Forgetting belongings	5 (2.5)
	Vomiting	3 (1.5)
	Police charges	3 (1.5)
	No response	137 (68.5)
Estimate of number of bottles consumed per day	1	86 (43.0)
	2	80 (40.0)
	3	24 (12.0)
	4	10 (5.0)
	5	--
Estimated amount spent per day	<1250.00 N	64 (32.0)
	1251.00 – 1500.00 N	86 (43.0)
	1501.00 – 1750.00 N	40 (20.0)
	1751.00 – 2000.00 N	8 (4.0)
	2001.00 – 2500.00	2 (1.0)
	>2500.00 N	--
Any encounter with law enforcement agents	Yes	159 (79.5)
	No	25 (12.5)
	No response	16 (8.0)

Analysis of data in Table 2b showed that nine and half percent (9.5%) of respondents believed that taking CCCS is not associated with any problem. On the other hand, others associated it to problems like 'excessive sleep' (8.0%), 'excessive laughter' (3.5%), 'forgetfulness' (2.5%), and missing of lectures (2.0%). Estimated consumption of 1 bottle (43.0%) and 2 bottles (40.0%) per day was prevalent amongst the users of CCCS. Majority (79.5%) admitted an encounter with law enforcement agents.

Discussion

The proportion of female (29.5%) users of CCCS in relation to male is considered high in Kano Urban areas when compared to some cities of the North-Eastern Nigeria like Maiduguri and Askira.[11] It was reported that the use of codeine-containing substances is on the increase among women and girls, particularly married women, and that female drug abusers are drawn to codeine simply because it does not smell like cannabis.[3] These findings are corroborated by anecdotal reports that emanated from the National Drug Law Enforcement Agencies (NDLEA). The agency lamented on the increasing involvement of married women in drug abuse, believing erroneously that these drugs enhance their sexual appetite. Investigations revealed that the stability of some homes is already being affected by this trend.[3]

Most of the users of CCCS are in their twenties and have attained senior secondary school educational qualification. A report by Dankani [12] showed that about 11% of youths in Nigeria rely on one form of drug or the other which poses a serious threat to sustainable youth development in the Northern Region.[12] As opposed to the findings of the present

work, the mean age of abusers of CCCS in Assam and Nagaland of India is 17 and 15 years respectively.[8] Similar study conducted in Maiduguri and Askira towns of North-Eastern Nigeria revealed that the clear majority of users of CCCS are in their twenties and they attained secondary school level of education.[11]

Majority of the respondents were either students or self-employed. This invariably means that effective educational pursuit may be hampered with amongst these users. Perhaps this explained why the majority of the users could not proceed beyond senior secondary school educational pursuit. Inability to proceed with educational pursuit after secondary school education could be a predisposing factor to the initiation of CCCS usage.

In describing the rewards attached to CCCS consumption, majority of the users admitted to increased concentration, comfort and feeling high. In line with the present findings, it was reported that there was massive consumption of CCCS as a means of getting high among youth in Northern Nigeria. Peer influence and depression/anxiety were the major factors contributing to consumption of drugs by the youths.[12] Even though above 50% of the respondents expressed their ability to go through a day without taking CCCS, the remaining respondents expressed various kinds of obnoxious feelings associated with the stoppage of CCCS. This suggests an establishment of dependence on CCCS among the respondents.

Majority of the users take 1 to 2 bottles of CCCS in a day amounting to a total daily expenditure of one to two thousand naira. This amount is not likely going to be easily acquired from the unemployed or self employed on petty businesses. The likely resolution of a dependent user is to source the drug or money by any means available. Perhaps this explains the rising crime rate among youths and within homes in the study area. It was reported that the Government of Kano State lamented on the increasing rate of crimes in the State.[13]

Majority of the users did not express any perceived hazards or problems associated with taking CCCS. This suggests that they may be unaware of associated health hazards on one hand or they are aware of the hazards but are in self denial. The fact that the majority admitted having encountered problems with the law enforcement agencies suggests that the agencies are making impacts in their mission of curtailing the menace. However it is suggested that the Government and other related agencies should intensify efforts in ensuring that control drugs are only restricted to relevant health facilities.

Conclusion

The users of CCCS in urban areas of Kano State were mainly youths in their twenties with educational attainment of senior secondary school certificate followed by the self employed. There was a surge in the proportion of female users of CCCS and some of their personal expressions suggests some level of addiction.

Recommendation More strategies should be deployed by government to sanitize the distribution of CCCS and to encourage the drug law enforcement agencies towards wholistic approach to curtailing CCCS usage.

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The Role of Fingerprints' Ridge Breadths in Identification of Sex and Age Estimation of The Hausa Ethnic Group in Nigeria

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Abstract

Fingerprints are the most commonly used biometric techniques that are considered legitimate proofs of evidence in courts of law. Apart from the use of ridge density to determine sex differences, ridge breadth (RB) should be considered where only a fraction of ridges is available. This research was aimed at identifying sex and estimation of age of an individual using fingerprints' RB of Hausa ethnic group in Nigeria in case of mass disaster. Data was collected from 263 male subjects and 208 females of age range 12 to 24 years. Fingerprint scanner device was used to capture fingerprints. Both radial and ulnar ridges were obtained by the application. Independent-samples t-test was used in assessing differences between sexes and regression analysis was used in establishing models for age estimation. The result revealed a significant difference in fingerprints' RB between sexes, with females exhibiting narrower mean RB, 0.63 mm than males, 0.66 mm ($p = 0.017$) for subjects between the ages of 12-17 years. For subjects between the ages of 18-24 years, 0.65 mm in female and 0.68 mm in male ($p = 0.015$). The mean RB varies significantly between left and right of the thumb and between radial and ulnar aspects of a fingerprint pattern, where ulnar ridge compartments give a better result for sexual dimorphism. Age estimation was equally significant in both sexes, but the prediction power was weak, with the largest $R^2 = 0.031$. In conclusion, sex identification and age estimation from fingerprints' RB in Hausa population is possible, thus alleviating the challenges of forensic investigation.

Keywords: Fingerprints, Sex identification, Age estimation, Ridge breadth, Hausa

Introduction

The fact that fingerprint pattern has been of significant use over time is irrefutable. Fingerprint can be defined as an impression left by the friction ridges of the finger.[1] Various types of equipment, inks, scanning devices, and techniques are used to record friction ridge detail. Normally, a white card friction ridge can also be recorded digitally with a fingerprint reader using techniques called live scan. The science of fingerprints has been acclaimed and reputed as a panacea for individualization particularly in forensic investigations.[2] Security challenges and technological advancement in our environment brought about the increased interest in biometrics.[3] Biometrics is essentially more reliable than the traditional password or access card methods of identification.[4] Traditional methods are prone to fraud. The variability of epidermal ridge breadth in humans is substantial.[5]

Fingerprints features were found to differ between sexes, ethnic groups and age categories.[6] Like many traits, genes influence pattern formation indirectly by contributing to the timing of the onset of friction ridge skin, the timing of the onset of volar pad regression, the growth rate of the foetus among others. Stresses across small areas of skin are not inherited, but to some extent, they represent one of many environmental factors that influence pattern formation.[7] It is well established that friction ridge patterning is also affected by the intra uterine environment.[8] Therefore, fingerprint ridges develop under the influence of genetic and environmental factors.[9] This is the reason behind the variation seen in fingerprints among individuals including identical twins.[10] Ethnic differences in ridge breadth among Yoruba students (Nigeria), English and Jews were found to be substantial in both sexes. [11]

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Within individuals, the breadth of fingerprint ridge varies within the hand and between right and left, but the difference is quite small, on the order of 0.05 mm and less.[6]

Indirect method was used for determination of ridge breadth: the number of ridges crossing a defined line transversely was counted and the ridge breadth is a result of dividing the number of ridges by a length of the transversely defined line.[6] It was evident that the epidermal ridge breadth varies considerably between different dermatoglyphic regions and also between the sexes. Ridges on the palm were coarser than on the fingertips. Digit I had a higher ridge breadth than all the other digits, the order of the decreasing ridge breadth was: I > II > III > V > IV and in the palm: thenar > first inter-digital region > hypothenar > inter-digital II > inter-digital IV > inter-digital III. The ridges on the right hand are coarser.[6] The ridge breadth co-varies with other dermatoglyphic features. In fingers, the ridges are coarser in the ulnar loops than in whorls.[6] Sexual dimorphism was also observed in fingerprint pattern distribution.[12] [13] It was observed that males have higher ridge breadth defined as the distance between the centres of two adjacent valleys, than females. [5] Females were observed to have a higher ridge thickness to valley thickness ratio compared to males. Fingerprints ridge breadths of two samples of the Argentinian population were analysed; the result revealed that females consistently exhibit narrower epidermal ridges than males. [14] Ridge breadth was found to be 9% greater in males than in females, where mean epidermal ridge breadth (MRB) under 0.39 mm signifies a sub-adult individual less than 15 years of age and MRB values over 0.52 mm came solely from adult males. [5] However, age changes of ridge breadth in teenagers overlap with adult sexual dimorphism. [5]

The real fingerprints were collected from different age groups such as 15-20 years and 20- 60 years of the rural and urban people. According to the experimental observation, soft biometric information can be used significantly to improve the recognition performance of the biometric system. The overall performance of the proposed method was found to be satisfactory and more competitive. [15] Estimation of age based on fingerprint was proposed, in which extraction of features via 2D Discrete Transforms and Principal Component Analysis was done, where a dataset of 400 fingerprints of the age of 12-60 years was collected and the overall success rate of classification in age estimation was around 68%. [16] In a conducted study of ridge breadths, it was stated that, at the age of more than 16 years the increase in ridges breadth was small. The difference between 16–19 years old and adults over 20 years old was not significant in either sex. [17]

Sex and age determination of an unknown individual can guide investigators to the correct identity among the large number of possible matches. It can also improve crime detection in forensic investigations through reducing the number of suspects by considering the only sex and age detected in a crime investigation. Security of life and property is very important in any society. Despite the efforts made by police and other security agencies through various means of crime detection, some criminals go undetected because of challenges of overwhelming population in forensic investigations and the ever increase in different crime activities such as cyber-crime. Apart from the common use of ridge density, ridge pattern or minutiae to determine sex differences, it is important that ridge breadth (RB) should be considered in a situation where only a fraction of ridges is available in a small area. This research was therefore conducted to determine sex differences in fingerprints ridge breadths, and to determine the age of individuals of Hausa ethnic groups using fingerprint ridge breadth.

Materials and Methods

Study Area

This research was conducted in Bayero University Kano. Data was collected from students of the Faculties of Basic Medical Sciences, Clinical Sciences, Allied Health Sciences, Dentistry, Arts and Islamic Studies of Bayero University, Kano, and Bayero University Staff Secondary School. For individuals below the age of 18 years, in the informed consent, their participation was approved by their parents or guardians. For a participant to be selected for the study, both of his/her paternal and maternal grandparents must be of Hausa ethnic group.

Study population

Stratified probability sampling technique was applied in data collection. The study population was stratified in to two strata; Secondary school and

undergraduate students. The first stratum was classified into four levels, junior secondary school III, senior secondary school I, II and III. The second stratum was also classified into four levels, level I, II, III and IV. Four hundred and seventy-one (471) participants were randomly selected from the eight levels, with 263 males and 208 females. The ages rang of the participants was 12-24 years.

Capturing fingerprints

An application system designed by ZKTeco Inc., China was modified by a software programmer to capture the fingerprints of sampled population through a fingerprint scanner device interfaced to the computer system via Universal Serial Bus (USB). The application was created using Microsoft Visual Basic 6.0 integrated development environment (IDE). Fig. 1 (left) shows how fingerprint was captured to the computer system. Fig. 1 (right) shows the runtime environment of the application where data is collected, Fig. 2 (left) shows the fingerprint analysis environment page of the application. The "next picture" button is clicked to select and upload a fingerprint for analysis. The radial and ulnar areas were designed such that by clicking the "move area", areas were dragged to a point of reference for counting. The ridges count on mouse click on the ridge under the selected area. "Save" button was clicked to save the information obtained appropriately and transported to Microsoft Excel for further analysis.

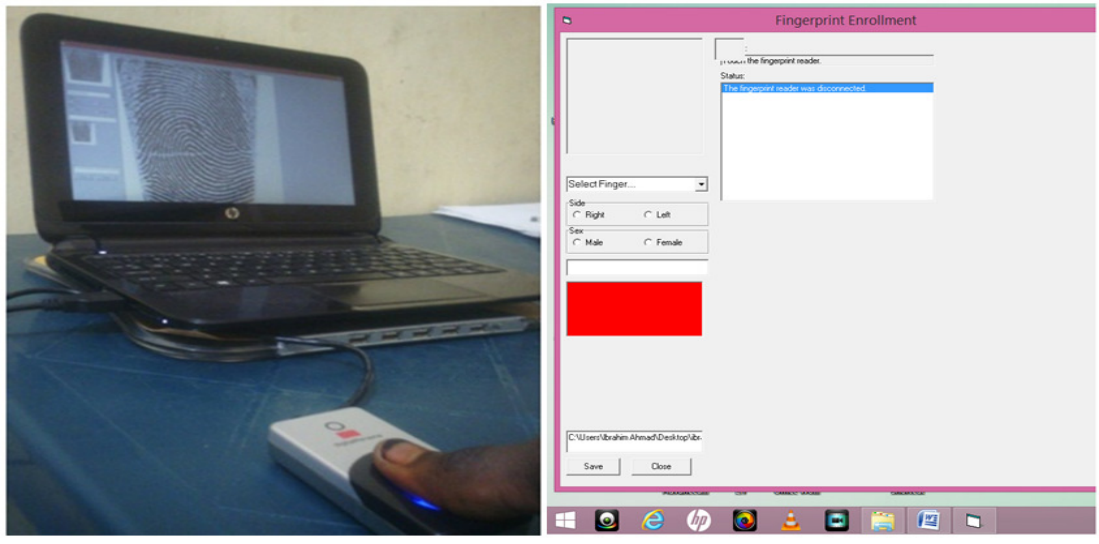

Figure 1: Thumb-printing through fingerprint reader and the runtime environment of the application

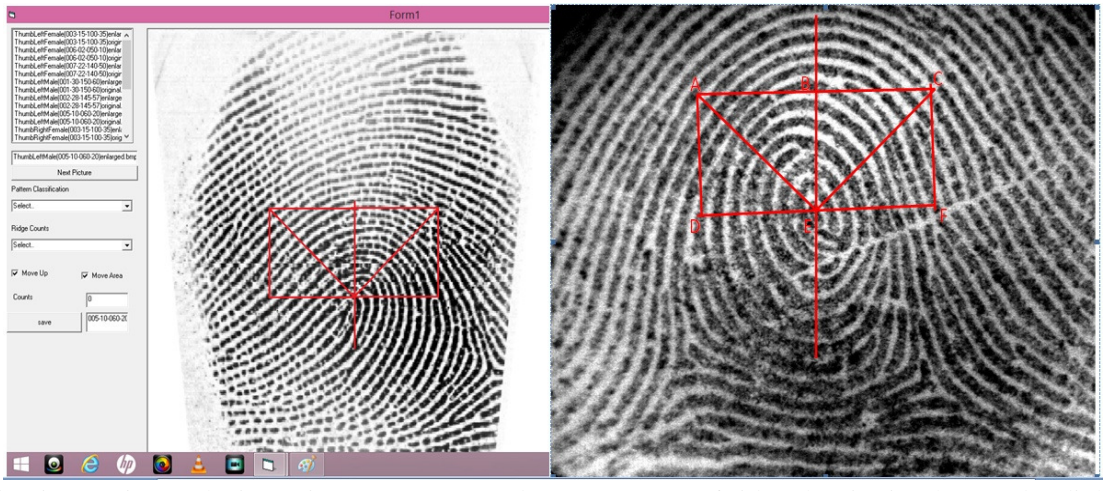

Figure 2: Fingerprint analysis environment page and measurement of ridge density in ulnar and radial area

Determination of ridge breadth

Mean ridge breadth (MRB) was determined through the method developed by Penrose [18]: By measuring the distance between the centre of one epidermal furrow and the centre of the next furrow along a line at right angles to the direction of the furrows. However, to locate a specific area for the measurement, the method developed by Acree [19] in determining ridge density was applied; where ulnar and radial ridge compartments were considered separately. The diagonals (E-A & E-C fig. 2 right) of each of the 25 mm^2 of fingerprint on both the ulnar and the radial sides crossed perpendicular to the ridges. MRB was obtained by dividing the length of the diagonal which, according to Pythagoras theorem is = $\sqrt{(5^2+5^2)}$ by the number of ridges along the diagonal (RD). Thus, MRB= $\sqrt{(5^2+5^2)} \div$ RD. To be more specific, MRB on both radial and ulnar compartments and of the left and right fingerprints was determined separately and recorded.

Statistical analysis

The data was expressed as mean ± SD. Independent-samples *t*-test was used in assessing differences between sexes where, the population under study was divided into two groups : group 1; within the age of 12-17 years and group 2; within the age of 18 to 24 years. Simple linear regression analysis was used to determine age from fingerprint ridge breadth. $p < 0.05$ was set as significant difference in the mean scores on the dependent variable for each of the two groups.

Results

Descriptive statistics revealed that, for males, the minimum ridge breadth in mm on the radial and ulnar of both sides (right and left) was 0.49, but the maximum ridge breadth in mm on the radial and ulnar of both sides was 0.92, except on the radial compartment of the right thumb that has 1.07 mm with a mean of 0.67 throughout except ulnar ridge breadth (URB) of the left thumb where a mean of 0.66 ± 0.08 was observed (Table 1). In the case of female subjects, the maximum ridge breadth in mm on the radial and ulnar count of both sides was 0.92, and the minimum ridge breadth was 0.43 on the ulnar compartment of the left, and 0.46 on the remaining (Table 1). The mean of 0.65 was observed on both right and left radial ridge breadth (RRB), 0.63 on the left Ulnar RB, and 0.64± 0.08 on right Ulnar RB (Table 1).

Table 1: Descriptive statistics of basic biometrics/thumbprint of the participants

Sex		Side	Mean ± SD	Minimum	Maximum
Male (n=263)	Ulnar ridge breadth	Right	0.67±0.08	0.49	0.92
		Left	0.66±0.08	0.49	0.92
	Radial ridge breadth	Right	0.67±0.09	0.49	1.07
		Left	0.67±0.08	0.49	0.92
Female (n=208)	Ulnar ridge breadth	Right	0.64±0.08	0.46	0.92
		Left	0.63±0.08	0.43	0.92
	Radial ridge breadth	Right	0.65±0.08	0.46	0.92
		Left	0.65±0.09	0.46	0.92

An independent-samples *t*-test was conducted on the entire sample to compare the ridges breadths (RB) for males and females. There was a significant difference in RB between the two sexes. For the subjects of 12-17 years (Table 2), the mean URB on the right thumb of males was 0.66 ± 0.09, and for females it was 0.63 ± 0.08, (t=2.40, p=0.017). The magnitude of the differences in the means was small (eta squared=0.030). The males mean RRB was 0.66 ± 0.08 and that of females was 0.66 ± 0.08 (t=0.15, p=0.88). For the sample population of 18-24years (Table 2), the mean URB on the right thumb of males was 0.68 ± 0.08, and for females it was 0.65 ± 0.08, (t=2.45, p=0.015). The magnitude of the differences in the means was small (eta squared=0.02). The males mean radial ridges breadth (RRB) was found to be 0.67 ± 0.09 and that of females was 0.65 ± 0.08 (t=1.98, p=0.049). The magnitude of the differences in the means was very small (eta squared=0.014). The result of the analysis on the left thumb (Table 2) was equally the same. It is obvious from the results below, that there were significant differences in FRB on both age groups except on the RRB of both right and left thumbs of the 12-17 age groups.

Table 2: Sex difference in right and left thumb-prints ridges breadth across different age groups of Hausa ethnic group

			Male	Female		
Side	**Age (Years)**	**Variables**	Mean ± SD (mm)	Mean ± SD (mm)	t	P
Right	**12-17**	Ulnar ridge breadth	0.66±0.09	0.63±0.08	2.40	0.017
		Radial ridge breadth	0.66±0.08	0.66±0.08	0.15	0.880
	18-24	Ulnar ridge breadth	0.68±0.08	0.65±0.08	2.45	0.015
		Radial ridge breadth	0.67±0.09	0.65±0.08	1.98	0.049
Left	**12-17**	Ulnar ridge breadth	0.66±0.08	0.63±0.08	2.15	0.033
		Radial ridge breadth	0.64±0.08	0.66±0.09	-1.03	0.304
	18-24	Ulnar ridge breadth	0.66±0.08	0.63±0.08	3.33	0.001
		Radial ridge breadth	0.67±0.09	0.65±0.09	2.18	0.030

It was observed in male participant that; only radial ridge breadth (RRB) of the left presented significant models, with R^2; 0.026 which is expressed in percentage and therefore explained 2.6 % of the variance in age. Thus, RRB can predict age by only 2.6 %. The result for the model was statistically significant (p = 0.009) (Table 3). In the case of female it was observed that, only URB of the right presented significant models, with R^2= 0.031 which is expressed in percentage and therefore explained 3.1 % of the variance in age. Thus, URB can predict age by only 3.1 %. The result for the model was statistically significant (p = 0.011)(Table 3).

Table 3: Simple linear regression analysis for prediction of age from fingerprint ridges breadth in Hausa ethnic group

Sex	**Side**	**Model**	**R**	**R^2**	**R^2 (%)**	**SEE**	**F**	**P**
Male	**Right**	Age=URB (2.649) + 17.594	0.078	0.006	0.6	2.87	1.55	0.214
		Age=RRB (2.541) + 17.686	0.076	0.006	0.6	2.87	1.46	0.228
	Left	Age=URB (0.875) + 18.764	0.024	0.001	0.1	2.87	0.15	0.700
		Age=RRB(5.446) + 15.730	0.161	0.026	2.6	2.83	6.88	0.009
Female	**Right**	Age=URB (6.158) + 13.624	0.177	0.031	3.1	2.67	6.63	0.011
		Age=RRB (1.767) + 16.436	0.052	0.003	0.3	2.71	0.54	0.463
	Left	Age=URB (1.060) + 16.893	0.031	0.001	0.1	2.71	0.19	0.663
		Age=RRB(1.710) + 16.446	0.055	0.003	0.3	2.71	0.62	0.433

R; Pearson correlation coefficient R^2; square of Pearson correlation coefficient (coefficient of determination), SEE; standard error estimation, URB; ulnar ridge breadth, RRB; radial ridge breadth, n= 471

Discussions

Several studies have been conducted on fingerprint but, mainly for ethnic determination, [12] genetic inheritance and sexual dimorphism of ridge pattern. [20] The present study has been conducted to expand the scope of ridge breadth i.e. estimation of age and sex differences in fingerprint ridge breadth. This study showed that males of Hausa population compared to their female counterparts have significantly thicker ridges. It also showed a significant difference across certain age groups. This may be due to the fact that males tend to have less ridges per given area, [21] compared to their female counterparts, and that, ridge breadth is likely to have indirect relationship with ridges density, because thicker ridges may be fewer than thinner ridges in a given area. Interestingly from the analysis conducted in this study, the mean ridges breadth varies significantly across specific age groups, side of the thumb and even radial or ulnar aspect of a fingerprint pattern. In the right thumb, it was found that both mean ulnar and radial ridge breadths increase with age in the case of male individuals, but in the case of females, only mean ulnar ridge breadth

of the right thumb increases with age. In fact, the mean radial ridge breadth was found to be slightly narrower in both thumbs of those between the ages of 18 – 24 years. In the left thumb, mean ulnar ridge breadth did not differ between the two age groups in both sexes, but mean radial ridge breadth in male of 18 – 24 years is higher as compared to that of 12 – 17 years. It was observed that fingerprints show a constant variation from one age group to the next. [22] This may be due to the fact that fingerprint ridges complete development toward the end of 2nd trimester and remain fixed permanently, [23] with growth, each ridge increases in breadth.

These analyses revealed that there were significant differences in fingerprint ridge breadth between the two sexes across age groups. Mean URB for males was higher than that of females. A similar result was obtained from the left, the result is in conformity with what was observed, that females consistently exhibit narrower epidermal ridges than males. [14] But, it was observed that mean epidermal ridge breadth (MRB) values over 0.52 mm came solely from adult males. [5] This study contributes to a resolution of otherwise, because it is clear that MRB of female can exceed this value and this may be attributed to ethnicity of the subject, whether thumb ridge or all fingers are considered, and side of the fingerprint read.

It was found that RRB did not present any significant difference between the two sexes of 12-17 year category, and even in 18-24 year category there was rather less significant difference between the two sexes in RRB compared to URB, implying that ulnar ridge compartment is far better for determination of sex. The reason behind it remains unclear. However, it could be a genetic marker that may be related to hormonal activity which regulates sexual characteristics. It could be concluded from this study that ulnar ridge compartments give a better result for sexual dimorphism than radial ridge compartments.

Regarding age estimation from fingerprint ridges breadth, it was observed that there was no statistically significant contribution to the models in both ulnar and radial ridges breadth of male right thumb. It was reported that at the age 16 years and above, the increase in ridges breadth was small, such that the difference between 16–19 years old and adults over 20 years old was not significant in either sex. [17] However, there was a statistically significant contribution to the models in male radial ridges breadth of the left thumb. This may be due to dermatoglyphic asymmetry and fingerprint ridges compartment. The reason could also be as a result of relationship between ridges breadth with height and weight, and that maximum height is usually attained at the early stage of this age category with little or no gain in body weight after, so the changes in ridges breadth is likely less significant. The result of this study indicated that radial ridges breadth of the left hand can best be used in estimating the age of a male participant, unlike the sexual dimorphism where ulnar ridge compartment was better. In the case of female, only ulnar ridges breadth of the right contributed significantly to the models in Hausa population. Here again, for females ulnar ridges breadth, not radial ridges breadth, and of the right, not the left that seems to contribute significantly. The mechanisms behind it are not clear, but could be a genetic factor related to sex. It was found that even though the results for some models were statistically significant, the prediction power is weak; this is because the largest R^2 in the whole models was found to be 0.031, meaning it can only predict age by 3.1%.

In conclusion, sex difference, which is an important tool in forensic science exist in fingerprint ridges breadth, but prediction of age from fingerprint ridges breadth is difficult despite reaching statistical significance because the prediction power was weak.

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Effect of Methanol Leaves Extract of *Cordia Africana* on Blood Glucose, Lipid Peroxidation and Oxidative Stress Biomarkers in Alloxan-Induced Diabetic Wistar Rats.

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Abstract

This study investigated the effects of methanol leaves extract of Cordia africana on blood glucose and oxidative stress biomarkers in alloxan-induced diabetic Wistar rats. Diabetes in the rats weighing between 120-150 g was induced with alloxan 150 mg/kg intraperitoneally. The rats were grouped into six groups of five animals per group. Group 1 is the normoglycaemic control group. Group 2 served as the diabetic negative control, Group 3 served as positive control and was treated with glibenclamide, Group 4, 5 and 6 were treated with 100 , 200 and 400 mg/kg body weight of methanol leaf extract of Cordia africana respectively. The extracts were given to the animals orally for a period of 4 weeks. At the end of the experimental period, the rats from each experimental group were euthanized with chloroform and the serums were collected and used for the determination of the oxidative stress biomarkers. The results revealed that there was a significant decrease ($p<0.05$) in the blood glucose levels as compared with the control. The highest activity resides at 4 weeks of administration of the methanol extract of cordia africana at the doses of 100, 200 and 400 mg/kg respectively significantly decreased the blood glucose levels as compared with the control untreated. However, in relation to the lipid peroxidation, there was a significant increase in the level of malondialdehyde when compared to control. Consequently, there was a significantly decreased the oxidative stress biomarkers (superoxide dismutase, catalase and glutathione peroxidase) as compared with the control untreated. The preliminary phytochemical screening of the methanol extract revealed the presence of tannins, saponins, flavonoids, alkaloids, cardiac glycosides, cyanogenic glycosides, Resins, Steroid/Terpenoids and Carbohydrates. The LD_{50} was found to be greater than 5000 mg/kg orally.

Keywords: Methanol, *Cordia africana* Blood glucose, Oxidative stress, Lipid peroxidation

Introduction

Diabetes mellitus (DM), one of the most challenging health pandemics of the 21st century currently affects about 347 million people worldwide. [1] This number is rapidly increasing and is expected to double by the year 2030, making diabetes the 7th leading cause of death in the world. DM is a complex metabolic disorder, characterized by high blood glucose levels (hyperglycaemia) and impaired lipid, carbohydrate and protein metabolism as a result of defects in insulin secretion, insulin action or both.[2 ,3] As a consequence of these metabolic alterations, at least 50 % of people with diabetes develop one or more microvascular (viz. retinopathy, neuropathy and nephropathy) or macrovascular (viz. myocardial infarction, heart failure and stroke) complications.[2] These complications are common to both types of diabetes, despite the difference in the pathogeneses of the diseases.[3] Poor control of blood glucose levels is the key contributing factor to the associated complications and treatment of hyperglycaemia is therefore, the main target in the prevention of these diabetes-related complications.[4,5]

Hyperglycaemia plays a critical role in the development and progression of diabetic complications by numerous mechanisms, including increased oxidative stress, decreased nitric oxide bioavailability, glucose autoxidation and non-

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enzymatic protein glycation.[6] It is also well known that oxidative stress develops when reactive oxygen-derived free radical production exceeds the antioxidant defense mechanism of the cell[6,7]. Furthermore, altered levels of blood lipids (dyslipidemia), mainly triglyceride and cholesterol are involved in the pathogenesis of cardiovascular disease in both type 1 and type 2 diabetes.[2,3] The detection and treatment of dyslipidemia could, therefore reduce the risk of cardiovascular disease and its consequences in diabetic patient.[2] Current therapies used in the treatment of DM include, insulin and pharmaceutical oral hypoglycaemic/anti-diabetic agents such as sulphonylureas, biguanides and glinides which have their own limitations and undesirable side-effects such as hypoglycaemia, gastrointestinal disturbances, lactic acidosis and liver toxicity.[8,9,10] In recent years, the search for alternative therapeutic agents in the treatment of diabetes has been the focus of scientific research. Notably in 1980, the WHO Expert Committee on DM recommended further scientific investigations into the therapeutic efficacy of medicinal plants due to their perceived minimal side-effects. Many herbal medicines and medicinal plants with diverse actions have been used traditionally for the control, management and/or treatment of DM in many parts of the world.[3]

Cordia africana is a medium-sized evergreen tree, it is about 30 meters high, has branches with an umbrella-shape. Leaves are alternate, simple, ovate shape, about 7.5-17.5 cm long and 3.5-10.2 cm broad, it is leathery dark green. Its generic name was honoured to a German biologist in 16th century, *Valerius Cordus*, "*africana*" means from Africa, its synonyms '*abyssinica*' means the plant was described from Ethiopia.[11] *Cordia Africana* English common names are East African *cordia* or large-leafed cordia or Sudan teak.[12] *Cordia africana* which is an important indigenous timber tree of Ethiopia is a multi-purpose tree species. Like many deciduous trees, it exhibits repeated branching.[13] It is called "*alulluba"* in Hausa language. Fruits have a sweet, edible pulp. The plant provides bee forage, because the flowers yield a lot of nectar. Beehives are often found in the trees.[14] The fresh, juicy bark of *Cordia africana* is used to tie a broken bone, this material is replaced occasionally with a fresh one until the bone is healed.[14]

The aim of this study is to determine the effect of Methanol leaves extract of *Cordia africana* on blood glucose level, lipid peroxidation and oxidative stress biomarkers in alloxan-induced diabetic Wistar rats.

Materials and Methods

Collection Material

Fresh leaves of *Cordia africana* were collected from Basawa town, Sabon-Gari Local Government Area of Kaduna State, Nigeria in August, 2016. The plant was authenticated at the Herbarium Section in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria. A voucher specimen (No 620) was deposited at the herbarium for future reference.

Chemical/drug used

Alloxan monohydrate and glibenclamide were purchased from Sigma Chemicals (St Louis, U.S.A.). All chemicals used were of analytical grade.

Preparation of Extract

The *Cordia africana* leaves were air dried under shade for twenty one days and then size-reduced into powder with a pestle and mortar. About 100 g of the powdered leaves was macerated with 500 ml methanol for 72 hour with occasional shaking. The extract was concentrated *in vacuo* affording a yield of 13.6 % w/w and subsequently referred to as methanol leaves extract of *Cordia africana*. Solutions of the extract were prepared freshly for daily administration.

Preliminary phytochemical Screening

The screening was carried out in accordance with the standard protocol as described by Trease and Evans.[15]

Acute toxicity study

The median lethal dose (LD_{50}) of the plant extract was determined by the method of Lorke[16] 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 hours for signs of toxicity. In the second phase, 4 groups containing one mouse each were injected with four more specific doses of the extract orally. The median

lethal dose (LD_{50}) was calculated using the second phase.

Experimental animals

Thirty (30) Wistar rats of both sexes weighing between 150-200 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,* and housed in polypropylene cages at room temperature throughout the study. These studies were carried out in Ahmadu Bello University in accordance with the rules governing the use of laboratory animals as accepted internationally.

Induction of Diabetes Mellitus

The Wistar rats were fasted for about 16-18 hours, after which diabetes was induced by a single intraperitoneal injection of Alloxan monohydrate dissolved in 0.9% cold normal saline solution at a dose of 150mg/kg body weight.[17] Alloxan produces fatal hypoglycaemia and to prevent this, the rats were treated with 20% glucose solution orally for 6 hours. After which they were placed on 5% glucose solution for 24 hours.[18] Blood was collected from the tail vein of the rats after 72 hours of Alloxan injection. The rats having fasting blood glucose level greater than 200mg/dl were selected for the study.

Determination of Blood Glucose Levels

Fasting blood glucose levels were determined by using the glucose oxidase method [19] with ONE TOUCH BASIC® Glucometer (LIFESCAN, Inc 2001 Milpitas, CA 95035, USA). Rats with blood glucose levels 200 mg/dl were considered for the study.

Experimental Design In the experiment, a total of thirty Wistar rats were used. The animals were randomly divided into six groups of five rats each as follows:

Group 1: Normoglycaemic control and administered (0.5 ml/kg body weight) distilled water

Group 2: Diabetic control and administered (0.5 ml/kg body weight) distilled water

Group 3: Diabetic administered 2 mg/kg body weight glibenclamide for 4 weeks orally

Group 4: Diabetic administered100 mg/kg body weight methanol leaves extract *Cordia africana* for a period of 4 weeks orally.

Group 5: Diabetic administered200 mg/kg body weight methanol leaves extract *Cordia africana* for a period of 4 weeks orally.

Group 6: Diabetic administered 400 mg/kg body weight methanol leaves extract *Cordia africana* for a period of 4 weeks orally

Blood Sample Collection and Serum Preparation

After the treatment, all animals were euthanized with chloroform and 5 mL of blood sample were collected into specimen bottles and allowed to clot and separated by centrifugation at 3000 *g* for 10 minutes using a centrifuge (Hitachi Universal 32, Germany). The supernatant obtained were used for the determination of lipid profile and liver enzymes.

Determination of Oxidative Stress Biomarkers

Glutathione Peroxidase

The NWLSS™ Glutathione peroxidase Assay kit was used which is an adaptation of the method of Paglia and Valentine.[20] Glutathione peroxidase catalyses the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG was then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$ (resulting in decrease absorbance at 340 nm) and recycling the GSH. Since GPx is limiting, the decrease in absorbance at 340nm is directly proportional to the GPx activity. The absorbance was read at 1, 2 and 3 minutes against reagent blank. The absorbance for blank was subtracted from the sample reading to give the corrected value. Thus, GPx activity was calculated using 8.412 as the extinction coefficient:

GPx(U/L) = 8.412 x Δ A 340/min

U/L = unit activity per liter

Δ A 340/min = change in absorbance at 340 per minute.

Superoxide dismutase activity Superoxide dismutase (SOD) activities were measured by the method of Misra and Fridovich.[21] Plasma (0.5 mL) was diluted to 1.0 mL with distilled water, and 250 µl of chilled ethanol and 150µl of chilled chloroform were added. The mixture was shaken and centrifuged.

The supernatant was used for the assay of enzyme activity. To 1.2 mL of the supernatant, 1.5 mL of 0.1

mol/L carbonate-bicarbonate buffer, pH 10.2, containing 0.2 mmol/L EDTA were added. The contents were mixed, and the reaction initiated by adding 200 µl of epinephrine (pH 3.0, 3 mmol/L) to the buffered reaction mixture. The changes in optical density per minute were measured at 470 nm.

Catalase activity

Catalase (CAT) activities were assayed by the method of Sinha.[22] About 0.1 mL of Plasma and 1.5 mL of phosphate buffer was added. To this, 0.4 mL of hydrogen peroxide was added and the reactions were arrested after 30 and 60 second by the addition of 2.0 mL dichromate acetic acid reagent. A control was also carried out simultaneously. All the tubes were heated in a boiling water bath for exactly 10 min, cooled and absorbance read at 620 nm. Standards in the range of 2-10 Mmoles were taken and processed as the test. The activities of catalase were expressed as µmoles of hydrogen peroxide consumed/min/mg of protein (unit per milligram of protein).

EstimationofMalondialdehyde (MDA)

Lipid peroxidation can be evaluated by the thiobarbituric acid reactive substances method.[23] Serummalondialdehyde (MDA) levels were measured by the double heating method of Draper and Hadley[24] using Malondialdehyde Assay kits from Northwest Life Sciences Specialities (NWLSS™, product NWK-MDA01 USA). Butylated hydroxytoluene (BHT) in methanol reagent was used as the control. The method is based on the spectrophotometric measurement of the purple colour generated by the reaction of thiobarbituric acid (TBA) with MDA at 532 nm. The MDA formed will therefore be quantified using an extinction coefficient of 1.56×10^5/mole/cm.[25] The amount of MDA formed in the control samples is subtracted from the amount in the experimental samples to obtain the amount of MDA in each sample. Since absorbance is directly proportional to the concentration, thus; concentration of MDA in each sample = Absorbance in sample – Absorbance in control x 10^5nmol/ml ÷ 1.56×10^5 $M^{-1}CM^1$

Statistical Analysis

Data obtained from each group were expressed as mean ± SEM. The data were statistically analysed using (ANOVA). All statistical analyses were evaluated using SPSS version 17.0 software and Microsoft Excel (2007). The values of $p \leq 0.05$ were considered significant.

Results Preliminary phytochemical screening

The preliminary phytochemical screening of the extract revealed the presences of tanin, saponin flavonoid, alkaloid cardiac glycoside, resin and cardiac glycoside.

Acute toxicity study (LD_{50})

The sign of toxicity were first noticed after 8-10 hours of extract administration. There was decreased locomotor activity, decreased feed intake, and prostration after 8 hours of extract administration. The median lethal dose (LD_{50}) in rats was calculated to be greater than 5000 mg/kg body weight orally.

Effect on Blood glucose level

Administrations of various doses of the methanol extract of *corda africana* extract (100,200 and 400 mg/kg) significantly decrease the blood glucose levels at week 1 of administration when compared with the diabetic control untreated. However there was a significant decrease in the blood glucose levels in the groups administered the standard drug 2mg/kg glibenclamide, 200 and 400 mg/kg *Corda africana* as compared to control diabetic untreated at week 1 and 2 respectively. Consequently, there was a significant decrease in the blood glucose level in all the treatments given at week 4 as compared to control diabetic untreated. As regards to the normoglycaemic group there was a significant increase in the blood glucose levels when compared with the diabetic treated groups as showed in table 1.

Effect on Serum Malondialdehyde Concentration

The result of serum MDA concentration was shown in Table 2. The MDA concentration, an index of lipid peroxidation was lower ($P < 0.05$) in the control (1.33± ± 0.05), compared to the various extract treated groups 100, 200 and 400 mg/kg (3.52 ± 1.02; 3.82 ± 0.10 3; 85 ± 0.08) respectively. However there was a significant increase with the group administered the standard drug glibenclimide as compared to control. Also there was an increase in the normoglyceamic control (4.20 ± 1.02) as compared to diabetic untreated control.

Effect on Serum Superoxide Dismutase Activity (SOD)
The superoxide dismutase activity was significantly ($P < 0.05$) higher in the untreated diabetic control (4.88 ± 1.62). However, administration of various doses of the extract (100, 200 and 400 mg/kg) respectively, significantly decreased the SOD level as compared to control as shown in table 2.

Effect on Serum Catalase Activity (CAT)
Table 2 shows a significant ($P < 0.05$) increase in the activity of serum CAT (65.10 ± 2.02) in untreated control. However, there was a significant ($P < 0.05$) decrease in CAT activity in the group administered 100,200 and 400 mg/kg of the extract when compared to the diabetic control untreated

Effect on Serum Glutathione Peroxidase Activity(GPx) Table 2 shows a significant ($P < 0.05$) increase in the GPx activity in the diabetic control untreated. Furthermore, there was a significant ($P < 0.05$) decrease in GPx activity in the group administered 100,200 and 400 mg/kg of the extract as compared to the diabetic control untreated

Table1: Effect of methanol leaves extract of *Cordia africana* on blood glucose levels in alloxan-induced diabetic Wistar rats

Group/Treatment	Week 0 (mg/dl)	Week1 (mg/dl)	Week2 (mg/dl)	Week3 (mg/dl)	Week4 (mg/dl)
Group I (Normoglycaemic)	72.0 ± 1.21	73.80 ± 1.80	79.40 ± 2.63	71.0 ± 2.21	70.2 ± 2.21
Group II (Diabetic untreated)	312.0 ±2.28	308.20 ± 3.22	320.40 ± 2.35	307.40 ± 1.53	315.20 ± 3.32
Group III (2mg/kg Glibenclamide)	310.60 ± 3.21	259.00 ± 3.27 [ns]	198.60 ± 2.54[a]	90.80 ± 1.94 [a]	98.40 ± 0.20 [a]
Group IV (100 mg.kg *Cordiaafricana*)	306.20 ± 1.66	286.30 ± 2.02[ns]	229.60 ± 2.13[ns]	222.00 ± 1.87[ns]	120.40 ± 1.03 [a]
Group V (200 mg/kg *Cordiaafricana*l)	301.80 ± 3.21	256.0 ± 2.07 [ns]	127.40 ± 3.20 [a]	131.2 ± 2.26 [a]	110.20 ± 2.12 [a]
Group VI (400mg/kg *Cordiaafricana*)	310.2 ± 3.11	206.20 ± 2.02[a]	197.00 ± 1.07[a]	129.40 ± 3.14 [a]	102.00 ± 1.81[a]

Values are expressed as mean ± SEM; n = 5. [a] Value considered statistically significant when compared to diabetic untreated control group ($p \leq 0.05$); [ns] Value considered statistically significant when compared with diabetic untreated control group.

Table 2: Effect of methanol leaves extract of *Cordia africana* on lipid peroxidation and oxidative stress biomarkers in alloxan-induced diabetic Wistar rats

Group/Treatment	MDA (nmol/L)	SOD (IU/L)	CAT (IU/L)	GPx (IU/L)
Group I (Normal)	4.20 ± 1.02	2.82 ± 0.83	48.60 ± 1.63	42.10 ± 1.09
Group II (Diabetic Control untreated	1.33± 0.05	4.88 ± 1.62	65.10 ± 2.02	72.10 ± 1.15
Group III (2mg/kg Glibenclamide)	3.68 ± 1.07 [a]	2.46 ± 1.34 [a]	38.00 ± 1.14 [a]	34.40 ± 0.81[a]
Group IV (100 mg/kg *Cordia Africana methanol extract*)	3.52 ± 1.02 [a]	2.61 ± 2.16 [a]	31.22 ± 0.22 [a]	32.00 ± 1.14[a]
Group V (200 mg/kg *Cordia Africana* methano extract)	3.82 ± 0.10 [a]	2.78 ± 2.02 [a]	32.60 ± 0.20 [a]	30.40 ± 1.02 [a]
Group VI (400 mg/kg *Cordia Africana methanol extract*)	3.85 ± 0.08[a]	2.81 ± 1.24 [a]	40.30 ± 0.04 [a]	38.30 ± 1.00[a]

Values are expressed as mean ± SEM; n = 5. [a] Value considered statistically significant when compared to diabetic untreated control group ($p \leq 0.05$); [ns] Value considered statistically significant when compared with diabetic untreated control group.

Discussion

Alloxan- induced diabetes is characterized by hyperglycaemia, severe body weight loss, polydipsia, polyphagia and polyuria. Management of diabetes with agents devoid of any side effects is still a challenge to the medical system. This has led to an increase in the demand for natural products with antihyperglycemic activity and fewer side effects. Plants may act on blood glucose through different mechanisms, some of them may have insulin-like substances and some may inhibit insulinase activity [26].The result on blood glucose levels showed a significant decrease in the group administered 400 mg/kg *Cordia africana* extract at one week of treatment, while there was no significant change in the groups administered of 100 and 200 mg/kg as compared with the diabetic control.

However, at 2, 3 and 4 weeks of administrations there was significant decreased in the groups administered 200 and 400 mg/kg extract as compared with diabetic control, while there was no significant change in the group administered 100 mg/kg. extract at 3 week of treatment as compared to control. As regards to the reference drug glibenclamide 2mg/kg, there was a significant decrease as compared with diabetic control. Also in relation to the normoglycaemic control, when compared with the diabetic treated groups there was a significant decrease in the normoglycaemic and increases in the treated diabetic groups.

The activity of the *Cordia africana* extract might be due to its secondary metabolites' present as revealed in the preliminary phytochemical screening such as flavonoids, tannin alkaloid resin and cardiac glycosides. Some plants are involved in the stimulation of cells to produce more insulin [27; 28] and others may increase cells in the pancreas by activating regeneration of pancreatic cells [29]. Hyperglycemia is a main cause for elevated free radical levels, followed by production of ROS, which can lead to increased lipid peroxidation and

altered antioxidant defense and further impair glucose metabolism in biological system[30] . An imbalance between oxidation and antioxidant status has been shown to play an important role in mediating oxidative stress[31]. Overwhelming free radicals generated due to oxidative stress may develop several adverse effects commonly seen in diabetes such as neuropathy, nephropathy, retinopathy, and vascular disorders[32]. The major antioxidant enzymes, including SOD, CAT, and GPx, are regarded as the first line of the antioxidant defense system against ROS generated *in vivo* during oxidative stress and act cooperatively at different sites in the metabolic pathway of free radicals[33]. From the study in relation to the oxidative stress biomarkers (SOD, CAT, GPx), there was significant decrease in the superoxide dismutase, catalase and glutathione peroxidase levels as compared with the diabetic control untreated with the various doses administered.

Superoxide dismutase serves as the first line of defence against the deleterious effect of Reactive oxygen species. Therefore, the increased activity of GPx might be a protective mechanism in response to increased concentrations of H_2O_2 and other lipid peroxides. GPx is an antioxidant enzyme involved in the detoxification of hydrogen and lipid peroxides and also acts as a peroxynitrite reductase[34]. A significant decrease in GPx activity might be accompanied by a significant increase in lipid peroxidation. The function of intracellular Superoxide dismutase is to scavenge superoxide (O_2^-) produced by cellular metabolism, which catalyse dismutation of superoxide to oxygen (O_2) and hydrogen peroxide [35; 36]. However, treatment with the methanol extract of *Cordia africana* at doses of 100, 200 and 400 mg/kg respectively, significant decreases all the oxidative stress biomarker (Superoxide dismutase, catalase and glutathione peroxidase) as compared with the diabetic control.

Consequently, there was a significant increase in the malondialdehyde (MDA) concentration in the groups administered the various doses of the extract as compared to diabetic untreated control. In conclusion the methanol extract *Cordia africana* decreases blood glucose levels and ameliorated oxidative stress biomarkers levels in the alloxan-induced diabetic Wistar rats.

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Eucalyptus camaldulensis L. Herit (Myrtaceae) Leaf Extract Possess Antiseizure Potentials: Preliminary Studies

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Abstract

Leaves of *Eucalyptus camaldulensis* (EC) has been used extensively as herbal remedy for epilepsy and other ailments. The aim of this study is to screen the methanol leaf extract for antiseizure actions. LD_{50} was performed using method of Lorke while qualitative phytochemical screening was carried out to identify the secondary metabolites. Maximal electroshock test (MEST) and pentylenetetrazole (PTZ)-induced seizure were used as primary screening models while 4-Aminopyridine and strychnine for mechanistic studies. LD_{50} was found to be 565.85 mg/kg, alkaloids, glycosides, saponins, tiriterpenes and tannins were found to be present. The extract at all the doses tested did not significantly ($P \leq 0.05$) delay the time of recovery of the convulsed chicks nor delayed the onset of seizures in both the MEST and PTZ models as against the standard drugs phenytoin (20 mg/kg) and sodium valproate (200 mg/kg) which protected all the animals against seizures induced by MEST and PTZ respectively. However, the extract at the doses of 75 mg/kg and 37.5 mg/kg delayed the mean onset of seizures in both the strychnine (STN) and 4-aminopyridine (4-AP) significantly ($P \leq 0.05$) as against normal saline treated group. This study therefore validate the use of EC leaves in traditional medicine and justifies its usage for the treatment of epilepsy.

Keywords: *Epilepsy, Seizure, Eucalyptus camaldulensis, Antiseizure*

Introduction

Epilepsy is a neurological disorder that stands unique among myriads of brain disorders. This is not unconnected with the myth, superstition and discrimination that are associated with it. It affects about 70 million people around the world with majority having limited access to treatment.[1] It is characterized by intermittent, synchronous and often unpredictable electrical discharges in the brain, more commonly termed as "seizures".[2] Epilepsy is the second most common neurological disorder; consequently, it should be given considerable attention.[3] From the outset, various forms of complimentary treatment of epilepsy has been documented.

Herbal medicine has been an integral component of this alternative practice.[4] From the evolution of bromide as a synthetic anticonvulsant to the discovery of levetiracetam in the beginning and end of the 20th century, this broad armamentarium of drugs in epilepsy have reasonably reduced the burden of morbidity and mortality of this disorder. Nonetheless, the current library of drugs in clinical circulation still present with untoward effects, that limits maximal utilization of their benefits.[5] Herbs and medicinal plants still offer huge potentials in the discovery of lead molecules to treat variety of diseases and disorders afflicting mankind.[6] Similarly, since 8 out of 10 people in the developing countries still utilize medicinal plants for their health care needs.[7] It is worthwhile to validate the claims of medicinal plants with potentials for use in epilepsy for their possible journey into lead compounds and drugs for treatment of this brain disorder. *Eucalyptus camaldulensis* (EC) is a tree that grows up to 45 m tall with leaves that are stalked, it belongs to the family: Myrtaceae. The adult leaf is dull green in colour containing many oil glands.[8] The leaves have been used to treat various ailments such as: childhood seizures, joint pain, osteoarthritis, whooping cough, respiratory tract infections, asthma, and pulmonary tuberculosis, among others in ethnomedicine. [8] While the popularity of the leaf decoction in the treatment of epilepsy is well entrenched in the Traditional Medical Practice among the Hausa/Fulani communities of Nigeria, to the best of our knowledge, no scientific evaluation of this claim has

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been reported. The aim of this study was therefore to screen the leaf extract of this plant using scientific anticonvulsant protocols.

Materials and Methods Source of Plant Materials
Fresh leaf of *Eucalyptus camaldulensis* were collected in the wild bush of Giwa town, Kaduna state around October, 2015. It was identified and authenticated in the Herbarium unit of the Department of Biological Sciences of Ahmadu Bello University, Zaria by comparing it with existing voucher specimen number 2767.

Experimental Animals
Swiss Albino mice of either sex (18-22 g) were obtained from the Animal House of the Department of Pharmacology and Therapeutics, ABU Zaria, while day old cockerels were procured from National Animal Production Research Institute (NAPRI) Shika. The animals were placed in propylene cages in a well ventilated room, they were made to acclimatize to the environment before the commencement of the studies, standard feeds and water were provided *ad libitum*. All experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.[9]

Chemicals and Drugs
Aluminum chloride (BDH Ltd Poole England), Diazepam 10mg in 2ml (Roche®, Pakistan), Methanol (Sigma-Aldrich, St. Louis U.S.A), 4-Aminopyridine (Merck-Schuchadt, Germany), Pentylenetetrazole (Sigma Chemical Co. Ltd, USA), Phenobarbitone (Bayer, UK), Phenytoin sodium (Parke-Davis and Co. Ltd), Sodium valproate (Sanofi Synthelabo, Onslow St. Surrey, Canada).

Preparation of Plant Extract
Maceration method of extraction was adopted. The leaf of *Eucalyptus camaldulensis* (EC) were washed and air dried under shade at room temperature until they were completely dried. The air dried plant material was then crushed into a coarse powder with the aid of a mortar and pestle. The resultant powdered plant was macerated with (70% methanol: 30% water) for 72 hours using cold maceration technique. The extract was filtered using Whatman filter paper No.25. The solvent was evaporated from the resulting filtrate on a water bath set at a low temperature (40^{o}C). The solutions of the extract were always freshly prepared for each study by dissolution of the appropriate amount required in normal saline.

Preliminary Phytochemical Screening
Phytochemical screening was carried out on each of the dried extracts using simple chemical tests to detect the presence of secondary metabolites according to standard protocol.[10]

Pharmacological Studies
LD_{50} determination
The method described by Lorke[11] was adopted. The study was divided into two phases. In the first phase, three groups of three mice were treated intraperitoneally (*i.p*) with the extract at doses of 10, 100 and 1000 mg/kg body weight. The animals were subsequently observed for signs of toxicity and death for a period of 24 hours after treatment. In the second phase, four groups each comprising one mouse was treated intraperitoneally with the extract of the plant using four specific doses (200, 400, 800, and 1600 mg/kg) of the extract which depend on the outcome of the first phase. The LD_{50} value was determined as the square root of the product of the lowest dose that caused death and the highest non-lethal dose i.e the geometric mean of consecutive doses for which 0 and 100% survival were recorded.

Anticonvulsant studies
Maximum electroshock-induced seizure test (MEST) in chicks
The method described by Swinyard and Kupferberg [12] was adopted. Fifty (50) one-day old ranger cockerels were randomly divided into five groups each containing ten chicks. The first group received normal saline (10ml/kg) via *i.p*. The second, third and fourth groups were administered (*i.p*) of (E.C =150, 75 and 37.5 mg/kg body weight) of the extract and the fifth group was treated with 20 mg/kg phenytoin sodium intraperitoneally.

Thirty minutes later, maximal electroshock was administered to induce seizure in the chicks using Ugo Basile Electroconvulsive Machine (Model7801) connected to Claude Lyons stabilizer with corneal electrodes placed on the upper eyelids of the chicks. The shock duration, frequency, current and pulse width were set and maintained at 1.6s, 150 pulse/sec, 80 mA and 0.8ms, respectively. Seizures were manifested as hind limb tonic extension (HLTE). The ability to prevent this feature or prolong the

latency and /or onset of the HLTE or reduce the recovery time was considered as an indication of anticonvulsant activity. [13]

Pentylenetetrazole induced seizure in mice Mice were divided into five groups of six mice each. Group 1 received normal saline (10 ml/kg), groups 2-4 received the extract (*i.p.*) at doses of 150, 75 and 37.5mg/kg while group 5 received standard drug sodium valproate at a dose of 200mg/kg intraperitoneally. Thirty minutes post administration; all groups received 100mg/kg pentylenetetrazole (PTZ) subcutaneously (*s.c.*). Animals were observed for the presence or absence of threshold seizures (an episode of clonic spasm of at least 5 second duration) and latency of convulsion. [13]

Strychnine-induced seizures in mice
Mice were divided into five groups of six mice each. Group 1 received normal saline (10 ml/kg), groups 2-4 received extract (*i.p.*) at doses of 150, 75 and 37.5 mg/kg while group 5 received phenobarbitone sodium at a dose of 30 mg/kg body weight intraperitoneally. Thirty minutes later, mice were administered 1 mg/kg body weight of strychnine subcutaneously and observed for incidence of convulsions. Prevention of tonic hind limb extensor jerk was considered as protection against seizures induced by strychnine. [14]

4-Aminopyridine-induced seizures in mice
The protocol used was described by Salih and Mustafa [15]. Mice were divided into five groups of six mice each. Group 1 received normal saline (10 ml/kg), groups 2-4 received (*i.p.*) of 150, 75 and 37.5mg/kg while group 5 was treated with phenobarbitone sodium (30 mg/kg). Thirty minutes post treatment; 4-Aminopyridine was administered at a dose of 15 mg/kg body weight to each mouse, subcutaneously (*s.c.*). Absence of tonic hind limb extension or prolongation of the latency of the hind limb tonic extension was considered as indication for anticonvulsant activity.[15]

Statistical Analysis
Data obtained were expressed as Mean ± Standard Error of Mean (Mean ± SEM) for time of onset of convulsion and recovery, and analysed using SPSS version 23. Data were analysed using one way analysis of variance (ANOVA), followed by Dunnett's post hoc test. $p \leq 0.05$ was considered statistically significant.

Results

LD_{50}
The LD_{50} value of the extract of EC was estimated to be 565.68 mg/kg.

Phytochemical constituents
Alkaloids, glycosides, saponins, tiriterpenes and tannins were found to be present (Table 1) in the leaf of *E. camaldulensis.*

Effect of Methanol Leaf Extract of EC on MEST-Induced Seizures in Chicks
The extract at all the doses tested did not reduce the recovery time of the convulsed chicks when compared with standard drug phenytoin 20 mg/kg that completely protected the chicks (Figure 1).

Effect of Methanol Leaf Extract of EC on PTZ-Induced Seizures in Mice
The extract of EC at all the doses had no effect on the mean onset of seizures when compared with standard drug sodium valproate 200 mg/kg that protected all the mice against seizures (100%) (Figure 2).

Effect of Methanol Leaf Extract of EC on STN-Induced Seizures in Mice
The extract of EC at a dose of 75 mg/kg prolonged the mean onset of seizures significantly (p 0.05) like the standard drug Phenobarbitone 30 mg/kg (Figure 3).

Effect of Methanol Leaf Extract of EC on 4-AP-Induced Seizures in Mice The extract of EC at a dose of 37.5 mg/kg significantly (P 0.05) prolonged the mean onset of seizures in the treated mice in the same manner as does the standard drug Phenobarbitone 30 mg/kg (Figure 4).

Table 1: Phytochemical constituents of *Eucalyptus camaldulensis* leaf

Constituents	Inference
Glycosides	+
Saponins	+
Steroids	-
Triterpenes	+
Tannins	+
Flavonoids	+
Alkaloids	+
Anthraquinone	-

Key; + Present and – Absent

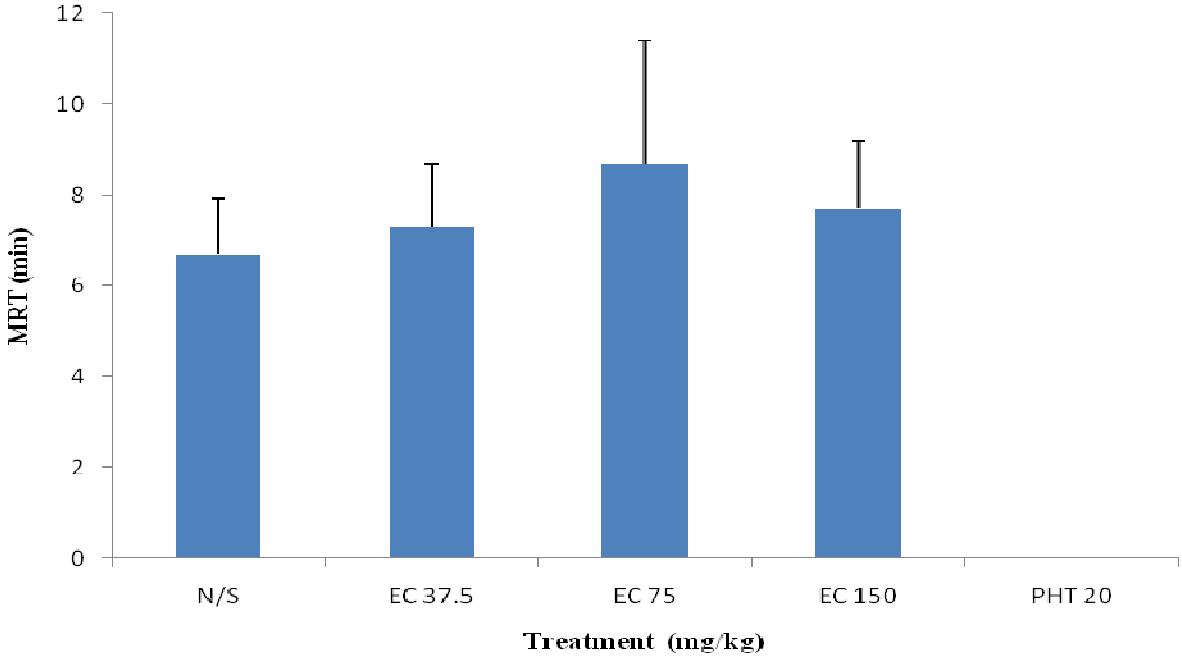

Figure 1: Effect of *Eucalyptus camaldulensis* Leaf Extract on MEST induced seizures in chicks. MRT= Mean Recovery Time, N/S=Normal saline, PHT= Phenytoin, n=10, Data expressed as mean ± SEM

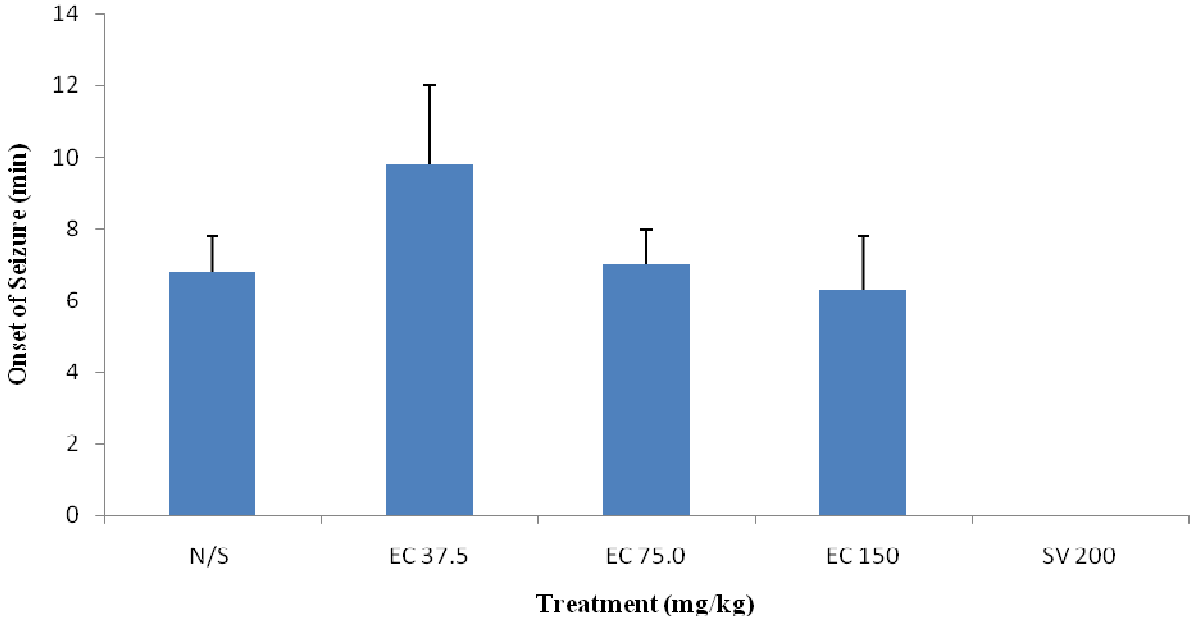

Figure 2: Effect of Leaf Extract of *Eucalyptus camaldulensis* on pentylenetetrazole-induced seizures in mice. n=6, Data expressed as mean ± SEM, SV= Sodium valproate

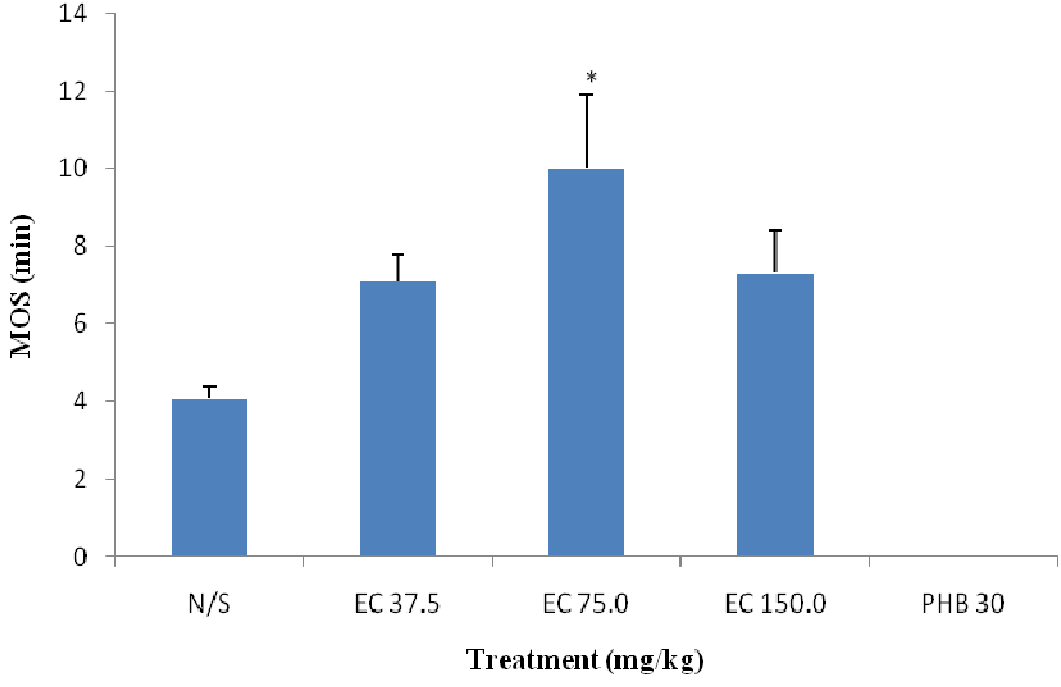

Figure 3: Effect of leaf extract of *Eucalyptus camaldulensis* on strychnine-induced seizures in mice. Data expressed as mean ± SEM, MOS= Mean Onset Seizures, n=6, *=p 0.05, PHB= Phenobarbitone

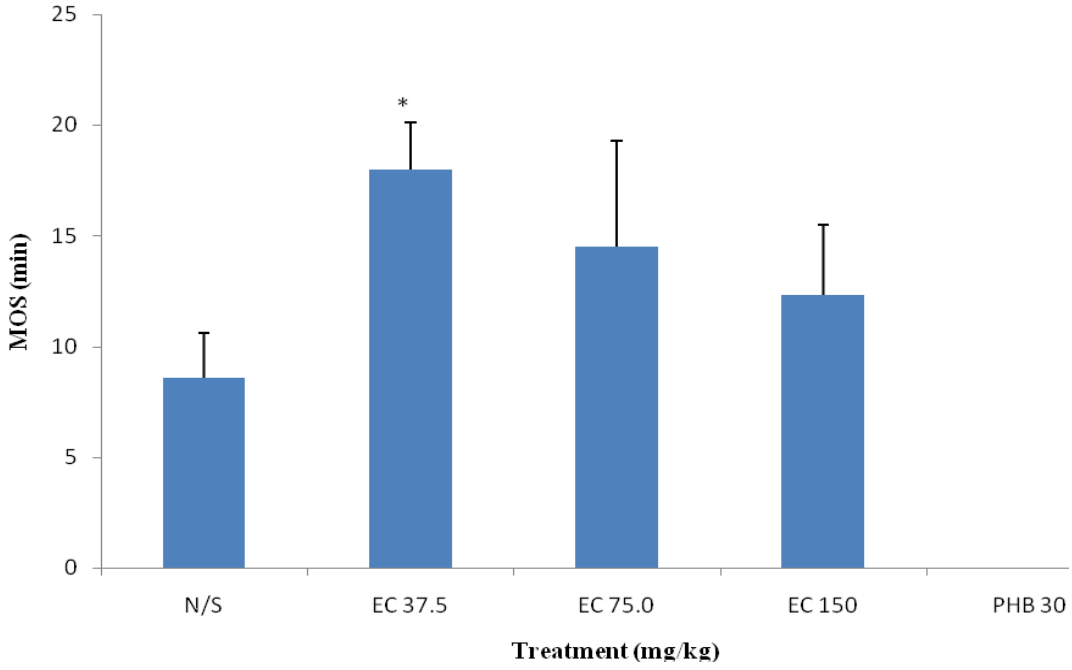

Figure 4: Effect of *Eucalyptus camaldulensis* leaf extract on 4-aminopyridine-induced seizures in mice. MOS= Mean Onset of Seizures, N/S= Normal saline, *p 0.05, n=6, Data expressed as mean ± SEM

Discussion

The phytochemical analysis of methanol extract of *Eucalyptus camaldulensis* (EC), revealed the presence of some chemical constituents which might be responsible for the observed pharmacological activities of the extracts. Steroids, triterpenes, saponins, flavonoids, and tannins were reported to be present in this plant.[17] The phytochemical constituents provide basic information about the different classes of the secondary metabolites present in the extract which are relevant for the treatment of various ailments.[6]

In the anticonvulsant screening carried out, MEST is one of the models used. This model is a non-mechanistic seizures model that has clearly defined end points such as inhibition of limb tonic extension (HLTE). In this model, the electrographic and behavioural seizures produced in the chicks are consistent with human disorder.[13] The methanol leaf extract of EC, did not show protection of the chicks against MEST induced seizures when compared to the standard drug phenytoin (offered 100%). The protection of seizures against HLTE in MEST-induced chicks will predict anticonvulsant activity of the extract.[12] Drugs such as carbamazepine and phenytoin suppress HTLE in MEST.[17]

Secondly, another chemoconvulsant used to identify anticonvulsant activities of this extract is the pentylenetetrazole (PTZ). PTZ has been shown to diminish the Gamma Aminobutyric Acid receptor tone by the inhibition of benzodiazepine (BDZ) site of the GABA receptors. [18] Gamma amino butyric acid (GABA) is a major inhibitory neurotransmitter in the brain, and the inhibition of its neurotransmission is correlated to the epileptogenesis. [19] The methanol leaf extract of EC at all doses tested did not protect the Swiss albino mice against PTZ-induced seizures when compared with standard drug (sodium valproate 20mg/kg) which offered 100% protection against PTZ-induced seizures. At all the doses for EC none of the dose offered a significant protection. The pharmacological profile of PTZ induced seizures are similar to the symptoms observed in absence seizure and drugs such as valproate, diazepam, and ethosuximide are useful in the treatment of absence seizure.[20] Leaf extract of EC did not show any observable protection against PTZ-Induced seizures.

Even though, The MEST and PTZ induced seizures are the primary screening models for hypothetical antiseizure compounds that are useful for generalized tonic clonic seizures and absence seizures respectively.[6, 21] Absence of activity in these models does not mean lack of clinical usefulness in epileptic disorders, as levetiracetam, a useful anticonvulsant failed the basic MEST and PTZ test but is still useful in refractory epilepsy and as adjunct in both Generalized Tonic Clonic Seizures (GTCS) both primary and secondary; this is because it has activity against kindling mouse model.[6, 21]

Thirdly, the convulsant strychnine (STN) was also used to screen the anticonvulsant activities of the extract. Strychnine acts as selective competitive antagonist of glycine which acts by blocking inhibitory effects of glycine at all glycinergic receptors.[22] Glycine is an inhibitory neurotransmitter to motor-neurons and interneurons in the spinal cord. Subcutaneous administration of STN produces hind limb tonic extensor jerks in mice.[22] Previous studies have shown that drugs or compounds (extracts) that abolish STN induced seizures mediate their activity by enhancing inhibitory neurotransmission in the brain and in the spinal cord.[23] The methanol leaf extracts of EC 75mg/kg showed significant delay in the mean onset of seizures and this may possibly suggest their effectiveness to manage convulsive episodes. Finally, 4-aminopyridine (4-AP) is a K^+ channel antagonist and Ca^{2+} channel blocker, both voltage-gated,[24, 25] and is administered subcutaneously (SC) to induce seizure in mice that would lead to tonic hind limb extension. Any compound or drug, which show protection of seizure or delay the hind limb tonic extension was considered as indication for anticonvulsant activity and acting via these channels.[26]

Conclusion

The leaf extract of *Eucalyptus calmudulensis* exhibited anticonvulsant activity at doses tested against 4-AP and STN models that justify its usage in ethnomedicine in the treatment of epilepsy.

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Study of the Effects of Placental and Umbilical Cord Parasitisation on Foetal Nutrition in Kano State, Nigeria

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Abstract

The major causes of many adverse pregnancy outcomes especially in developing countries include poor nutritional status of the mother, hypertension, malaria and other infections during pregnancy. This study was designed to evaluate the effects of placental and umbilical cord parasitisation on some umbilical cord nutrients**.** A total of 500 participants were recruited for the study. Maternal socio-demographics variables were obtained using questionnaire. Placental and umbilical cord malarial parasitisation status were determined by pathologist using Giemsa stain. Umbilical cord blood levels of iron, zinc, magnesium and calcium were determined using atomic absorption spectrophotometer. The results showed that 189 (37.8%) and 33 (6.6%) of the newborns had placental and umbilical malarial infection respectively. The mean umbilical cord blood levels of iron, zinc, magnesium and calcium in mothers with placental parasitisation, umbilical cord parasitisation and those without parasitisation were 4.92 ± 1.35, 4.8 6 ± 1.24, 4.59 ± 1.17, 0.08 ± 0.04, 0.07 ± 0.04, 0.06 ± 0.03, 0.07 ± 0.04, 0.06 ± 0.03, 0.03 ± 0.02 and 0.16 ± 0.42, 0.14 ± 0.40, 0.10 ± 0.34 respectively. Of the 189 mothers with placental parasitisation, the mean umbilical cord blood levels of iron, zinc, magnesium and calcium according to class of histological evaluation of placenta was, Class 1 4.82 ± 1.41, 0.07 ± 0.07, 0.06 ± 0.05, 0.16 ± 0.42, Class 4 4.61 ± 1.19, 0.06 ± 0.08, 0.05 ± 0.04, 0.13 ± 0.04 and Class 5 (control) 4.93 ± 1.37, 0.08 ± 0.06, 0.08 ± 0.03, 0.20 ± 0.45. However, the p- values were not statistically significant, thus the placental and umbilical cord parasitisation showed no effect on umbilical cord blood levels of iron, zinc, magnesium and calcium.

Keywords: Umbilical cord, Foetal nutrition, Placenta, Parasitisation

Introduction

Congenital malaria occurs when malaria parasites cross the placenta either during pregnancy or at the time of delivery.[1] It is usually defined as the presence of asexual forms of malaria parasites in the peripheral blood within the first seven days of life,[2] or later if there is no possibility of postpartum infection by a mosquito bite as would be the case in non-malaria endemic areas.[3] This could occur with or without accompanying clinical manifestations. However, congenital malaria is frequently reported as the sole presence of parasites in cord blood.[3]

The major causes of many adverse pregnancy outcomes especially in developing countries include the poor nutritional status of the mother, hypertension, malaria and other infections during pregnancy.[4] There is considerable evidence supporting the role of various micronutrients in determining pregnancy outcomes such as low birth weight (LBW) and prematurity. While some nutrients have been studied extensively much less is known about others.[4] Nigeria is a malaria endemic country, and pregnant women are especially vulnerable to malaria,[5,6,7,8,9] which can adversely affect the outcome of pregnancy. Although foetal micronutrients malnutrition is more common than LBW in Nigeria,[10,11] few studies had specifically relate placental and congenital malaria infection to foetal micronutrients malnutrition among Hausa population of Kano state. foetal micronutrients malnutrition is common in Nigeria and other developing countries,[11] and it has been shown to affect body composition and impair brain development and behaviour in experimental animals.[12,13,14] It is therefore important to evaluate the factors responsible for foetal micronutrients malnutrition in Kano State so that preventive strategies can be mapped out to mitigate the documented associated adverse neurologic effects.[12,13,14] The aim of the study was to evaluate the

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effects of placental and umbilical cord parasitisation on some umbilical cord micronutrients Kano state**.**

Materials and Methods Study

Location and Duration

The study was conducted at Department of Obstetrics and Gynaecology, Murtala Muhammad Specialist Hospital Kano (MMSH). It was a prospective study design which lasted from September 2015 to October 2016. The study includes referrals to the MMSH from various hospitals in the State. The Obstetrics and Gynaecology department has average deliveries of 35 to 60 per day as obtained from the record units of the department.

Study Population

Data were collected from 500 mothers (mean age 24.84 ± 5.34) and their newborns (males = 232 and females = 268), delivered at Murtala Muhammed Specialist Hospital Kano, using simple random sampling technique. Pregnant women with singleton gestation and all newborns delivered within the study period were included for the study. Pregnant women with diabetes mellitus, HIV, Hepatitis B and C, sexually transmitted infections, chronic diseases, newborns with physical abnormalities and Rhesus negative mothers were excluded for the study.

Materials

1. Paraffin block, Hematoxylin and Eosin, Paxgene, Giemsa stain (VWR International, Pennsylvania, USA and BDH chemicals Ltd, Pool, England),
2. Ethylene diamine tetraacetic acid (EDTA) and non-EDTA bottles,
3. Digital USB microscope camera (Amscope, California, USA),
4. Questionnaire and other relevant materials were used for the study.

Methodology

The diagnosis of malarial parasitisation was based on the presence or absence of parasites in the cord blood and placental tissue and of malaria pigment in monocytes or in fibrin deposits on the maternal side of the placenta [15]. With the aid of questionnaire maternal age, parity, ethnicity and level of education were recorded.

Histological Techniques

The tissue preparation method for the histological analysis that was employed involved six stages namely; fixation, tissue processing, sectioning, staining, and photomicrography.[16, 17, 18]

Cord Blood and Placental Parasitological Analyses

Cord clamps were used to prevent mixing of cord and maternal blood. Two millilitres of cord blood were collected using 21 gauge hypodermic needle and syringe and the blood was placed in a 5ml EDTA specimen collection tube then stained using Giemsa stain for a demonstration of the malarial parasite. [1] Four tissue samples were taken per placenta (maternal side), which were fixed separately in pax gene, then stained using Giemsa stain for a demonstration of malarial parasites at Histopathology laboratory Ahmadu Bello University Teaching Hospital. Digital USB microscope camera (Amscope, California, USA) was used to capture images under the microscope for analysis.

Placental Examination

A detailed protocol for the examination of placental samples was prepared according to the method described by Kassam *et al*., [19] Placental malarial infection was classified according to Rogerson *et al.* [15] Class 1: Malarial parasites presence but no pigment in monocytes or in intervillous fibrin deposits. Class 2: Malarial parasites and pigments presence in monocytes, with or without pigment in fibrin. Class 3: Malarial parasites presence in monocytes and pigments in fibrin. Class 4: Malarial pigment presence only in monocytes and fibrin, without parasites and Class 5: No parasites or pigments presence in monocytes and fibrin.

Umbilical Cord Nutrient Analysis

Another 2ml of the cord blood was collected and used for umbilical cord micronutrient analysis. The cord blood was centrifuged for 15 minutes at 3500 rpm and the serum was separated and kept in trace elements free tubes and stored at -20^{0}C. The cord blood serum levels of iron, zinc, calcium and magnesium were determined using atomic absorption spectrophotometer (AAS) after wet digestion of the samples with nitric-perchloric acid mixture in the ratio 650:80 [20] at multi- user laboratory, Department of Chemistry, ABU Zaria.

Ethical Consideration

The ethical clearance was obtained from Kano State Hospital Management Board Research Ethics

Committee with the reference number HMB/GEN/488/Vol1. The following protocols were observed; There was no intervention; drugs and invasive procedures in babies and mothers and diseases in newborns were reported to the Neonatologist. The procedure was not carried out without the consent of the participants or family concerned.

Statistical Analyses

The data were expressed as mean ± standard deviation. After normality test (Kolmigorov-smirnov test, $p > 0.05$) one way analysis of variance and a Turkey Post-hoc test were used to compare mean values of the umbilical cord nutrients based on sites of parasitisation and class of histological evaluation of placenta. Pearson correlation was used to investigate relationship between maternal age and umbilical cord nutrients. P-value less than <0.05 was considered significant. Data analyses were carried out using SPSS version 20.0 statistical software (IBM, New York, USA).

Results and Discussion

Plate I is a micrograph showing parasitised umbilical cord blood smear, section shows the presence of parasites in the cord blood. Plate II is a micrograph showing normal umbilical cord blood smear, section shows the normal cytoarchitecture of the cord blood with no parasite. Plate III is a micrograph of class 1, early placental malaria parasitisation. Parasites are seen in the maternal side of the placenta in the intervillous space in early infection. Plate IV is a micrograph of Class 4, past/old placental malaria parasitisation. Haemozoin (malaria pigment) are seen in intervillous fibrin deposits on the maternal side of the placenta in past infection. Plate V is a micrograph class 5, Normal placental tissue. Neither malarial parasites nor pigment is seen in the maternal side of the placenta at both the intervillous and perivillous spaces.

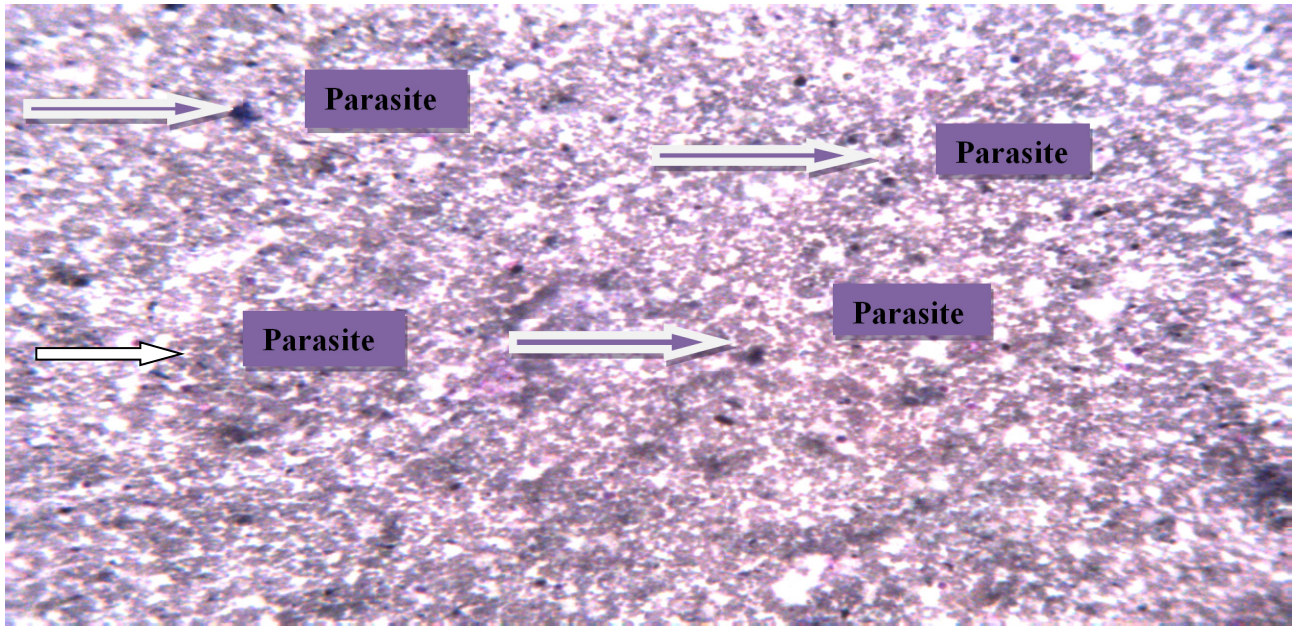

Plate I: Micrograph of parasitised umbilical cord blood smear, section shows the presence of parasites in the cord blood (Giemsa ×100).

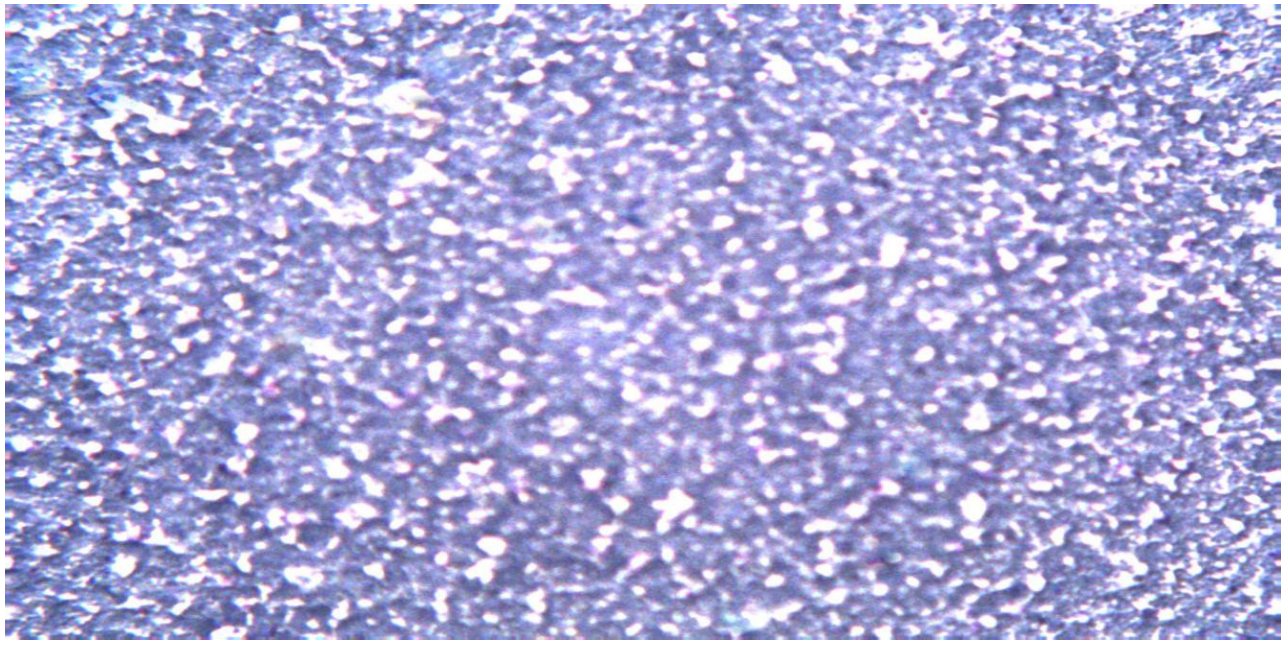

Plate II: Micrograph of normal umbilical cord blood smear, section shows the normal cytoarchitecture of the cord blood with no parasite (Giemsa ×100)**.**

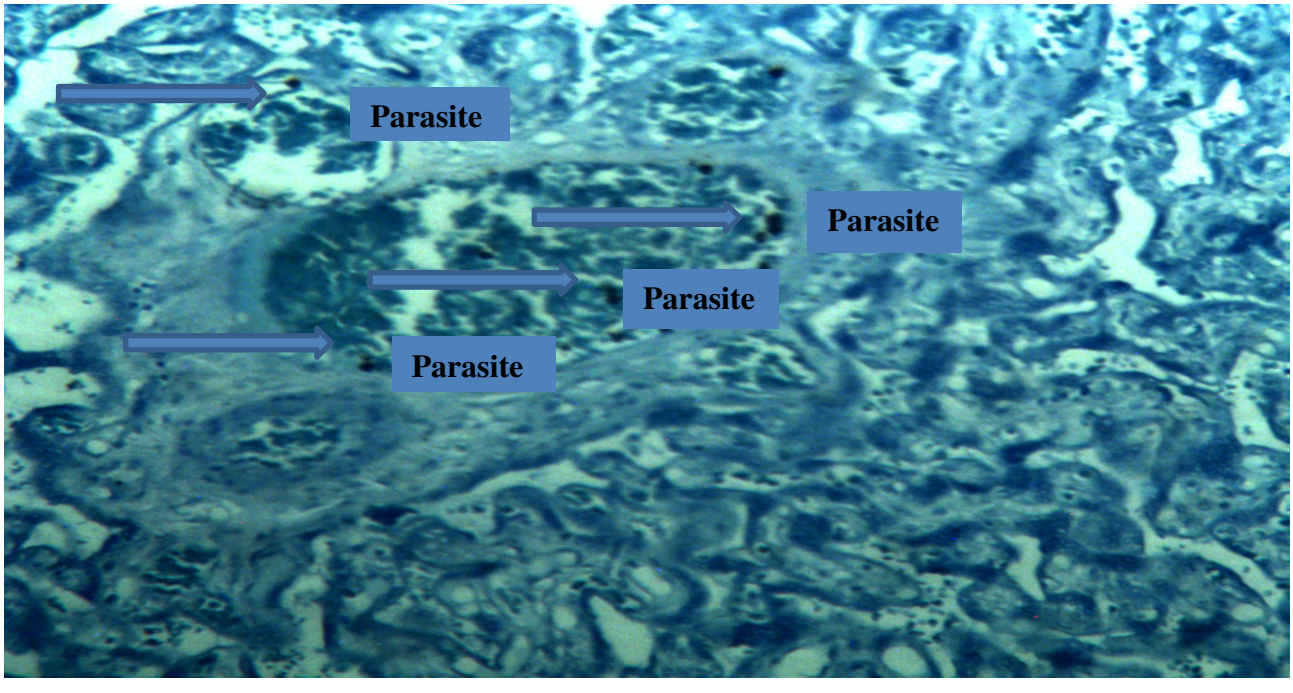

Plate III (Class 1): Micrograph of early placental malaria parasitisation shows malarial parasites but no pigments in monocytes or in intervillous fibrin deposits (Giemsa ×250).

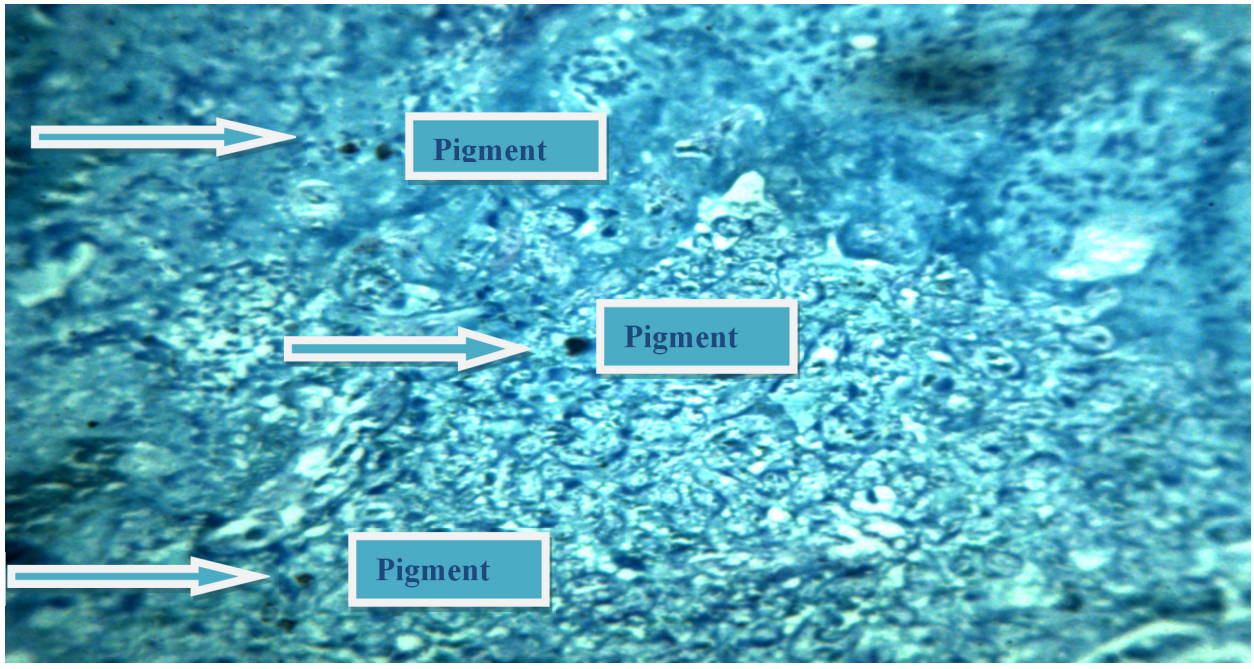

Plate IV (Class 4): Micrograph of past/old placental malaria parasitisation showing malarial pigment only, without parasites (Giemsa ×250).

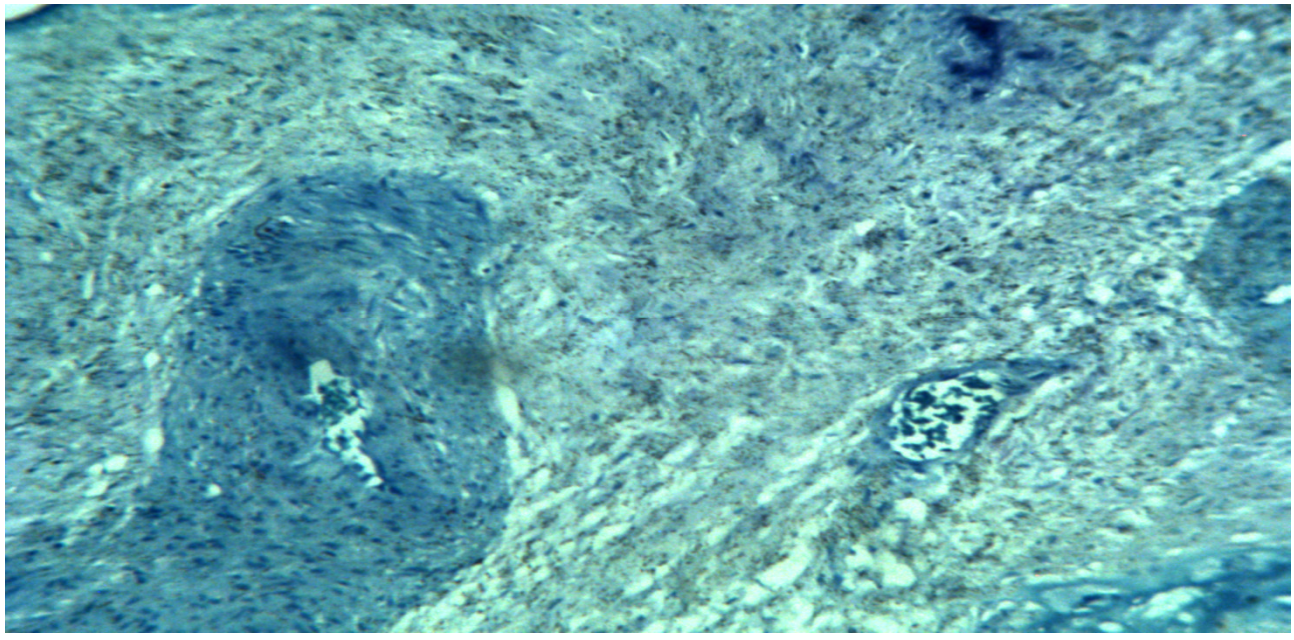

Plate V (Class 5): Micrograph of normal placenta shows the normal cytoarchitecture of the placenta with neither parasite nor pigment (Giemsa ×250).

From Table 1 it was observed that the average maternal age as well as the umbilical cord micronutrients level were relatively within the normal rage based on world health organization (WHO) standard.[21] This may be due to the fact that majority of the subjects (>70%) had no malarial parasitisation in their placenta and umbilical cord blood. Preliminary findings from the centre for disease control (CDC) United State of America pregnancy nutrition surveillance system (PNSS) suggest that malaria during pregnancy may be associated with low level of umbilical cord

micronutrients.[22] They found that women who had malarial infection were 40 times more likely to have lower cord micronutrients and at risk of delivering infant prematurely with intrauterine growth restriction (IUGR). [23] The respective mean differences of the umbilical cord micronutrients were not statistically significant in all the groups (Table 2). This finding is in contrary to the work of Kassam *et al*., and Griepink *et al*., [19,24] who reported that, malarial parasitisation had deleterious effect on foetal micronutrients nutrition. The placental parasitisation rate of 37.8% obtained in this study was within the ranges previously reported from other parts of Nigeria [6,25] and West Africa.[26] These include 40% in Ibadan,[7] 26.2% in Calabar, [27] 33.2% in Lagos, [7] 20.2–48% in Gambia.[26] A much higher rate of 56.7% previously recorded in Ile-Ife [28] The findings in this study are in support of the view that placental malaria parasitemia is a common observation among pregnant women in Nigeria and other developing countries.[2,6,29]

Table 1: Descriptive statistics of maternal and umbilical cord blood parameters (n=500).

Parameters	Mean±SD	Minimum	Maximum	Variance
Maternal Age (years)	24.82±5.34	15.00	40.00	0.52
Iron (mg/dl)	4.90±1.38	1.47	7.93	1.31
Zinc (mg/dl)	0.06±0.39	0.02	0.33	0.01
Magnesium (mg/dl)	0.07±0.44	0.01	0.18	0.01
Calcium (mg/dl)	0.15±0.41	0.51	1.70	0.11

SD; standard deviation.

Table 2: Biochemical characteristics of the newborns according to sites of parasitisation normal placenta and umbilical cord blood tissue

Parameters	Normal Mean±SD (n=278)	Placental Mean±SD (n=189)	Umbilical Mean±SD (n=33)	F- value	P- value
Iron (mg/dl)	4.92±1.35	4.86±1.24	4.59±1.17	0.11	0.90
Zinc (mg/dl)	0.08±0.04	0.07±0.04	0.06±0.03	1.15	0.35
Magnesium (mg/dl)	0.07±0.04	0.06±0.03	0.03±0.02	0.10	0.91
Calcium (mg/dl)	0.16±0.42	0.14±0.40	0.10±0.34	0.47	0.63

SD; standard deviation.

The prevalence based on the class of histological evaluation of the placenta was higher among class 1 (72.5%) than class 4 (27.5). However, the respective means of the umbilical cord micronutrients according to class of histological evaluation of the placenta were not statistically significant at P<0.05 (Table 3).

The findings of this study disagree with World Health Organisation report that, class 1 of the histological evaluation of the placenta is more common in pregnant women than other classes. [21] Globally, by WHO criteria, 52% of pregnant women from the developing countries were infected with class 1 compared with 20% from industrialized nations. Moreover, the finding of this study is consistent with other reports as in the highest prevalence was among pregnant women in India (88%), followed by Africa (50%), Latin America (40%), and the Caribbean (30%) [21]. Similar cases were also reported in Koro and Bandiagara of Mali.[30]

The result showed a positive significant correlation between maternal age, iron and magnesium as indicated by the double asterix (**) in Table 4. It also reveals a negative significant correlation between iron and calcium and a positive relationship between zinc magnesium and calcium. The relationship between umbilical cord micronutrients and maternal age may imply that maternal age and malaria are major factor in determination of foetal nutrition in Kano state, Nigeria. It seems that they are responsible for normal and abnormal growth in the uterus. This finding was supported by a report which posits that maternal age, nutritional factors and malarial infection both prior to pregnancy and during pregnancy accounted for more than 50% of

the levels of intrauterine growth retardation seen in rural areas of developing countries. [4,31] Lower maternal age is a factor found in this study to be significantly related with increased risk of malarial parasitisation, a finding consistent with reports of previous studies [2,3]. The reasons for this may be attributed to immunity-related factors.[3]

Table 3: Biochemical characteristics of the newborns according to class of histological evaluation of the placenta

Parameters	Class I Mean±SD (n=137)	Class IV Mean±SD (n=52)	Class V Mean±SD (n=278)	F-Value	P-value
Iron (mg/dl)	4.82±1.40	4.61±1.19	4.93±1.37	0.35	0.71
Zinc (mg/dl)	0.07±0.07	0.06±0.08	0.08±0.06	1.51	0.22
Magnesium (mg/dl)	0.06±0.05	0.05±0.04	0.08±0.03	0.38	0.68
Calcium (mg/dl)	0.16±0.42	0.13±0.04	0.20±0.45	0.19	0.83

SD; standard deviation.

Table 4: Pearson correlation between maternal and umbilical cord micronutrients

Parameters	Iron	Zinc	Magnesium	Calcium
Maternal age	0.317**	0.218**	0.126**	0.141**
Iron	-	0.010	0.19**	-0.145**
Zinc			0.005	0.098
Magnesium				0.440**

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed).

The placental and umbilical cord parasitisation had no effect on umbilical cord blood micronutrients levels despite the high prevalence of malarial parasitisation recorded within the study period. Therefore, giving the magnitude of this prevalence and its possible long-term effects, efforts must be made to reduce malarial transmission through provision of antenatal care to all pregnant women with micronutrients supplements inclusive. This will help to reduce perinatal problems and malaria infections during pregnancy.

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Conflict of interests The researchers hereby declare that, there was no conflict of interest in any part of this research.

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32. Foetal nutrition, malaria, placenta, umbilical cord.

Assessment of Liver Oxidative Stress Status of Sub-Chronic Lead Intoxicated Rats Supplemented with Folic Acid and Vitamin C

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Abstract

Lead induced oxidative stress is the chief mechanism by which lead affects every part of the body, especially the liver. This study examined the liver oxidative stress status of sub-chronic lead intoxicated rats supplemented with folic acid and/or vitamin C. Forty (40) male albino rats weighing 160-190g were randomly divided into eight groups containing five (n=5) experimental rats each. Lead acetate was administered to groups (I-IV), for a period of six weeks, followed by folic acid and/or vitamin C supplementation to groups (II-VII) for another period of four weeks. Findings from this study revealed statistically significant ($p \leq 0.05$) decrease in catalase (CAT) and superoxide dismutase (SOD) activities in the non-supplemented lead intoxicated rats as compared to the control, folic acid and/or vitamin C supplemented groups. The significant ($p \leq 0.05$) reduction in the activities of CAT and SOD correlates negatively with the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), while the significant increase of malondialdehyde (MDA) level in the non-supplemented lead intoxicated rats correlate positively with the activities of AST. More so, significant positive correlation exists between the liver MDA versus serum ALT activities in the lead intoxicated group, co-supplemented with folic acid and vitamin C. The co-supplementation (folic acid and vitamin C) distinctively lowered MDA level, indicating that the co-supplemented nutrients might have reversed the lead induced liver dysfunction.

Keywords: Lead, Folic acid, Vitamin C, Oxidative stress, Liver emzymes

Introduction

Lead is present in our environment particularly in the industrial-based environment, [1, 2] and posed tremendous medical problem and public health concern world over. [3] It has wide industrial applications, such as production of rodenticides and batteries, [4, 5, 6] and because of being used recklessly, it contaminates the air, water and soils which makes it one of the dangerous environmental pollutants to plants, animals and humans in both urban and rural areas. [7]

Vitamin C is related to glucose structurally, consisting of both L-ascorbic acids and L-dehydroascorbic acids, both of which are inter-convertible. [8] It is widely distributed in nature and found mostly in green leafy vegetables, fruits and to a larger extent in kidney and liver of animals. [9] Ascorbic acid plays a vital role against toxicological manifestation exhibited by lead. [4] Unlike ascorbic acid, the role of folic acid on lead induced toxicity has not been extensively studied, despite being one of the targeted nutrients of lead intoxication. Folic acid deficiency is associated with high blood lead levels, and folate deficient children are highly susceptible to lead toxicity. [10, 11, 12]

Lead intoxication decreases the body antioxidants status, [13] thereby prompting various degrees of injuries, yet most research is geared towards protective approach using exogenous antioxidants, and this lacks substantive scientific basis to tackling lead toxicity. This is based on the fact that most people are exposed to lead, especially chronic and sub-chronic lead intoxication, unknowingly and unwillingly resulting to irreversible complications. As such research effort should be tailored towards reversibility of oxidative stress induced by lead. The reason allured the attention of the researchers to examine the role of combined supplementation of folic acid and vitamin C on lead-induced oxidative stress in albino rats.

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Material and Methods

Experimental Design

Forty (40) male albino rats weighing 160-190g were used in this study. The albino rats were acclimatized and fed *ad libitum* for two weeks. Ethical clearance for the use of the experimental rats was approved by the ethical committee of the Ahmadu Bello University, Zaria. The albino rats were divided into eight groups of five each. The experimental rats were subjected to the same quantity of food and water intake. All treatment and supplementation were given by oral gavage. Lead acetate was administered to groups (I-IV), for period of six weeks, followed by folic acid and/or vitamin C supplementation to groups (II-VII) for another period of four weeks. Group I was administered lead acetate (60mg/kg body weight/day) Group II was administered lead acetate (60mg/kg body weight/day), supplemented with folic acid (500µg/kg body weight/day) Group III was administered lead acetate (60mg/kg body weight/day), supplemented with vitamin C (60mg/kg body weight/day). Group IV was administered lead acetate (60mg/kg body weight/day), supplemented with both folic acid (500µg/kg body weight) and vitamin C (60mg/kg body weight/day). Group V was given folic acid (500µg /kg body weight/day). Group VI was given vitamin C (60mg/kg body weight/day). Group VII was given both folic acid (500µg /kg body weight) and vitamin C (60mg/kg body weight/day). Group VIII serves as the control, and was given normal saline (1ml). At the end of the experiment, the experimental animals were fasted overnight, sacrificed, followed by collection of samples (blood and liver) via surgical process.

Collection and sample preparation

The samples (blood and liver) were collected into EDTA tubes. Prior to the analysis, the livers collected were rinsed in an ice-cold saline (0.9% w/v NaCl) and blotted dry. The tissues were homogenized using 10mmol/L phosphate buffer saline. The homogenate was centrifuged at 4000rpm for 20 minute, followed by collection of the supernatant fractions which was stored at appropriate temperature. The blood sample collected was also centrifuged at 4000rpm for 20 minute, and the supernatant was kept at 4^{o}C.

Determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) AST and ALT activities were determined spectrophotometrically using Randox commercial test kits (GOT[AST]-LQ and GPT[ALT]-LQ respectively) purchased from Chemelex S.A. (Barcelona, Spain).

Estimation of malondialdehyde (MDA)

MDA was determined using the method of Placer *et al.* (1966), [14] as described by Prakask *et al.* (2010). [15] MDA is the most extensively studied, and is used as a biochemical marker for the assessment of lipid peroxidation. The reaction depends on the formation of pink colour complex between MDA and thiobarbituric acid reagent. Two tubes were set up for the blank and the test respectively; 0.5ml of distilled water and 3.0ml of thiobarbituric acid reagent were pipetted into the blank tube, while 0.75ml of each sample and 3.0ml thiobarbituric acid reagent were also pipetted into the test tubes. All samples in the test tubes were boiled in a water bath for 15 minutes. The tubes were allowed to cool, centrifuged for 10 minutes at 3000 rpm and absorbance of supernatant was read at 535nm.

Where extinction coefficient (ε) = 1.56×10^5

Catalase determination

Catalase activity was determined using the method described by Aebi (1984). [16] As catalase decomposes hydrogen peroxide (H_2O_2), the absorption decreases with time and from this decrease, catalase activity was measured at 240nm. The analysis was carried out by adding 2ml of sample and 1ml of H_2O_2 solution into the sample test tubes while 2ml of the blank solution and 1ml of hydrogen peroxide were added to the blank test tube. The change in the absorbance of test sample against blank at 240nm was recorded every 15 seconds using a UV–Visible Spectrophotometer.

Concentration (u/l) = Abs_1 is absorbance at t = 0 second Abs_2 is absorbance at t = 15 seconds
Where 0.2 and 0.00693 are constant

Superoxide dismutase (SOD) determination SOD was determined by the method described by Fridovich (1989). [17] The ability of the superoxide dismutase to inhibit auto oxidation of adrenaline at pH 10.20 forms the basis of this assay. Exactly

0.2ml of the serum was added to 2.5ml of 0.05M carbonate buffer, followed by addition of 0.3mM adrenaline. The absorbance was taken over 30seconds up to 150 seconds at 450nm. 1 unit of SOD activity is the quantity of SOD necessary to elicit 50 percent inhibition of the oxidation of adrenaline to adenochrome in 1minute.

Statistical analysis

In each analysis, the data obtained was expressed as mean (n=5) ± standard deviation. Data obtained was subjected to analysis of variance (ANOVA) using (SPSS version 20.0). The level of significance (P≤0.05) was taken statistically using Duncan multiple range test.

Results The results shown in Table 1 depict the activities of ALT and AST in the sub-chronic lead intoxicated rats, treated with folic acid and/or vitamin C supplementation. The results showed statistical significant (p≤0.05) difference in the activity of AST of lead intoxicated group with no supplementation compared to the activity observed in the folic acid, vitamin C, folic acid plus vitamin C supplemented and control groups respectively (Table 1). Similarly, the activity of ALT in the group subjected to lead, with no supplementation differ significantly (p≤0.05) from the level observed in the control, lead intoxicated groups treated with folic acid and/or vitamin C supplementation (Table 1).

Table 1: Serum activities of ALT and AST of sub-chronic lead intoxicated rats supplemented with folic acid and/or vitamin c

Group	ALT (U/L)	AST (U/L)
Lead	74.05±0.69[a]	82.51±1.53[a]
Lead+FA	63.65±1.53[b]	68.23±1.24[b]
Lead+Vit C	56.01±2.78[c]	61.66±1.36[c]
Lead+FA+Vit C	44.45±2.01[d]	43.15±1.50[d]
FA	41.65±1.40[e]	40.48±1.40[e]
Vitamin C	41.73±1.32[e]	40.25±2.77[e]
FA+Vit C	40.60±1.46[e]	39.20±1.99[e]
Control	40.25±1.75[e]	39.83±2.28[e]

Values are expressed as mean ± Standard deviation (n=5). Values down the group having different superscript differ significantly. FA: folic acid, Vit C: vitamin C More so, the activity of ALT in the non-lead intoxicated groups supplemented with folic acid and/or vitamin C differs significantly (p<0.05) compared to the lead exposed groups supplemented with folic acid and/or vitamin C (Table 1).

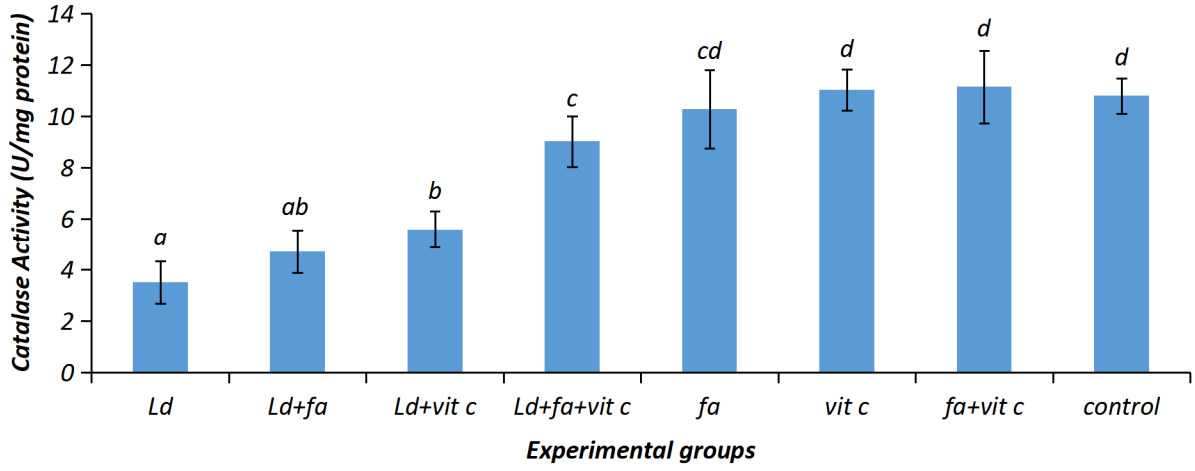

Figure I: Liver Catalase Concentration of Sub-Chronic Lead Intoxicated Rats Supplemented with Folic acid and Vitamin C. Bars with different superscript differ significantly (p≤0.05). Ld // lead, FA // folic acid, Vit C // vitamin C.

The liver CAT activity showed statistical significant ($p \leq 0.05$) difference in the lead intoxicated group co-supplemented with folic acid and vitamin C compared to the activity observed in the control (Figure 1). The SOD activity was significantly ($p \leq 0.05$) lower in the non-supplemented lead intoxicated group compared to the activity observed in the control, folic acid and/or vitamin C groups (Figure 2). The MDA level was significantly ($p \leq 0.05$) higher in the lead intoxicated, co-supplemented group (folic acid and vitamin C), compared to the level observed in the non-supplemented lead intoxicated groups (Figure 3).

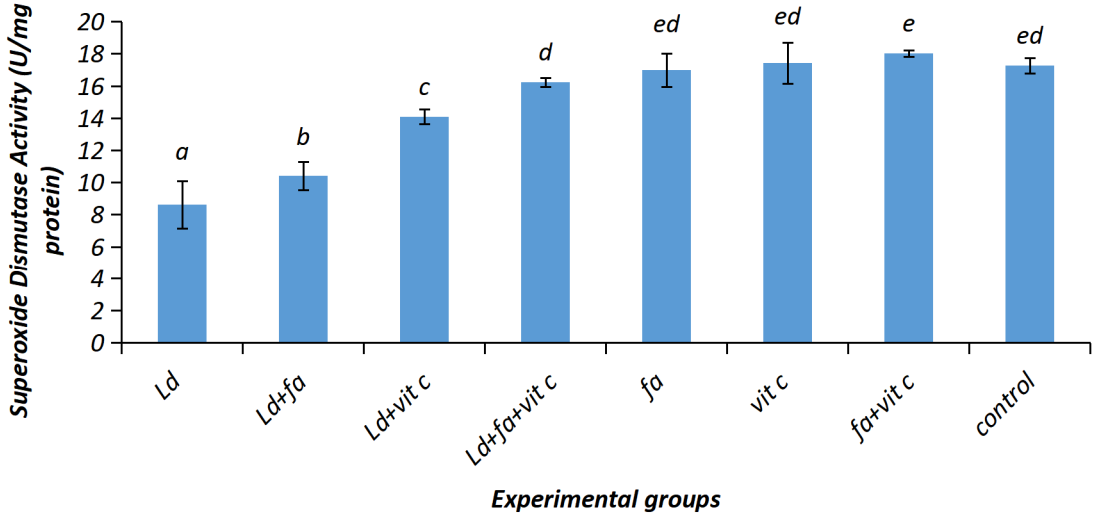

Figure 2: Liver Superoxide Dismutase (SOD) Activity of Sub-Chronic Lead Intoxicated Rats Supplemented with Folic acid and Vitamin C. Bars with different superscript differ significantly ($p \leq 0.05$). Ld – lead, fa – folic acid, vit c – vitamin C.

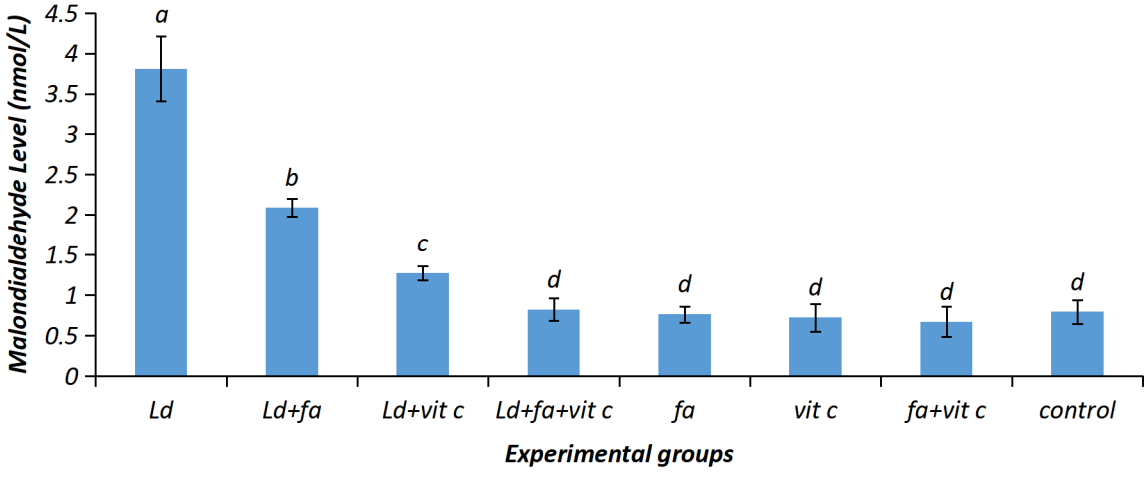

Figure 3: Liver Malondialdehyde Concentration of Sub-Chronic Lead Intoxicated Rats Supplemented with Folic acid and Vitamin C. Bars with different superscript differ significantly ($p \leq 0.05$). Ld – lead, fa – folic acid, vit c – vitamin C. Furthermore, statistical significant decrease was observed in the lead intoxicated groups supplemented with either folic acid or vitamin C as compared to the non-supplemented lead intoxicated group (Figure 3).

Table 2: Correlation between liver MDA, CAT, SOD and the activities of AST and ALT.

Group	MDA vs AST	MDA vs ALT	CAT Vs AST	CAT Vs ALT	SOD Vs AST	SOD Vs ALT
Lead	0.946(0.015)	0.284(0.644)	-0.589(0.269)	-0.943(0.016)	-505(0.385)	-0.553(0.334)
FA	0.412(0.491)	-0.515(0.375)	0.279(0.649)	0.588(0.297)	0.277(0.651)	0.484(0.409)
Vit C	-0.471(0.423)	-0.364(0.547)	0.032(0.960)	-0.468(0.434)	-0.731(0.161)	0.849(0.069)
Lead+FA+Vit C	0.077(0.902)	0.953(0.012)	-0.149(0.811)	-0.624(0.260)	0.809(0.097)	0.132(0.833)
FA	-0.231(0.709)	0.003(0.997)	-0.066(0.916)	0.325(0.593)	0.147(0.813)	0.466(0.429)
Vit C	0.473(0.421)	-0.694(0.194)	0.259(0.674)	0.136(0.827)	-0.687(0.200)	-0.460(0.436)
FA + vit C	0.658(0.228)	0.086(0.099)	-0.107(0.864)	-0.327(0.590)	-0.578(0.307)	-0.616(0.208)
Control	0.295(0.630)	0.405(0.499)	-0.379(0.536)	-0.079(0.849)	-0.611(0.274)	-0.452(0.445)

Correlation is significant at $p \leq 0.05$. Values in bracket represent statistical significant values. Non-bracket values represent correlation coefficient ("r"). . 0.00 – No correlation, 0.01 to 0.19 Very weak correlations, 0.20 to 0.39 Weak correlations. 0.40 to 0.59 Moderate correlations, 0.60 to 0.79 Strong correlations, 0.80 to 0.99 Very strong correlations. Vit C – Vitamin C, FA – Folic acid.

The correlation between the liver MDA and the activities of AST depicts very strong positive (0.946) correlation in the non-supplemented lead intoxicated group (Table 2). The correlation between CAT and ALT revealed very strong negative correlation, while between CAT and AST there was moderate negative correlation in the non-supplemented lead intoxicated group (Table 2). On the other hand, the SOD showed moderate negative correlation with AST and ALT in the non-supplemented lead intoxicated group (Table 2).

Discussion

Liver is a vital organ that performs numerous metabolic functions such as synthesis and degradation of molecules and converting molecules to another form. Exposure to toxic substance such as lead could impair liver working capacity. It was observed in this study that sub-chronic lead intoxication to the experimental rats induced increase in the serum levels of AST and ALT. Our observation is similar to previous report that lead administration to experimental rats elevated the serum levels of these enzymes.[18] Such elevation is an indication of liver dysfunction. However, co-supplementation of folic acid and vitamin C restored the hepatic dysfunction as evident from the insignificant difference between the control and co-supplemented group. As such, they could be used to mend liver dysfunction in sub-chronic lead exposure. Serum ALT and AST are very sensitive biomarkers in the diagnosis of hepatic damage, as both are cytoplasmic enzymes released into the circulation upon hepatic cellular damage. .[19] Exposure to lead can act by directly damaging the hepatocytes mainly by destroying the permeability of the cell membrane, resulting in the release of AST and ALT from the hepatocytes to the blood.

Unlike folic acid which has not been extensively studied in relation to lead induced hepatic damage, ascorbic acid had been reported to ameliorate hepatic damage. [20, 21, 22] More so, information on the combined effect of folic acid and vitamin C on lead induced hepatic damage is either little or not available, although vitamin C had been used with other vitamins, such as vitamin E, in ameliorating lead induced liver injury. For instance a study revealed that ascorbic acid may have liver protective effect, though tends to do better when co-administered with other agents. [8]

Catalase is found in nearly all living organism, and very vital to health, especially in oxygen metabolic processes. The significant decrease in catalase activity in the non-supplemented lead intoxicated rats could be attributed to inhibition of metabolic synthesis of a key component (heme) of catalase. Exposure to lead inhibits enzymes of heme biosynthesis, [23] especially ferrous chelatase that catalyses the introduction of iron into the central cavity of porphyry ring, and this may affect the catalytic concentration of catalase in the experimental rats. However, folic acid and/or vitamin C supplementation to the lead intoxicated rats significantly mends the catalytic concentration of catalase. Previous research findings revealed increased catalase concentration upon vitamin C supplementation amongst lead exposed workers. [24]

The co-supplementation of folic acid and vitamin C significantly mends the MDA level, a measure of lipid peroxidation, in the lead intoxicated group. Similar findings also revealed that combined supplementation of vitamin C and E to chronic hepatotoxicity mitigate lipid peroxidation in albino rats. [25] SOD is one of the active enzymes in oxygen metabolism. The decreased level of SOD is similar to previous report following exposure to lead. [26] SOD contains calcium and zinc both of which are responsible for the stability and functionality of the enzyme, and alteration of these divalent cations may affect the catalytic function.

Lead induced increase in the lipid peroxidation level could be responsible for liver damage as evident in the significant positive correlation between the liver lipid peroxidation levels and AST level in the non-supplemented lead intoxicated rats. However, increase serum AST is not only specific to liver damage. On the other hand, insignificant positive correlations between MDA levels versus ALT level do not establish any relationship. Nonetheless, lead induced oxidative stress in any part of the body has been postulated to be one mechanistic approach by which lead induces tissue injury. [27] Catalase has one of the highest turnovers that can decompose hydrogen peroxide to oxygen and water. As such, lead induced decrease in the catalase concentration could lead to liver injury as evident from the significant negative correlation between catalase and ALT activities in the non-supplemented lead intoxicated rats.

Conclusion

Lead induced imbalance between the production of free radicals and the biological system ability to detoxify the reactive intermediate immeasurably contribute to liver damage. Sub-chronic lead intoxication to the experimental rats induced increase in the level of MDA which correlate positively to AST and ALT activities. However, co-supplementation of folic acid and vitamin C distinctively lowered the MDA level, thus mends liver dysfunction in the sub-chronic lead intoxicated rats, suggesting co-supplementation of folic acid and vitamin C might serve as nutrient supplements to restore liver dysfunction.

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Effects of Dichlorvos Inhalation on the Histology of Blood and Selected Haematological Indices in Adult Wistar Rats

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Abstract
Dichlorvos is an organophosphate commonly used as insecticide and pesticide. It constitutes the active ingredient of the locally formulated mosquitoes control solution, Madarar piapia or Ota piapia. It is an anticholinestrase that inhibits the action of enzyme acetylcholinestrase. Studies on dichlorvos focused mainly on neurotoxicity, less attention was paid to its effect on blood histology and its indices. The aim of this study was to evaluate the effects of dichlorvos solution on the blood histology and haematological indices in adult Wistar rats. Twenty five apparently healthy adult Wistar rats were procured, randomly selected and divided into five groups. The group I was used as control, group II was treated with 2.5mg/m^3 ethanol while, groups III, IV &V were used as treatments and exposed to 11.25, 7.50 and 3.75 mg/m^3 doses of dichlorvos in ethanol solution respectively and experimented for twenty eight days. Twenty-four hours after the last exposure, blood samples were collected in heparinized bottles for serum analyses and slide smear preparation. The haematological indices were expressed as mean ± SEM and one way analysis of variance (ANOVA) was conducted using Minitab (version 16) statistical software. There was significance difference, ($p < 0.05$) in the values the WBC, Hb, PCV, MCV, MCHC, RDW and PLT between the control and treated groups. Dichlorvos inhalation alters the significantly haematological indices and blood histology of Adult Wistar Rats.

***Keywords**:* Blood, Dichlorvos, Histology, Inhalation, Wistar rats.

Introduction
Dichlorvos, (2, 2, - dichlorovinyl dimethyl phosphate; DDVP), is both a household and agricultural pesticide traded under different names such as Nu*van, Sniper* and *Ota-piapia*. In addition to its use as control for insects on crops, household, and stored products, dichlorvos is also used to treat external parasitic infections in farmed fish, livestock, and domestic animals.[1] *Ota-piapia* is the common name in Nigeria, and is hawked around and used for agricultural and domestic purposes to kill various insects and to protect stored products from insects. Dichlorvos is a volatile organophosphate which is preponderantly active pesticide ingredient in the local formulation of *Ota-piapia* in Nigeria.[2] Dichlorvos is widely accepted in Nigeria due solely to its cheap production, efficacy, accessibility, and affordability.[3] The use of DDVP has been long since the early 1960s and has been the subject of many toxicity studies and review articles.[4] It is rapidly absorbed through gastrointestinal and respiratory tracts and skin, and enters human system via inhalation, dermal or oral routes, and it is metabolised by the liver and excreted by the kidney.[4, 5] Most of organophosphate (OP) compounds are highly lipid-soluble agents and are well absorbed from the skin, oral mucous membranes, conjunctiva, gastrointestinal and respiratory tracts.[6] The organophosphate insecticides have relatively short biological half-lives and are fairly rapidly metabolised and excreted.[7] The mechanism of action for the dichlorvos is mainly by blocking of acetylcholinesterase – an enzyme which in turn decomposes acetylcholine [8, 9]. Overdose of this OP leads to symptoms which include weakness, headache, tightness in chest, blurred vision, salivation, sweating, nausea, vomiting, diarrhoea, respiratory failure, and abdominal cramps being an acetylcholinesterase inhibitor [10]. It is mainly metabolised by esterase to dimethylphosphate and

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dichloroacetaldehyde. Dimethylphosphate is excreted in the urine, while dichloroacetaldehyde is rapidly metabolised via two pathways to dichloroethanol glucuronide, hippuric acid, urea and carbon dioxide, and excreted in the urine and expiration.[5]

The clinical signs and symptoms of dichlorvos are generally attributable to acetylcholine accumulation.[11] These signs and symptoms are commonly divided into three groups; muscarinic, nicotinic and central.[12] There have been two clinical reports describing four patients suffering from severe poisoning from dichlorvos, taken orally, who survived after treatment and who showed delayed neurotoxin effects.[11] Thus, although the possibility of neuropathy in human beings cannot be excluded, it is likely to occur only after almost lethal oral doses.[12] When dichlorvos was administered orally to human volunteers (single or repeated doses of a slow-release PVC formulation), significant inhibition of red blood cell ChE activity was found at 4 mg/kg body weight or more.[11] At 1 mg/kg body weight or more, plasma ChE activity was significantly inhibited. Daily oral doses of 2 mg dichlorvos/ person for 28 days reduced plasma ChE activity by 30%, but red cell ChE activity was unaffected.[12]

Many studies conducted on dichlorvos toxicity focused on its effects on the brain and brain related tissue. The reason perhaps might not be far – fetched from the fact that dichlorvos, being anticholinestrase inhibits acetylcholine thereby leading to its accumulation in the synaptic junctions, which as a result causes the animal to manifest parasympathetic symptom both on the central and peripheral nervous systems. However, despite these numerous studies, there is paucity of data on the effect of this chemical on the histology of blood and its indices. The aim of this study was to evaluate the effects of dichlorvos inhalation on the histology of blood and selected haematological indices in adult Wistar rats.

Materials and Method

Experimental Animals Twenty five apparently healthy adult Wistar rats consisted of both sexes and weighed about 195 – 400g were purchased from the Pharmacology Department, Aminu Kano Teaching Hospital (AKTH) Kano, Nigeria and allowed to acclimatize for two weeks in laboratory condition before subjected to experimentation. The animals were housed in well-ventilated rectangular aluminium cages (290 × 320 × 390 mm) bedded with soft saw dust and maintained under standard laboratory conditions with proper illumination under natural condition. The animals had free access to food (Vital feed) and water *ad libitum*. The animals' sanitation and husbandry were in accordance with "Guide for the Care and Use of Laboratory Animals." [13] The animals were then divided into five groups (i.e. I, II, III, IV and V) with each group containing at least equal number of rats.

Chemical and Dose Preparation

A stock concentration of 1000 g/l of dichlorvos (Delvap Super ®) was purchased from the vendors of insecticides, pesticides and other Agro Allied Chemicals at Sabon-Gari market, Kano, Kano State, Nigeria. The lethal concentration (LC_{50}) of dichlorvos which was reported as15mg/m^3 [14] was taken as a reference value. The study was carried out with sub-lethal doses equivalent to 75% (11.25mg/ m^3), 50% (7.50mg/m^3) and 25% (3.75mg/m^3) of the reference LC_{50}dose.

Experimental Design

Five poorly ventilated 1m x 1m cubed wooden boxes labelled A, B, C, D and E, each with a rectangular sliding glass pane measured about 0.2m x 0.1m for entrance at the top was constructed for the exposure of the animals. About 2 ml each of the graded solutions were drawn separately using 4 mls hypodermic syringes and then sprayed thoroughly into the boxes every day before the animals were exposed. The animals in group I were used as control and exposed to ambient air, while those in group II were treated with 2 mls of 2.5 mg/m^3ethanol solution (96.5% purity), groups III, IV and V were treated with 11.25 mg/m^3, 7.50 mg/m^3, and 3.75 mg/m^3 concentrations of dichlorvos in ethanol solution that is sprayed in the boxes respectively. The exposure lasted for 2 hours in all the groups every day for twenty eight days.

Samples Collection

Twenty four hours after the last exposure, 2 ml of blood sample was randomly collected in EDTA and heparin blood containers from each group immediately after decapitation.

Determination of haematological Indices

The blood samples collected were analysed each for Packed cell volume (PCV), Red blood cell (RBC),

White blood cell (RBC), Hemoglobin (Hg), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), and Platelet counts (PLT), using automated hematology analyser (Mindray, Model BC-2800Vet).

Slide Preparation

The blood smears were prepared using modified method of Leishman [15] and viewed under *Olympus* light microscope to which fitted *Celestron*© (Digital microscope imager, USA) with an inbuilt 15x magnifying lens at 150 and 600 magnifications respectively. **Statistical Analyses** The values of haematological indices were expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by *Tukey's* post- hoc test was conducted to assess the level of the mean differences between groups using Minitab (version 16) statistical software was used for statistical analyses. $P < 0.05$ was considered as level of significant. **Results** Table 1 showed mean ± SEM of selected haematological indices of adult Wistar rats exposed to 11.25, 7.50 and 3.75 mg/m^3 doses of dichlorvos through inhalation. The mean WBC, RBC, Hb MCHC and PLT counts were higher in the group III compared to the control group that were exposed to ambient air. The lowest values of the haematological indices for WBC, MCV, PCV, MCHC, and PLT were recorded in group V (i.e. the lowest dichlorvos exposed group).

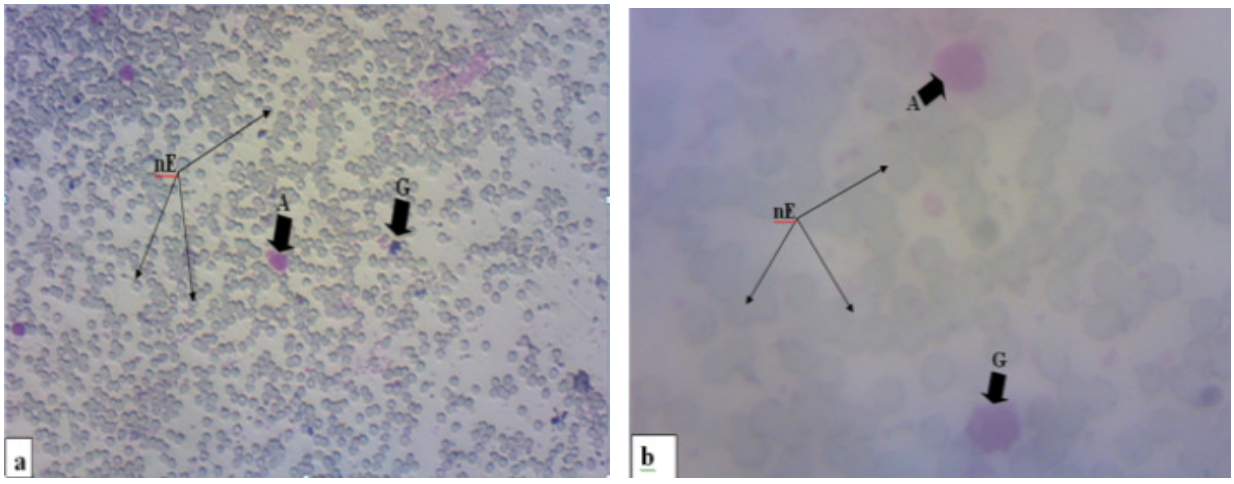
Significant increase in the mean WBC and PLT (P 0.005) was observed in the group with highest chemical exposure, this was then followed by dose dependent decrease in the WBC compared to the control group. The Hb, PCV, and MCV counts also increased significantly in all the treated (P 0.001) compared to the normal groups. Dose dependent increase and decrease in the MCHC and RDW count (P 0.005) was also observed respectively in the treated groups except for the group V.

Table 1: haematological indices of adult Wistar rats exposed to dichlorvos

Haematological Indices	Group I Ambient air	Group II Ethanol (96.5%)	Group III (11.25mg/m^3)	Group IV (7.50mg/m^3)	Group V (3.75mg/m^3)
WBC (10^3/mm^3)	7.73 ± 0.71	5.56 ± 0.67*	11.14 ± 1.32*	6.00 ± 1.17*	3.38 ± 0.47*
Hb (g/dL)	10.09 ± 0.64	10.82 ± 1.09	12.68 ± 0.79*	12.25 ± 0.94*	12.67 ± 1.30*
RBC (10^6/mm^3)	7.12 ± 1.08	7.65 ± 0.92	8.55 ± 1.02	7.61 ± 0.50	7.99 ± 0.61
PCV (%)	36.09 ± 1.05	39.45 ± 0.67*	39.79 ± 0.48*	41.90 ± 0.28*	41.85 ± 0.48*
MCV (fL)	50.07 ± 0.26	52.60 ± 0.77*	46.19 ± 1.06*	54.11 ± 0.53*	51.76 ± 0.52*
MCH (pg)	14.32 ± 0.70	15.15 ± 1.10	14.55 ± 0.83	15.38 ± 0.94	14.80 ± 0.60
MCHC (%)	28.16 ± 0.69	29.11 ± 0.54	30.84 ± 0.76*	29.97 ± 0.95*	29.46 ± 1.5
RDW (%)	17.07 ± 0.95	17.05 ± 0.65	15.83 ± 0.89*	15.81 ± 0.92*	17.36 ± 0.65
PLT (L^{-1})	480.01 ± 1.11	445.37 ± 0.97*	673.03 ± 0.94*	607.06 ± 0.70*	353.35 ± 1.35*
MPV (fL)	9.14 ± 0.48	9.47 ± 0.90	8.71 ± 0.69	9.02 ± 0.90	10.18 ± 1.41
PDW	15.35 ± 1.27	15.41 ± 0.74	14.69 ± 1.02	14.92 ± 1.06	15.27 ± 1.11

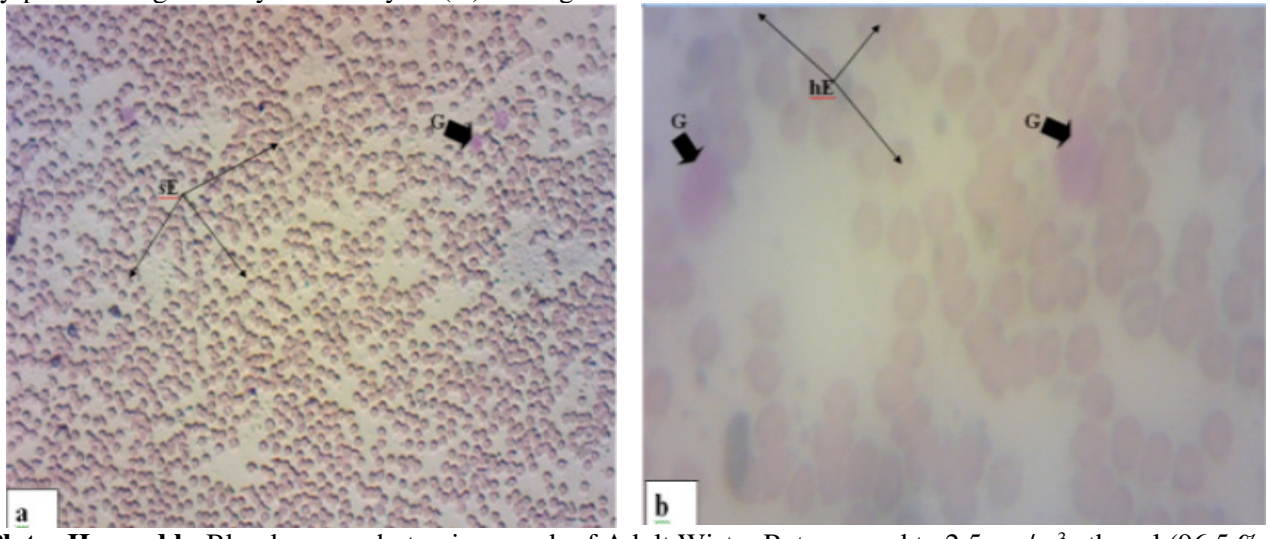
WBC= white blood cell; **Hg**= Hemoglobin; **RBC**= Red blood cell; **PCV**= Packed cell volume; **MCV**= Mean corpuscular volume; **MCH**= Mean corpuscular haemoglobin; **MCHC**= Mean corpuscular haemoglobin concentration; **RDW**= Red blood cells distribution width; **PLT**= Platelet **MPV**= Mean platelet volume; **PDW**= Platelet distribution width. * $p < 0.05$

Plates I a and b are H and E photomicrographs of adult Wistar rats exposed to ambient air (normal group) at lower and higher magnifications respectively. The plates showed histology of blood smears containing biconcave normochromatic erythrocytes (nE) with central pallor interspersed by patches of both granulocytic (G) and agranulocytic (A) leucocytes. At higher magnification, the photomicrographs were rather clear showing lymphocytes and patches of platelets.



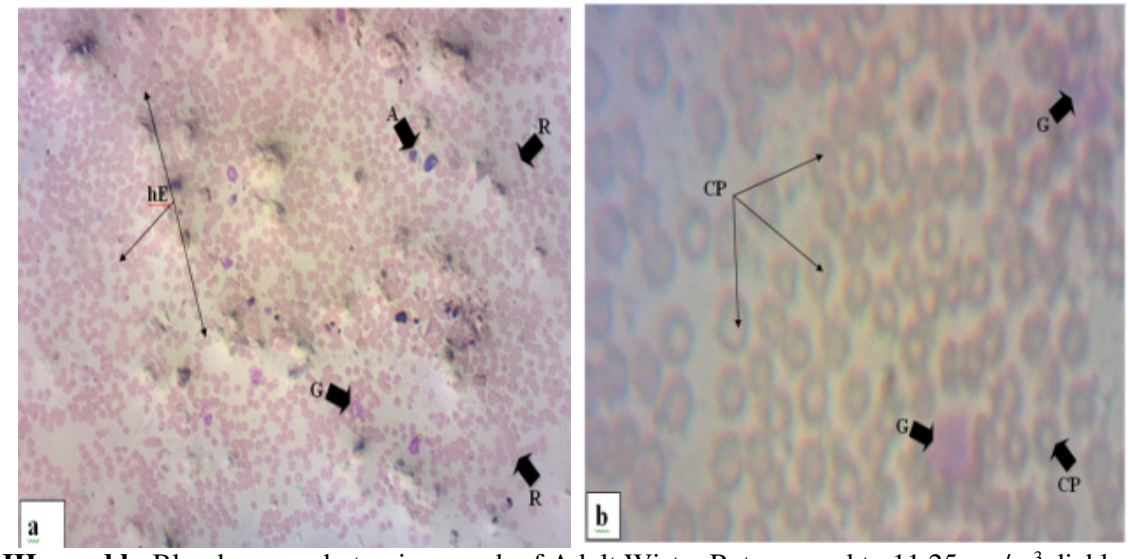
Plates I a and b: Blood smear photomicrograph of normal Adult Wistar Rat exposed to ambient air as control at (a) x150 and (b) x600 magnifications respectively (Positive control). **A=** Agranulocytic leukocyte; **G=** Granulocytic leukocyte; **nE=** Normochromatic erythrocytes.

Plate IIa and b are H&E photomicrographs of the animals exposed 2 ml of ethanol (96.5% purity). At lower magnification, the plate A showed slightly spherocytic films of erythrocytes (sE) interspersed by patches of granulocytic leucocytes (G). At higher magnification, the patches were more evident and still featuring the hyperchromatic erythrocytes (hyE) bearing relatively normal central pallor and granulocytic leucocytes.



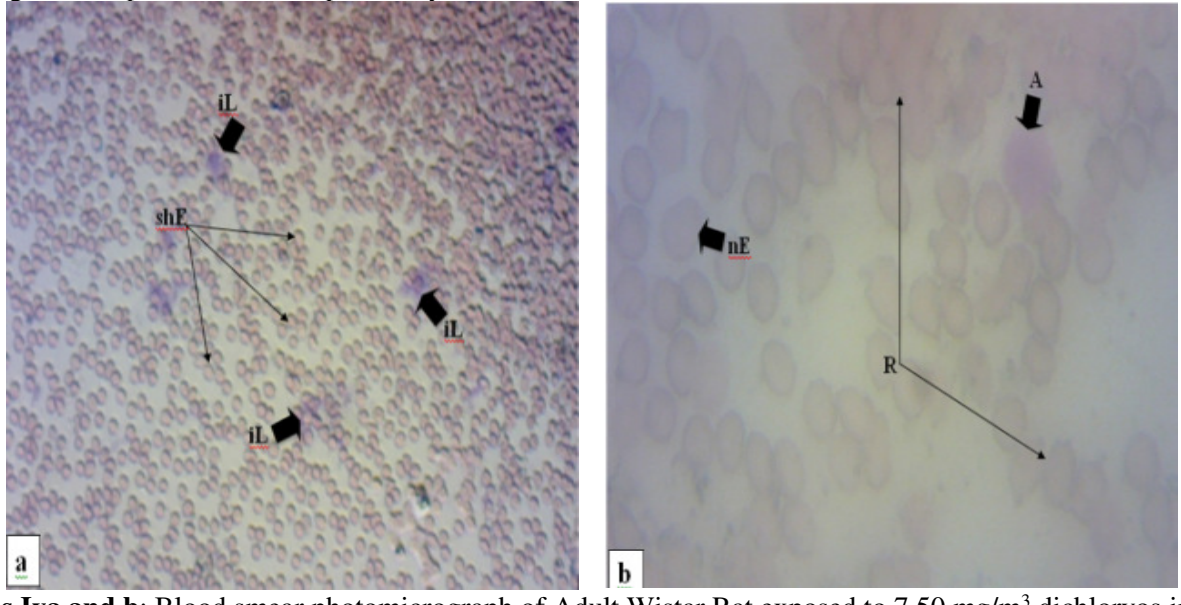
Plates II a and b: Blood smear photomicrograph of Adult Wistar Rat exposed to 2.5 mg/m^3 ethanol (96.5 % purity) as negative control at (a) x150 and (b) x600 magnifications respectively. **G** = Granulocytic leucocytes; **hE** = Hyperchromatic erythrocytes; **sE =** Spherocytic erythrocytes.

Plates III a and b are H and E photomicrographs of blood smears of adult Wistar rats exposed to 11.25mg/m^3 dichlorvos in ethanol solution. At lower magnification, the photomicrograph showed slightly hypochromatic (hE) erythrocytes bearing relatively wider central pallor. Some of the erythrocytes that stacked on top of one another to form rouleaux (R), were interspersed by patches relatively granulocytic (G) and agranulocytic (A) leucocytes. The platelet patches were rather abundant compared to the control group. At higher resolution, the photomicrograph clearly showed the wider central pallor (CP) and patches of granulocytic (G) leukocytes.



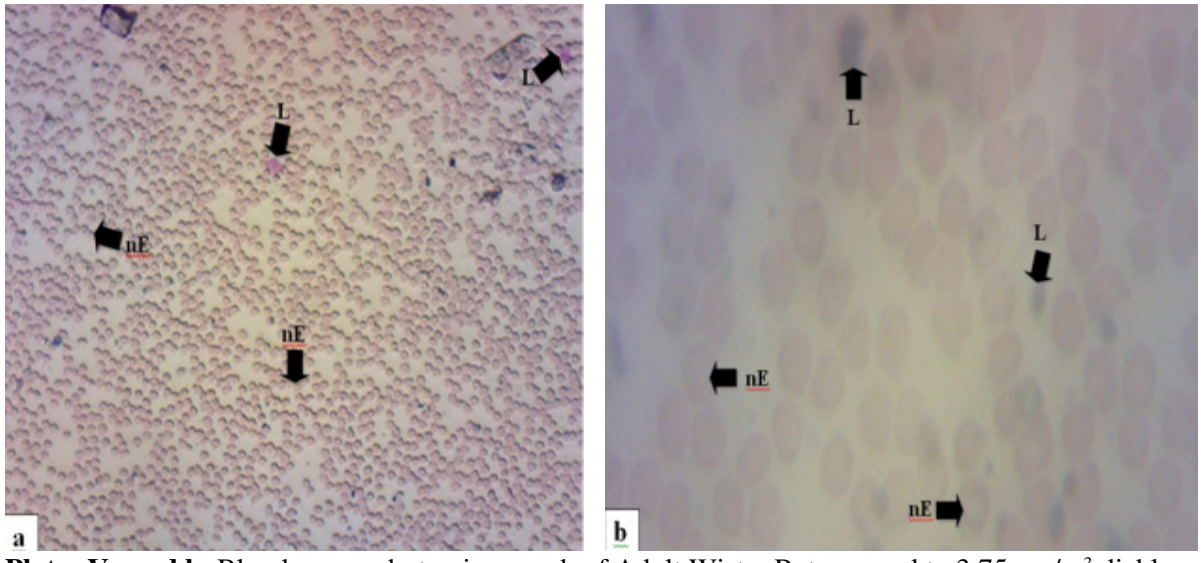
Plates III a and b: Blood smear photomicrograph of Adult Wistar Rat exposed to 11.25 mg/m^3 dichlorvos in ethanol solution at (a) x150 and (b) x600 magnifications respectively. **A** = Agranulocytic leucocytes; **CP** = central pallor; **G** = granulocytic leucocytes; **hE** = Hypochromatic erythrocytes; **R** = Rouleaux formation.

Plates IVa and b showed the photomicrographs of adult Wistar rats exposed to 7.50 mg/m^3 dichlorvos. The plate with lower magnification showed spherocytic and hyperchromatic erythrocytes (shE) lacking central pallor and interspersed by indistinct leucocytes (iL), which at higher magnification showed spots of agranulocytic (A) leucocytes entangled in rouleaux formation (R).



Plates Iva and b: Blood smear photomicrograph of Adult Wistar Rat exposed to 7.50 mg/m^3 dichlorvos in ethanol solution at (a) x150 and (b) x600 magnifications respectively. **A** = Agranulocytic leucocytes; **iL** = indistinct leucocytes; **nE** = Normochromatic erythrocytes; **shE** = Spherocytic and hyperchromatic erythrocytes **R** = Rouleaux formation.

The photomicrographs of Plates Va and b were smears of adult Wistar rats exposed to 3.75 mg/m^3 dichlorvos in ethanol solution showing relatively normal erythrocytes (nE), bearing central pallor and patches of leucocytes (L). At higher magnification, the cells spread out with no rouleaux formation, still indicating elements of leucocytes (L).



Plates Va and b: Blood smear photomicrograph of Adult Wistar Rat exposed to 3.75 mg/m^3 dichlorvos in ethanol solution at (a) x150 and (b) x600 magnifications respectively. **L** = Leucocytes; **nE** = Normochromatic erythrocytes; **R** = Rouleaux formation.

Discussions

Dichlorvos exposure through inhalation is one of the common causes of its toxicity that is often unaware by the users. Although the liver and blood have the enzymes that neutralize the toxicity, prolong exposure may compromise the efficiency of these organs thereby leading to ill health consequently. Significant difference in the mean values of WBC and PLT was observed in the treated groups compared to the control. This likely revealed that the rats' defense mechanism most have been stimulated to act against the toxic effect of the xenobiotic effect of dichlorvos or its metabolites. In a related study by Brown *et al.*[16] however found significant decrease and increase in WBC and PLT values respectively in dose dependent manner. The discrepancies in the mean values could probably be due to the dose concentration and genetic strains of the Wistar rats used in the experiment. The increase in the Hb counts might probably be due to the muscarinic effect of the dichlorvos on the respiratory system, which could have led to hypoxia thereby causing significant inhibition of red blood cell ChE. In a compensatory mode, the bone marrow and spleen might have undergone erythopoiesis thereby producing more erythrocytes and haemoglobin, and by default caused the increase in the MCHC.

Evidently, the histology of the RBC in the treated groups (plate III – V) corroborated this finding by the presence of brighter stains compared to the control (plate Ia and b). The increase in the platelet (thrombocytosis) counts (PLT) might however have resulted due to trauma on the lungs, respiratory muscles and blood vessels while the animals struggled to adjust and maintain the normal the airways ventilation. This finding contradicted the results of Soltani *et al.* [17] and Pourgholam *et al.* [18], and Brown *et al.* [16] were decrease in Hb, RBC and PCV counts were reported following intraperitoneal exposure to dichlorvos and long-term exposure to sub-lethal concentrations of organophosphate and diazinon respectively. The contradiction might have arisen following the difference in the route of chemical exposure, the rats' immuno-competence and duration of exposure.

The significant increase in the PCV and MCHC as indicated in the table was not a surprise as the mean Hb increased perhaps in an effort to balance the partial pressure of oxygen that might have been unstable probably due to hypoxia caused by the dichlorvos and its metabolites. In a similar study [19] using similar organophosphate, diazinon reported significant decrease in RBC, Hb, HCT, MCV, MCH and WBC count following exposure of *C. mrigala* to diazinon for 30 days. The relatively insignificant values of RBC, MCH, MPV and PDW across the control and all the treated groups however, contradicted similar studies [20, 21] using diazinon as indicated by decreased Hb, RBC and WBC count and increased values of MCV and MCH after diazinon exposure in giant sturgeon (*Huso huso*) and African catfish, *C. gariepinus* respectively. The discrepancy of these findings might probably be due to the dose, duration and immune-competence of the species used. The various histomorphologic changes seen in the blood smears of the rats exposed to the graded doses of the dichlorvos might possibly have occurred due to the formation of reactive metabolites that attack cell membranes and result in peroxidation of polyunsaturated fatty acids thereby caused hypochromatic appearance, rouleaux formation and reduction of the intensity of the cells central pallor.

Conclusion Inhalation of dichlorvos is toxic to the histology of the blood smears and haematological parameters. Although the alterations in levels of the selected haematological parameters cut across virtually all the treated groups, significant differences were recorded in the WBC, PCV, MCV and PLT parameters. Equally, blood histology altered in dose dependant manner. It is therefore recommendable that prolong exposure to dichlorvos through inhalation should be avoided or minimized as this could temper with the normal functions of the haematological parameters.

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Conflict of Interest

The authors have not declared any conflict of interest.

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Lipid Profile, HMG CoA Reductase and Cholesterol Esterase Activities of *P. guajava* and *R. americanum* Aqueous Extract Treated Obesity Prone Rats

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Abstract

The antihyperlipidaemic activities of *P. guajava* and *R. americanum* leaves aqueous extract were studied in obesity prone rats fed high fat feeds and salted water. The effects of the aqueous extracts on lipid profile of rats fed high fat feeds and salted water show that the extracts significantly ($p<0.05$) increased HDL-cholesterol and decreased total cholesterol, triacylglyceride, LDL-cholesterol, VLDL-cholesterol and the atherogenic index of the treated groups when compared to the control. The pancreatic cholesterol esterase and liver HMG COA reductase activities of the extracts treated groups were significantly ($p<0.05$) reduced. However, there was no significant ($p>0.05$) difference between the antihyperlipidaemic activities of the two extracts in the rats. The study supported the antihyperlipidaemic activities of *P. guajava* and *R. americanum* leaves extracts.

Keywords: Lipid Profile, HMG CoA Reductase, Cholesterol Esterase, *P. guajava*, *R. americanum*

Introduction

The prevalence of obesity is markedly rising in modern societies and is of considerable concern given the strong association between obesity and cardiovascular diseases such as hypertension.[1] Experimental evidence demonstrates that excess weight gain increases blood pressure. Conversely, loss of weight in hypertensive patients lowers blood pressure [1]. Studies have also shown that an increase in weight increases the levels of total-cholesterol (TC) and LDL-cholesterol (LDL-C) and triacylglyceride (TG), and decreases HDL-cholesterol (HDL-C).

Endothelial dysfunction has been previously defined in blood vessels from rodent models of diet-induced obesity, which mobilizes lipids against the cells and blood vessels [1]. The association between cholesterol synthesis and increased production of TG-rich lipoproteins in obese individuals has been documented. [2,3] Excessive quantities or improper types of lipid-intake may result in hyperlipidemia which is characterized by an abnormal elevation in one or more of the serum lipids such as TC, LDL-C and TG. Hyperlipidemia is considered to be a major risk factor for cardiovascular diseases including atherosclerosis, myocardial infarction, heart attacks, and cerebrovascular diseases. [4] Today in most of the developed and developing countries, hyperlipidemia and thereby atherosclerosis is the leading cause of cardiac illness and deaths. Hypercholesterolemia and hyper triglyceridemia are independent risk factors that alone or together can accelerate the development of atherosclerosis and progression of atherosclerotic lesions [5].

The effect of blackcurrant extracts and anthocyanins on the lipid profile has been investigated. Varying results indicate positive [6] and negative [7] effects on cholesterol and triglycerides. Polyphenols, anthocyanins from the fruit have been demonstrated to inhibit inflammation mechanisms suspected to be at the origin of heart disease, cancer, microbial infections or neurological disorders like Alzheimer's disease in laboratory experiments. [8,9]

Psidium guajava is a large evergreen shrub or small tree that grows up to 15 m in height. The plant is cultivated from Asia to the west coast of Africa, with varieties originally introduced over the past 300 years from the United States. [10,11] *Psidium guajava* is used to treat many diseases, especially

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hypertension and diarrhoea in the central plateau of Burkina Faso. [12,13] This study was carried out to investigate hypolipidaemic effects of *P. guajava* and *R. americanum* leaves aqueous extract by studying the extracts effects on lipid profile, HMG-CoA reductase and cholesterol esterase of obesity prone rats.

Materials and Methods The Plants and Extraction Procedure

Ribes americanum and *Psidium guajava* leaves were collected from Bayero University campus and authenticated by taxonomist at the Faculty of Science, Bayero University, Kano. The leaves were washed and dried in a fully ventilated environment and away from direct sun light. The dried leaves were ground using a mechanical grinder.

Ribes americanum and *Psidium guajava* leaves aqueous extract were prepared according to Mittal *et al.* [14] and Fernando *et al.* [15] methods. Two hundred grams (200g) of powdered *R. americanum* or *P. guajava* leaves was mixed with one litre of distilled water in a 5 litre beaker and steamed at 65 ºC for one hour then allowed to cool and mixed vigorously. The mixture was filtered using Whatman No. 1 filter paper. The aqueous extract was then concentrated by evaporation at 60 ° C in water bath (OLS 200 water bath and shaker, Grand Instruments, Cambridge). Finally, the total yield of the aqueous extract was determined and the extract stored in air tight containers.

Induction of Hyperlipidemia

A modified method of Boustany-Kari *et al.* [1] was used to induce hyperlipidemia in rats. Fully grown Wister strain rats were exposed to high fat diet formulation for a period of 6 weeks. The diet was formulated by adding 25% animal fat and 5% palm oil to standard feeds (Vital Feeds, Bukuru Jos, Plateau) and body weights (BW) were recorded weekly. Segregation into obesity prone (OP) and obesity resistant (OR) groups was performed based on the weight gained by the rats in the six weeks. Rats with the highest BW gain (upper one-third, weight gain ≥ 66.67%) and the lowest BW gain (lower one-third, weight gain ≤ 33.33 %) were assigned to the OP and OR groups, respectively.

Sample Collection

At the end of the experimentation, the rats were sacrificed by decapitation and blood was collected for further studies. The kidney, liver and the pancreas were also harvested using standard procedures for further analysis.

Preparation of Tissue Homogenate

Tissue homogenate were prepared according Abdel-Wahab method. [16] A known weight of the tissue was washed in ice-cold isotonic saline containing 1 mM EDTA. The tissue was then homogenized separately in phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA using a homogenizer at 4 °C. The crude tissue homogenate was then centrifuged at 8000 rpm for 15 minutes and the supernatant was collected. HMG CoA reductase (HMG CoA and Mevalonate ratio) was assayed in liver homogenate while pancreas homogenate was assayed for cholesterol esterase.

Determination of Lipid Profile of Experimental Animals

The levels of total cholesterol [17] triacylglycerol and HDL-cholesterol [18] were assayed using Randox laboratories assay kits and procedure. The LDL-cholesterol, VLDL-cholesterol and the atherogenic index (AI) were calculated using Friedewald *et al*. [19] methods. LDL Cholesterol = Total Cholesterol – (HDL Cholesterol – VLDL Cholesterol) LDL-cholesterol level in serum was expressed as mg/dl or mmol/L**.** VLDL Cholesterol = Triglycerides/5 VLDL cholesterol level in serum was expressed as mg/dl or mmol/L.

$$\text{Atherogenic Index} = \frac{\text{Total Cholesterol} - \text{HDL Cholesterol}}{\text{HDL Cholesterol}}$$

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Determination of HMG CoA and Mevalonate as an Index of HMG CoA Reductase

HMG CoA was determined by the reaction with hydroxyl-amine at pH 5.5 and subsequent colorimetric measurement of the resulting hydroxamic acid by formation of complexes with ferric salts. Because mevalonate interferes in this estimation at acid pH, alkaline hydroxylamine was used to estimate specifically HMG CoA only. Possible interference by coenzyme A is also minimal when readings were taken at 540 nm [20]. Mevalonate was estimated by reaction with the same reagent, but at pH 2.1. At this pH, the lactone form of mevalonate readily reacts with hydroxylamine to form the hydroxymate. Tissue homogenate, 1ml was mixed with 1 ml of dilute perchloric acid (5ml/100ml) and allowed to stand for 5 minutes and centrifuged at 2000 rpm for 10 minutes. Then 1ml of filterate was added to 0.5ml of freshly prepared 1M alkaline hydroxylamine reagent (hydroxylamine reagent and sodium hydroxide(4.5M) 1:1 v/v), mixed and 1.5ml ferric chloride reagent (5.2g of TCA, 10g ferric chloride in 50 ml of 0.65 M hydrochloric acid and dilute to 100 ml) was added. The contents were shaken vigorously and absorbance read at 540 nm after 10 minutes. About, 1 ml of saline treated in similar manner was used as blank. HMG CoA reductase activity was determined as mevalonte ratio: HMG CoA ratio.

Determination of Pancreatic Cholesterol Esterase

The pancreatic cholesterol esterase was assayed spectrophotometrically [21]. Pancreatic homogenate was mixed with 5.16 mM taurocholic acid and 0.2 mM p-nitro phenyl butyrate in 100mM sodium phosphate buffer, 100 mM NaCl, pH 7.0. The absorbance was read at 405 nm after incubation for 5 minutes at 25 ° C.

Statistical Analysis

The results of the studies was presented as means ± SD and the difference between the means was analysed using statistical soft ware, Grapad-instant, version 3.0.

Results and Discussion

Table 1. Average weight gain of rats fed with standard feeds and high fat feeds (Obesity Resistant and Obesity Prone).

	Weight Gain in Grams					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Normal feeds	6.75±5.56[a]	7.81± 4.33[a]	8.38±5.97[a]	9.63±6.56[a]	10.56±5.44[a]	11. 22±6.22[a]
Obesity resistant	5.96 ± 3.44[a]	7.28±3.98[a]	8.00±4.65[a]	8.82±2.76[a]	10.02±3.54[a]	11.46±4.22[a]
Obesity prone	11.67±6.43[a]	15.90±4.44[b]	18.00±4.23[b]	22.36± 7.75[b]	25.45±6.22[b]	28.58±8.34[b]

Result presented as Mean ± SD. Values with different superscript along a column are significantly different

Table 2. Lipid profile of rats fed with standard feeds and high fat feeds (Obesity Resistant and Obesity Prone).

	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/I	LDL-C (mmol/L)	VLDL (mmol/L)	AI
Standard feeds	2.08±0.43[a]	0.80±0.33[a]	0.68±0.29[a]	1.56±0.21[a]	0.16±0.07[a]	2.05±0.56[a]
Obesity resistant	2.02±0.62[a]	0.72±0.29[a]	0.66±0.37[a]	1.50±0.31[a]	0.14±0.06[a]	2.06±0.61a
Obesity prone	3.26±0.78[b]	1.48±0.56[b]	0.48±0.19[b]	3.08±0.70[b]	0.30±0.11[b]	5.80±1.02[b]

Result presented as mean ± SD. n=5, Values with different superscript along a column are significantly different, TC = total cholesterol, TG = triacylglcerol, HDL-C = high density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol, VLDL = very low density lipoprotein, AI = atherogenic index

Table 3. Lipid profile of rats exposed to standard feeds, high fat feeds and salted water for 21 days

	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L	LDL-C (mmol/L)	VLDL (mmol/L	AI
Standard feeds	2.08±0.43[a]	0.80±0.33[a]	0.68±0.19[a]	1.56±0.21[a]	0.16±0.07[a]	2.05±0.48[a]
Standard feeds-salted water	2.22±0.31[a]	0.76±0.45[a]	0.65±0.18[a]	1.72±0.39[a]	0.15±0.06[a]	2.41±0.72[a]
High fat feeds	3.16±0.42[b]	1.48±0.56[b]	0.48±0.12[b]	2.98±0.67[b]	0.30±0.11[b]	5.58±0.91[c]
High fat feeds-Salted water	3.36±0.48[b]	1.47±0.31[b]	0.42±0.16[b]	3.23±0.65[b]	0.29±0.06[b]	7.00±1.05[d]

Result presented as mean ± SD, n=5. Values with different superscript along a column are significantly different, TC= total cholesterol, TG= triacylglcerol, HDL-C= high density lipoprotein-cholesterol, LDL-C= low density lipoprotein-cholesterol, VLDL= very low density lipoprotein, AI= atherogenic index

Table 4. Lipid profile of rats fed on high fat feeds, salted water and treated with *P. guajava* and *R. americanum* aqueous leaves extract for 21 days

	TC (mmol/L)		TG (mmol/L)		HDL-C (mmol/L)		LDL-C (mmol/L)	
	14days	**21days**	**14days**	**21days**	**14days**	**21days**	**14days**	**21days**
Standard feeds	1.69±0.63[a]	1.78±0.22[a]	0.75±0.34[a]	0.80±0.39[a]	0.74±0.22[a]	0.78±0.29[a]	1.10±0.19[a]	1.16±0.22[a]
Standard feeds + high salt	1.74±0.32[a]	1.84±0.18[a]	0.73±0.21[a]	0.77±0.15[a]	0.61±0.18[a]	0.56±0.11[a]	1.28±0.31[a]	1.39±0.19[a]
High fat feeds	2.59±0.43[b]	2.76±0.27[b]	1.34±0.23[b]	1.48±0.66[b]	0.42±0.18[bc]	0.38±0.13[b]	2.44±0.21[b]	2.88±0.43[b]
High fat + high salt	2.75±0.25[b]	2.82±0.26[b]	1.32±0.19[b]	1.54±0.21[b]	0.38±0.05[b]	0.35±0.09[b]	2.63±0.22[b]	2.78±0.35[b]
High fat feeds + 350mg/kg *P guajava*	2.26±0.51[c]	2.33±0.98[c]	1.18±0.33[b]	1.09±0.34[c]	0.52±0.17[c]	0.57±0.15[c]	1.97±0.19[c]	2.00±0.34[c]
High fat + high salt + 350mg/kg *P guajava*	2.32±0.33[c]	2.28±0.20[c]	1.22±0.13[b]	1.11±0.08[c]	0.49±0.09[c]	0.58±0.05[c]	2.07±0.20[c]	1.92±0.18 [c]
High fat feeds + 350 mg/kg *R. americanum*	2.27±0.66[c]	2.21±0.44[c]	1.08±0.26[b]	1.01±0.16[c]	0.58±0.16[c]	0.64±0.20[c]	1.91±0.22[c]	1.77±0.22[cd]
High fat feeds + salt + 350mg/kg *R. americanum*	2.19±0.12[c]	2.14±0.24[c]	1.12±0.18[b]	1.08±0.16[c]	0.62±0.22[c]	0.64±0.07[c]	1.79±0.22[c]	1.72±0.33[cd]
High fat feed + simvastatin	2.22±0.32[c]	2.00±0.20[c]	1.16±0.42[b]	1.06±0.32[c]	0.61±0.17[c]	0.65±0.12[c]	1.84±0.41[c]	1.56±0.15[d]
High fat feed + salt + + simvastatin	2.26±0.23[c]	2.08±0.21[c]	1.09±0.18[b]	0.98±0.10[c]	0.62±0.08[c]	0.68±0.18[c]	1.86±0.22[c]	1.60±0.18[d]

Result presented as mean ± SD, n=5. Values with different superscript along a column are significantly different, TC= total cholesterol, TG = triacylglcerol, HDL-C = high density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol

Table 5. Effects of *P. guajava* and *R. americanum* Aqueous Leaves Extract on HMG CoA Reductase and Cholesterol Esterase of Rats

	HMG COA/ Mevalonate ratio	Cholesterol Esterase
Standard feeds	3.98 ± 0.25^{ad}	765.50 ± 9.25^{a}
Standard feed + salt	3.85 ± 0.34^{ac}	730.00 ± 13.40^{b}
High fat feeds	3.05 ± 0.18^{b}	856.66 ± 9.56^{c}
High fat + salt	3.09 ± 0.21^{b}	860.46 ± 7.45^{c}
High fat + 350mg/kg P. guajava	3.51 ± 0.24^{c}	815.40 ± 11.30^{d}
High fat + 350mg/kg R. americanum	3.65 ± 0.38^{cd}	810.20 ± 8.14^{d}
High fat feeds + salt +350mg/kg P. guajava	3.89 ± 0.28^{cd}	780.75 ± 15.62^{e}
High fat feeds + salt + 350mg/kg R. americanum	3.90 ± 0.48^{cd}	800.90 ± 11.22^{f}

Result presented as mean ± SD, n=5. Values with different superscript along a column are significantly different at ($p \leq 0.05$), HMG CoA= Hydroxy methyl glutharyl coenzyme A

The weight gain per week of rats fed on standard feeds and high fat feeds and the subsequent segregation of the rats, into obesity prone and obesity resistant groups (Table 1) showed that, there was no significant ($p>0.05$) difference in the weight gain of the rats exposed to standard feeds and the obesity resistant rats. However, significantly ($p<0.05$) higher weight gain was observed in the obesity prone groups starting from week 2. The total weight gain of the rats exposed to high fat feeds (obesity prone) was also significantly ($p< 0.01$) higher than the total weight gained of the rats exposed to standard feeds and obesity resistant groups. This finding is in agreement with previous studies [22]. Rats fed on moderately high fat (MHF) diet were segregated into obesity prone (OP) and obesity resistant (OR), with considerable differences in their body weight (BW) and adiposity [22]. The increase in body weight after hyperlipidemia is generally known and it can be used as one of the aspect of the animal models to develop anti obesity agents similar to those of previous study [1,23].

The result for the lipid profile of rats fed on standard feeds and high fat feeds (obesity resistant and obesity prone) (Table 2) showed that, there was no significant ($p>0.05$) difference in the plasma levels of total cholesterol, triacylglycerides, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol and the atherogenic index of the rats exposed to standard feeds and the obesity resistant rats. However, significantly ($p<0.05$) higher plasma levels of total cholesterol, triacylglycerides, LDL-cholesterol, VLDL-cholesterol and the atherogenic index were observed in the obesity prone groups when compared with the rats exposed to standard feeds and the obesity resistant groups. Significant reduction ($p<0.05$) in HDL-cholesterol was also observed in the obesity prone rats as compared to the standard feeds and obesity resistant groups. These results confirm previous findings that weight is associated with increase in the levels of serum lipids and lipoproteins. Dattilo and Kris-Etherton [24] observed that weight loss subsequent to 10 week intervention program had a favourable effect on BMI and plasma lipid variables. Madhu *et al.* [25] reported a mean weight loss of 4.4 kg and correlated with plasma triglycerides (TG), total, and LDL cholesterol (LDL-C) were reduced by 31.8, 9.9, and 11.9%, respectively.

The lipid profile of rats fed on standard feeds, high fat feeds and salted water (Table 3) showed that, there was no significant ($p>0.05$) difference in the plasma level of total cholesterol, triacylglycerol, HDL-cholesterol, LDL-cholesterol, and VLDL-cholesterol of the group fed on standard feed and standard feeds and salted water. There was also, no significant ($p>0.05$) difference in the plasma level of total cholesterol, triacylglyceride, LDL-cholesterol,

and VLDL-cholesterol of the groups fed on high fat feeds only and high fat feeds-salted water and there was significant ($p<0.05$) difference in the HDL-cholesterol and atherogenic index of the two groups. Significantly ($p<0.05$) higher level of total cholesterol, TG, LDL-cholesterol, VLDL-cholesterol and atherogenic index were observed in the groups fed on high fat feeds and high fat feeds-salted water, when compared to standard feeds and standard feeds-salted water group. The HDL-cholesterol level of the high fat feeds-salted water group was significantly ($p<0.05$) less than that of the other groups. The result of the study showed that increased salt intake increased total cholesterol, LDL cholesterol, VLDL cholesterol and atherogenic index, however, the increase was not statistically significant. However, the combine effects of high fat and salt had significantly ($p<0.05$) increased the lipid parameters.

In a world health organization review on the effects of reduced sodium intake on blood pressure, renal function, blood lipids and other potential adverse effects, total cholesterol, reduced sodium intake relative to usual sodium intake had no effect on total cholesterol. [26, 27, 28, 29] However, reduced sodium intake relative to usual sodium intake reduced HDL cholesterol concentration by 0.01 mmol/L, with borderline statistical significance ($p< 0.05$). [27 29] Harsha *et al.,* [28] Gates *et al.,* [29] and Mc-Carron *et al.* [30] reported that reduction in sodium intake had no affect on LDL cholesterol while Sciarrone *et al.* [31] reported significant reduction in LDL cholesterol with reduced salt intake. Studies targeting individuals with hypertension, reported a decrease in triglyceride concentration with decrease in salt intake. [27, 28, 29]

The lipid profile of rats fed on high fat feeds, salted water and treated with *Psidium guajava* or *Ribes americanum* leaves aqueous extract for 21 days (Table 4) showed that there was no significant ($p>0.05$) decrease in the total cholesterol, triacylglycerides and LDL-cholesterol levels of the treated groups and the group that was fed on high fat feeds by day 14. However, the plasma total cholesterol and TG levels of the treated groups was significantly ($p<0.05$) lower than the groups that were fed on high fat feeds and high fat feeds-salted water by day 21. The plasma LDL-cholesterol of the treated groups was significantly ($p<0.05$) lower than the high fat only and high fat -salted water group by day 14 and 21. There was no significant ($p<0.05$) difference in the plasma level of all the lipid parameters measured between the *P. guajava* and *R. americanum* aqueous extract treated groups. However, there was significant ($p<0.05$) difference between the LDL-cholesterol level of *P. guajava* and the standard drug used (Simvastatin®) and there was no significant ($p>0.05$) difference between that of the drug and *R. americanum* aqueous extract. The study showed that significant antihyperlipidaemic activity of both the plants extract was observed at 21 days.

The antihyperlipidaemic activity of *R. americanum* is supported by reports which demonstrated the influence of blackcurrants on serum lipid profile: increased HDL-cholesterol level and decreased triglyceride and total cholesterol [32], as well as reduced serum inflammatory markers. Frank *et al.* [33] and Tahvonen *et al.* [35] reported positive effects of blackcurrant extracts on cholesterol and triglycerides. The findings on *P. guajava* were also supported by Shubhangi *et al.* [23] who demonstrated that, the aqueous and methanolic extract of leaves of *P. guajava* exerted a hypolipidaemic activity in laboratory animals.

The effects of *P. guajava* and *R. americanum* leaves aqueous extract on liver HMG CoA reductase, measured as HMG CoA and mevalonate ratio and pancreatic cholesterol esterase of rats fed on high fat feeds and salted water (Table 5), show that there was no significant ($p>0.05$) difference in HMG CoA reductase activity between the standard feed and standard feed salted-water group. Likewise, there was no significant ($p>0.05$) difference in HMG CoA reductase activity between the high fat and high fat-salted water groups. However, there was significant ($p< 0.05$) increase in HMG CoA reductase activity of the high fat only and high fat salted water groups, when compared to the standard feed only and standard feed salted water groups.

There was significant ($p<0.05$) decrease in HMG CoA reductase activity of the groups treated with *P. guajava* and *R. americanum* aqueous extracts when compared to the high fat and high fat salted water groups. However, there was no significant difference in HMG CoA reductase activity between the groups that were treated with *P. guajava* and *R.*

americanum aqueous extract. The effects of *P. guajava* and *R. americanum* on cholesterol esterase showed that there was significant ($p<0.05$) decrease in cholesterol esterase activity of the groups treated with the extracts when compared to the high fat and high fat-salted water group. There was also significant ($p<0.05$) increase in the high fat and high fat-salted water groups when compared to the standard feeds and standard feeds-salted water groups. There was also significant decrease in the cholesterol esterase activity of the *R. americanum* extract when compared with the *P. guajava* extract treated group.

These observations were supported by previous literature such as Chan *et al.* [34] and Simonen *et al.* [36] The association between cholesterol synthesis and increased production of TG-rich lipoproteins in obese individuals has been documented. [35] Decreases in plasma concentrations of intermediate metabolites involved in the biosynthetic pathway of cholesterol were reported in subjects who lost substantial amounts of weight, [36] suggesting that reductions in cholesterol synthesis may contribute to the lowering of plasma cholesterol.

Conclusion

The outcome of this research demonstrate the potentials of *P. guajava* and *R. americanum* leaves extracts as antihyperlipidaemic agents and have potent antioxidant potentials. Hence the extract may ameliorate some cardiovascular ailments associated with free radicals (oxidants) and hyperlipidemia. However, further studies is required to fully establish the biochemical bases of the observed activities.

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Effect of Sprouting on the Oil Physicochemical Properties and Fatty Acids Composition of Two Bambara Groundnut (*Vigna subterranea*) Landraces

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Abstract

Bambara groundnut is a legume crop which has long been recognised as a protein-rich and drought tolerant crop that is widely used in sub-Saharan Africa. This study evaluated the effect of sprouting on the oil physicochemical properties and fatty acid composition of two Bambara groundnut landraces. Analysis of the physicochemical properties was carried out using standard techniques. The fatty acid profile was evaluated using GC-MS. Results revealed that oils of the two landraces contained both saturated and unsaturated fatty acids that vary in number and position of double bonds and chain lengths. Changes in the fatty acids composition were observed in both landraces following sprouting. Differences were also observed in the physicochemical properties of sprouted Bambara groundnut. Results indicated that unprocessed Bambara groundnut landraces had higher oils content (raw Niger cream 7.72 and raw Yobe black 6.96) compared to the sprouted samples. Significant differences ($p<0.05$) were observed in the iodine value and saponification value of the sprouted samples when compared to the raw sample.

***Keywords**:* Sprouting, Fatty acids composition, Physicochemical properties.

Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a type of legume of the family *Fabaceae*. It is an indigenous food crop in semi-arid Africa [1] and is widely produced, especially in the northern states of Nigeria. [2]. In Nigeria, Bambara groundnut is eaten by boiling fresh seeds, while dried seeds are processed by roasting or by soaking followed by cooking or processing into other local cuisines. According to Lacroix *et al.* [3], it is the third most important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) in Africa. Bambara groundnut plays an essential role as a staple food in meeting nutritional requirements. [4] Like most legumes, it is a rich source of protein which serves as an essential source of food security for less privileged households. The mature seeds are a rich source of protein and carbohydrate, but in comparison with groundnut, the lipid content is low. [5]

Bambara groundnut oil is reported to contain both saturated and unsaturated fatty acids. [6, 7] These fatty acids play an essential role in human health promotion. For instance, linoleic acid produces hormone like substances that regulates a wide range of functions such as; blood clotting, blood pressure control, inflammation response to injury infections and blood lipid-level. [8, 9] According to findings by Adelowo-Imeokparia and Ojo [10], Kim *et al.* [11] and Olanipekun *et al.*[9], processing methods such as sprouting and fermentation improves the nutritional properties of foods. This research is therefore aimed at assessing how sprouting affects the physicochemical properties and fatty acids composition of oil extracted from seeds of two Bambara groundnut landraces.

Materials and Methods

Seeds Sample

Seeds of two Bambara groundnut landraces were purchased locally from Gwadabe market Minna, Niger State and Sunday market, Yobe State, Nigeria. Based on the place of collection and colour, the seeds were respectively designated as Niger cream and Yobe black. The seeds were identified at the Department of Plant Biology, Bayero University, Kano with a voucher number '0509'. For each landrace, a portion of the seeds was used for sprouting while the unprocessed seeds were used as control in the experiments.

Sprouting Procedure

Sprouting was carried out according to Shah *et al.* [12] with some modifications.

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The seeds were soaked by submerging in distilled water in glass containers for 4 hours (Niger cream) and 8 hours (Yobe black) at room temperature. The seeds were removed at 72 hours and 92 hours respectively for Niger cream and Yobe black landraces. The seeds were dried in an oven at 80^{o}C for 6 hours. The dried seeds were then milled into flour and stored in glass containers prior to analysis.

Extraction of Oil

A portion of sample (100g) was placed into a porous cellulose thimble. The thimble is placed in an extraction chamber suspended above the flask containing 300ml petroleum ether and below a condenser. The flask was heated at 60^{o}C; when the solvent (petroleum ether) was boiling the vapour rises through the vertical tube into the condenser at the top where it is converted into liquid. This was allowed to continue for six hours. The solvent in the flask was then evaporated leaving behind the oil. [13]

Percentage oil yield

The percentage oil yield was calculated by from the following relation. [14].

% Oil yield =

Physicochemical Analysis

Iodine Value

Iodine value was determined according to the method of AOAC. [15]. Oil (0.1g) was weighed into a 250ml flask and 15ml of carbon tetrachloride added and swirled to ensure that the sample completely dissolved. Wij's solution (12.5ml) was then pipetted into the flask containing the sample. The flask was stopped and swirled to ensure complete mixing. The sample was then placed in the dark for 30 minutes at room temperature. The flask was removed from storage and 10ml of 10% KI solution added, followed by75ml of distilled water. The mixture was titrated with 0.1N NaS_2O_3 solution until the yellow colour had disappeared. A blank determination was conducted simultaneously. The iodine value was calculated using the equation;

Iodine value = X 100

1ml 0.1N NaS_2O_3 = 0.127g I_2

Saponification Value

Saponification value was determined by tritrimetric method of AOAC. [15] A portion of the of oil (0.26g) was weighed into a flask and 12.5ml of alcoholic KOH pipetted and allowed to drain for about 1 minute into the mixture. A blank was prepared and determined simultaneously with the sample. It was then covered with cotton wool and was placed in a water bath.

The mixture in the flask was allowed to boil gently and steadily for 45minutes for complete saponification. The condenser was disconnected and 1ml of phenolphthalein indicator added to the content of the flask. The solution was titrated with 0.5N HCl until the pink colour disappeared. The saponification number was calculated using the equation below.

Saponification value =

Where 56.1 is equivalent weight of KOH

Acid Value

The oil (0.5g) was dissolved in 25ml ethanol, two drops of phenolphthalein indicator added and titrated to pink end point (which persisted for 15 minutes) with 0.1N KOH. [16] Acid value was calculated from the relation:

Acid value =

Where 56.1 is the equivalent weight of KOH.

FreeFatty Acid

The free fatty acid content was obtained by titration. A warmed portion of the oil (1g) was titrated with 0.14M KOH solution. Phenolphthalein was used as an indicator and constant shaking was ensured until a regular end point was achieved. [16] The free fatty acid content was obtained from the relation:

% Free Fatty Acid = 0

GC-MS Analysis

The analysis of fatty acids in the oil samples was done according to AOAC. [17] The method was followed to esterify the lipid extract. Fatty acid methyl esters of the sample oils were prepared by heating with methanolic NaOH first and then with BF3 methanol for esterification; 5 ml n-heptane was added to recover the methyl esters in organic phase. Saturated NaCl solution was then added to the mixture and the aqueous and organic layers separated using a profil separating funnel. The upper n-heptane phase was pipetted out and stored in 10 ml

glass vials and stored under sub-zero temperature till GC-MS analysis.

The gas chromatograph analysis of methylated fatty acids was performed on a Shimadzu QP2010 quadrupole Gas Chromatography Mass Spectrometer (GC-MS) instrument equipped with a carbowax capillary column (30 m × 0.25 mm ID;0.25im film thickness, Intercut DB5MS, Japan). Helium was used as the carrier gas and 1µl of the sample was injected into the capillary column. Injector and detector temperatures were set at 280°C. Injection was performed in split mode (1:30). The column temperature was programmed initially at 50°C for 1 minute and then set to increase at a rate of 5°C per minute to a final temperature of 280°C. Fatty acid methyl esters were separated at constant pressure (100 kPa) and peaks were identified by comparing the mass spectra with a spectral database. The identification of compounds was based on the comparisons of their mass spectra and retention time with NIST Library 2014.

Statistical Analysis

Data obtained were statistically analysed using Independent sample T-test with SPSS version 20.0 statistical package. Results were expressed as mean ±standard deviation of three replicates.

Results

The physicochemical properties of both raw and processed oils extracted from Bambara groundnut are presented in Table 1. Lower oil yields were seen in sprouted samples in both landraces studied. A significant difference ($p<0.05$) in iodine value was observed between raw and the sprouted samples. Similarly, the free fatty acid value of sprouted Niger cream was significantly lower ($p<0.05$) than that of the raw landrace, but the reverse was observed between raw and sprouted Yobe black landrace which happened to be statistically insignificant ($p>0.05$).The results of saponification value in the above table showed a decrease after sprouting in all the samples studied. The decreases in both cases were statistically significant ($p<0.05$)

Table 1: Physicochemical properties of oil from raw and sprouted bambara groundnut landraces.

Parameter	Raw Niger Cream	Sprouted Niger Cream	Sprouted Niger Cream	Sprouted Yobe Black
Yield (%)	7.72	5.94	6.96	4.87
Colour	light yellow	light yellow	light yellow	light yellow
Acid value	0.40 ±0.14[a]	0.46 ±0.17[a]	0.39±0.71[a]	0.45± 0.11[a]
Free fatty acid (mg g-1)	1.85± 0.07[d]	0.83± 0.02[b]	0.65± 0.07[a]	0.50± 0.57[a]
Iodine value (mg iodine g-1)	98.35 ±2.48[a]	124.60 ±4.24[b]	116.00 ±1.69[c]	121.2 ±1.98[d]
Saponification value (mg KOH g-1)	161.00 ±1.42[a]	127.00 ±1.41[b]	154.15± 4.03[c]	132.50± 2.12[d]

Values are mean ± standard deviation of duplicate determinations.
Means with different superscript along each row are significantly different ($p<0.05$).

From the analysis of fatty acid content of the extracted oils (Table 2), differences were observed in the types of fatty acids following sprouting in both landraces. With respect to raw samples, Niger cream contained margaric acid (saturated fatty acid) which was absent in raw Yobe black landrace. Stearic acid however was observed to be the prominent saturated fatty acid in raw Yobe black landrace and butyric acid was found common in both landraces following sprouting.

Table 2: Fatty acids composition of extracted oil from raw and sprouted bambara groundnut landraces.

RT(min)	Fatty acid	Common name	Formular	Mol.Weight	Area%
Raw Niger Cream					
50.295	Hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$	256	0.13
52.640	2,9-Hexadecanoic acid	Palmiteloic acid	$C_{16}H_{30}O_2$	254	1.20
56.120	2,9,17-octadecanoic acid	Oleic acid	$C_{18}H_{34}O_2$	282	0.77
58.282	9,12-octadecadienoic acid	Linoleic acid	$C_{18}H_{34}O_2$	294	16.20
07.066	Nonanoic acid	Pelargenic acid	$C_9H_{18}O_2$	158	00.09
87.590	Heptadecanoic acid	Margaric acid	$C_{17}H_{34}O_2$	270	77.16
Sprouted Niger Cream					
11.755	Butanoic acid	Butyric acid	C_3H_7COOH	88	0.09
23.992	Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$	200	0.12
35.568	Pentadecanoic acid	Pentadecyclic acid	$C_{15}H_{30}O_2$	242	0.09
41.068	Heptadecanoic acid	Margaric acid	$C_{17}H_{34}O_2$	270	0.28
41.171	Decanoic acid	Claprylic acid	$C_7H_{16}O_2$	144	0.09
42.30	Heptacosanoic acid	Heptacosylic acid	$C_{27}H_{54}O_2$	410	0.47
50.259	Hexadecanoic acid	Palmitic acid	$C_{16}H_3O_2$	256	0.47
58.282	9,12-Octadecenoic acid	Linoleic acid	$C_{18}H_{34}O_2$	256	87.76
Raw Yobe Black					
56.210	Octadecanoic acid	Stearic acid	$C_{17}H_{36}O_2$	284	1.40
59.211	9,12-Octadecanoic acid	Linoleic acid	$C_{18}H_{34}O2$	294	83.50
33.665	Nonadecanoic acid	Nondicyclic acid	$C_{20}H_{34}O_2$	298	0.23
35.568	Hexanoic acid	Caproic acid	$C_6H_{12}O_2$	116	0.13
23.992	Tridecanoic acid	Tridecyclic acid	$C_{13}H_{26}O$	214	0.22
10.876	Decanoic acid	Capric acid	$C_{10}H_{20}O_2$	172	0.26
Sprouted Yobe black					
10.876	Decanoic acid	Capric acid	$C_{10}H_{20}O_2$	172	0.27
11.755	Butanoic acid	Butyric acid	C_3H_7COOH	88	0.27
15.419	Undecanoic acid	Undecyclic acid	$C_{11}H_{22}O_2$	186	0.33
41.063	Heptadecanoic acid	Margaric acid	$C_{17}H_{34}O_2$	270	0.32
50.259	Hexadecanoic acid	Palmitic acid	$CH_3(CH_2)_{14}COOH$	256	0.30
58.502	9,12-Octadecenoic acid	Linoleic acid	$C_{18}(CH_2)_{34}COOH$	294	85.61

Discussion

Raw and sprouted Yobe black produced brown yellow oils, while raw and sprouted Niger cream gave a brown yellow and light yellow respectively. Variation in the oil colour seen in Niger cream landrace could be due to the effect of processing. Decrease in oil yield as observed in both landraces after sprouting might be due to leaching out of fat content during steeping, [18] or could be as a result of breakdown of glycerol and fatty acids during hydrolysis for the production of energy. [19] Moreover, the difference in yields within raw samples could be attributed to the intra-landrace variation. [20] Oil yield obtained in the present study is quite low when compared to other plants like cotton (24%), groundnut (46%) [21] and soybean (18%). [22] However, these results are in line with findings on some underutilised plant seed oil such as *Pearsea americana* (10.8%) [23] and *Detarium microcarpum* (7.42 %). [24]

Results in this study indicated higher iodine values in sprouted samples, implying a higher degree of unsaturation [25, 26] as compared to the raw samples. This also implies that the oils obtained from sprouted seeds are better choices as non-drying oil than oil from raw samples. [27] The lower the iodine

value, the greater the oxidative storage stability.Oils with higher iodine values may also be found useful as raw materials in the manufacture of many vegetable oil-based products. [28]

Acid value is a measure of the extent to which the triacylglycerides in oil have been decomposed; [29] it is often used as a general indication of the condition of edibility of the oil. Low level of acidity is an indicator of suitable quality of oil. [30] The low acid values obtained in both landraces can compare favourably with Bambara groundnut seed oil 0.82mg KOH/g reported by Aremu *et al.* [27] According to FAO/WHO, [31] acid value of oil suitable for edible purposes should not exceed 4 mgKOH/g of oil. The acid value of the seed oils studied fell within allowable limits for edible oils. The free fatty acids values obtained shows that sprouting has little effect on the free fatty acids composition, thus both oils are fit for consumption. Since free fatty acids are known to stimulate chemical oxidation to form off flavour component, [32] high concentrations of free fatty acids are undesirable in vegetable oils.

The saponification values obtained in this work decreased upon sprouting. However, high saponification value of the unprocessed samples indicates the presence of high proportions of short fatty acids which can be regarded as edible oils. [33] However the values obtained in the present study are lower than reported for some common oils like palm oil (196-205 mgKOH/g), groundnut oil (188-196 mgKOH/g) and corn oil (187-196 mgKOH/g). [34] Findings also indicate the presence of both saturated and unsaturated fatty acids that differ in position of double bonds and chain length. Linoleic acid was found to occur predominantly in raw Yobe black, sprouted Yobe black and sprouted Niger cream landraces. This essential fatty acid is used to produce hormone-like substances that regulate a wide range of biochemical functions. [35, 36] It is also known to increase the levels of high density lipoprotein (HDL) and decrease the levels of low density lipoprotein (LDL). [37]

The total unsaturated fatty acid was seen to be higher than the total saturated fatty acids in all the oils samples except in raw Niger cream sample, where margaric acid accounts for about 77% of the fatty acids. Plant oils contain mostly unsaturated fatty acid ranging from 73-94% of total lipids. [38, 11, 39] The prevalence of unsaturated fatty acids in this study is considered to be a plus from the nutritional point of view. The unsaturated fatty acids present (linoleic, oleic and palmiteloic acid) can confer health benefits like decreasing the risk of breast cancer, help in losing weight and relieve the pain of rheumatoid arthritis. [40] They also help the body absorb nutrients, especially the fat-soluble vitamins needed for normal growth and development in children, and keep brains and central nervous systems healthy. [37] Many of the oils seen in the present study have uses in, especially the food, and other industries. Lauric acid is a useful component in treatment for acne. [41] Capric acid is used in the manufacture of esters for artificial fruit flavours and pharmaceuticals. It is also used as an intermediate in chemical synthesis. [42] Butyric acid esters are used as fragrant and flavouring agents in beverages, food and cosmetic industries. [43] Linoleic acid is useful in beauty products industry because of its beneficial properties on the skin. [35, 36]

Following sprouting, it was observed that the concentration of saturated fatty acids generally decreased; in Niger cream and Yobe black pelagonic acid and stearic acid were not detected. However, butyric acid, a short chain saturated fatty, appeared after sprouting in both landraces. Nutritionally, butyric acid has been reported to have many beneficial effects, for example, it is thought to play several beneficial roles in the gastrointestinal tract. [44] In unprocessed samples, only Niger cream was observed to contain the unsaturated fatty acid oleic acid which was not detected in g sprouted samples whereas linoleic acid as investigated in both sprouted landraces increased during the processing. Similarly, sprouting has been reported to change fatty acid composition. [11] Changes in fatty acid composition following sprouting might be due to the increased metabolic activities in the seeds occurring during germination. [45].

Conclusion

Based on the findings of the present study, it can be concluded that sprouting affect the fatty acid composition of Bambara groundnut. The changes in fatty acid composition following sprouting might increase the nutritional quality of Bambara groundnut. The studied Bambara groundnut oils might also have medical and food uses.

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Detection of Penicillin Binding Protein among Clinical Isolates of Staphylococci for Evaluation of Multidrug-Resistant Resistance in Kano, Nigeria.

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Abstract

Staphylococci are one of the most common causes of human disease and their impact is enhanced by the development of antibiotic resistance, most notably methicillin-resistant *S. aureus* (MRSA) that is resistant to virtually all β-lactam antibiotics. The mechanism of such resistance in staphylococci is based on the production of an additional low affinity penicillin – binding protein (PBP2a/2′), which is encoded by the *mec*A gene. This study was aimed at detecting penicillin binding protein antigen among clinical isolates of staphylococci for evaluation of multidrug resistance. Three hundred and two (302) isolates of staphylococci were recovered from various sample sites and identified by standard bacteriological techniques at Aminu Kano Teaching Hospital, Kano. Methicillin-resistant *S. aureus* screening was carried out using disk diffusion method with cefoxitin(30µg) and oxacillin(1µg) discs which was confirmed with PBP2a latex agglutination test. The results were read and interpreted according to the manufacturer's instructions**.** One hundred and eighty four (60.93%) clinical samples were recovered from blood culture followed by wound swabs 66 (21.85%), the least were from semen and high vaginal swabs with 3 (1.0 %) each respectively. Eighty two isolates 82 (27.2%) were found to be MRSA with oxacillin, 65 (21.5%) with cefoxitin disk diffusion method of which 98.0% were positive for latex agglutination test. The PBP2a latex agglutination for MRSA screen test is rapid, easy to perform and reliable test for the detection of methicillin resistance in *S. aureus***.** The study showed that ciprofloxacin and clindamycin are best drugs for treating staphylococcal infections with resistant rate of only 8.9% and 9.6% respectively. All effort must also be made for an establishment of antimicrobial policy for successful implementation process that will be aimed at reducing the pool of antibiotic-resistant organisms like MRSA.

Keywords: Penicillin Binding Protein, isolates, Staphylococci, resistance, sensitivity

Introduction

Staphylococci are among the most common causes of human disease. Most staphylococci colonize the skin and mucous membranes of people without disease.[1] However, given the right conditions, staphylococci can cause superficial and systemic infections. Its impact is enhanced by the development of antibiotic resistance, most notably methicillin-resistant *S. aureus* (MRSA) that is resistant to virtually all β-lactam antibiotics.[2] Consequential effect of MRSA infection had resulted in, prolonged hospitalization, increased in medical expenses, and difficulty in patient treatment and management. The evolution of such resistance does not cause the organism to be more intrinsically virulent than strains of staphylococci that have no antibiotic resistance, but resistance does make MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous. MRSA began as a hospital-acquired infection, but has developed limited endemic status and is now sometimes community-acquired.[3]

The mechanism of resistance in staphylococci is based on the production of an additional low affinity penicillin – binding protein (PBP2a/PBP2′), encoded by the *mec*A gene.[4, 5, 6] Strain encodes PBP2a/PBP2′, which differs from other PBPs as its active site does not bind to β-lactam antibiotics. As such, PBP2a/PBP2′ can continue to catalyze the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of antibiotics.[1, 7]

Although methicillin is no longer produced, the name MRSA has persisted and can be regarded as referring to resistance to virtually all β-lactam

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antibiotics. Susceptibility testing now typically uses oxacillin or cefoxitin.

Penicillin-binding proteins (PBPs) are a group of proteins that are characterized by their affinity for and binding of penicillin.They are a normal constituent of many bacteria, the name just reflects the way by which the protein was discovered. They are all involved in the final stages of the synthesis of peptidoglycan, which is the major component of bacterial cell wall. Penicillin-binding proteins bind β - lactam antibiotics because they are similar in chemical structure to the modular pieces that form the peptidoglycan. When they bind to penicillin, the β-lactam amide bond is ruptured to form a covalent bond with the catalytic serine residue at the PBPs active site.[8]

Accurate detection of methicillin resistant staphylococci in clinical microbiology practice is very important both for approprite treatment of individual patients and for surveillance of multidrug resistance staphylococci. Although some studies on MRSA were done in Kano, Nigeria, however phenotypic studies alone are not enouph to give a true picture of MRSA but detection of PBP2a. The work was aimed at detecting the penicillin binding proteins 2a/2′ (PBP2a/PBP2′) among staphylococci isolates from various clinical samples for evaluation of multidrug resistance and antimicrobial sensitivity pattern in Kano, Nigeria.

Materials and Methods

Study Area

The study was carried out at Medical Microbiology Laboratory of Aminu Kano Teaching Hospital located in Kano metropolis. Ethical permission was obtained from the research and ethical committee of the hospital before the commencement of the study.

Sample Size The sample size of the study was define by an epidemiological formula put forward by [9] thus:

$$N = \frac{Z^2 \times P(1-P)}{d^2}$$

Where: n= number of samples

Z = statistic for level of confidence at 95% = 1.96

P = prevalence from recent past study = 25.0% (0.25) [16]

d = allowable error of 5% (0.05)

q = 1 – P $N = \frac{1.96^2 \times 0.25(1-0.25)}{0.05^2}$ N=288.12

(**Attri**tion 5% of 288) = 14.45 ≈ 14. Therefore. 288 + 14 = 302.

However, in order to improve on the quality of the work, this sample size was rounded to 302.

Microbiological Analysis and Identification

Sample collection and processing

A total of three hundred and two (302) non duplicate samples of Staphylococcal isolates were collected from various clinical isolates in Medical Microbiology Laboratory of Aminu Kano Teaching Hospital and subcultured onto chocolate, MacConkey and mannitol salt agar.

Media Preparation

All the media were prepared according to the manufacturer's instruction.

Gram Staining Gram staining of the isolated colonies was carried out to identify Gram reaction of the isolates as described by. [10]

Biochemical tests Staphylococcal isolates from various clinical samples were identified and confirmed using standard bacteriological procedures. [10] *Staphylococcus aureus* (ATCC 25923) was used as control strain.

Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci.[10]

Coagulase test

This test is used to identify *Staphylococcus aureus* which produces the enzyme coagulase.[10]

Heamolysis Activity

Heamolysin activity of *Staphylococcus aureus* isolates was detected using blood agar. A Blood Agar Base (Oxoid, UK) was autoclaved at 121°C for 15minutes. While the medium was still molten at 45 – 50°C, sterile sheep blood (17% defribrinated) was added and mixed. About 20ml volumes was poured in to petri dishes and dried off surface moisture. A colony of the *Staphylococcus* under test was touched with an inoculating loop and was streak – inoculated on the plate and incubated at

37°C for 24 hours. Plates were checked for heamolysis.

Lactose fermentation

Macconkey agar was prepared according to the manufactures direction. The isolate was subcultured on the medium incubated for 24hrs at 37°C . The plate was observed for fermentation of lactose by the isolates after over night incubation. [10]

Mannitol fermentation

Mannitol salt agar was prepared according to the manufactures direction. The isolate was subcultured on the medium incubated for 24hrs at 37°C . The plate was observed for fermentation of mannitol by the isolates after over night incubation.[10]

Screening for MRSA

Preparation of turbidity standard (0.5 MacFarland Standard)

Sulphuric sulphate (1% v/v) standard suspention was used as turbidiy standard which was prepared in accordordance with the method of Cheesbrough. [11] One percent (1%) solution of sulphiric acid was prepared by adding 1ml of concentrated sulfuric acid (H_2SO_4) in 99 ml of distilled water. One percent (1%) weight per volume solution of barium chloride in 1000ml distilled water. Exert 0.6ml of barium chloride solution will combined with 99.4ml sulphiric acid to yield 1.0% w/v sulphiric sulphate suspension. The turbid solution formed was transferred into test tube for comparison, as standard which match with 0.5 MacFarland standard.[11]

Cefoxitin disc diffusion

Cefoxitin disk (30µg) susceptibility testing was performed according to Clinical Laboratory Standards Institute. [12] Briefly a bacteria suspension adjusted to 0.5 MacFarland was inoculated onto Mueller – Hinton agar by streaking using swab stick. A filter paper disk containing 30µg Cefoxitin was placed on the inoculated Mueller – Hinton agar. All plates were incubated at 35°C for 24 hours and the zones of inhibition were measured.

Oxacillin Disc Diffusion

Oxacillin disk (1µg) susceptibility testing was performed according to Clinical Laboratory Standards Institute. [12] Briefly a bacteria suspension adjusting to 0.5 MacFarland was inoculated onto Mueller – Hinton agar by streaking using swab stick. A filter paper disk containing 1µg oxacillin was placed on the inoculated Mueller – Hinton agar. All plates were incubated at 35°C for 24 hours and the zones of inhibition were measured.

Detection of PBP2a/PBP2' The test was performrd according to the procedure adopted in previous works. [13, 14, 15]

Extraction of PBP2a from Staphylococcal Colonies

The isolates were subcultured onto Mueller Hinton agar and incubated at 35°C for 18hours to obtain fresh growth. To extract PBP2a from the colonies, a loopful of cells was suspended in 4 drops of extraction reagent 1 solution (0.2M NaOH)] and used in the test proper. The suspension containing the extract was placed in boiling water for 3 minutes and then allowed to cool to room temperature for about 10minutes, 1 drop of extraction reagent 2 [0.2N (HCl)] was added to the mixture and mixed well. The suspension was then centrifuged at 1,500 rpm for 5 minutes.[14]

PBP2a/PBP2' Test Proper

This was done according to the manufacturers instruction. Result for the test and control reaction were recorded at which agglutination was visible by eye.

Reading and Interpretation of Latex Agglutination Result

The reasult was interpreted according to manufacturer's guideline and also in accordance with the procedure by Kumurya *et al.* [14]

Data Analysis

The data generated in this study was analysed using the Statistical Package for Social Sciences (SPSS) for windows version 20.0 used for statistical analysis and data interpretation. The values were expressed as means and percentages as in descriptive statistic.

Results

For the total 302 of the clinical isolates, 184 (60.93%) were recovered from blood culture followed by 66 (21.85%) wound swab, and the least were from semen and high vaginal swabs with 3 (0.99%) each respectively (Table 1). All the 302 (100%) isolates were Gram positive cocci in pair and clusters, lactose fermenters and positive for catalase

test. Out of the 302 clinical isolates, 215 (71.2%) were positive for slide and tube coagulase test, 218 (72.2%) mannitol fermenters, and 84 (27.8%) did not ferment mannitol. Forty eight (15.9%) and 254 (84.1%) were beta and gamma heamolytic organisms respectively (Table 2). The result of the methicillin resistance screening test showed that 202 (66.9%) isolates were sensitive, 18 (6.0%) intermediate and 82 (27.2%) resistant to oxacillin. Two hundred and thirty seven (78.5%) were sensitive to cefoxitin while 65 (21.5%) isolates were resistant to the cefoxitin disc (Table 3). Of the entire isolates tested with oxacillin (1µg) and cefoxitin (30µg) only 58 (19.2%) and 50 (16.56%) of the coagulase positive *S. aureus* isolates were resistant to the two drugs respectively, while 24 (7.9%) and 15 (5.0%) of the coagulase negative *S. aurues* were resistant to oxacillin and cefoxitin respectively (Table 4).

Methicillin-resistant *S. aureus* screened based on oxacillin and cefoxitin disc diffusion methods showed that 26 (24.1%) isolates were resistant to cefoxitin but sensitive to oxacillin, while 46 (42.6%) isolates were resistant to oxacillin but sensitive to cefoxitin and 36 (33.3%) isolates were resistant to both cefoxitin and oxacillin (Table 5). Of the 50 isolates used for the detection of PBP2a/PBP2' using latex agglutination, 20 isolates were choosen from oxacillin sensitive but cefoxitin resistant strains and 20 isolates from oxacillin resistant but cefoxitin sensitive strains while the remaining 10 isolates were selected from oxacillin/cefoxitin resistant strains. Only one 1 (2.0%) isolate was found to be indeterminate from oxacillin/cefoxitin resistant strains while all the other isolates 49 (98.0%) were PBP2a/PBP2' positive (MRSA) (Table 6).

Table 1: Distribution of staphylococcal isolates based on the site of infection

Site	No. of isolates	Percentage
Blood culture	184	60.9
Wound sample	66	21.9
Eye swabs	23	7.6
Throat swabs	15	5.0
Cerebrospinal fluid	8	2.6
High vaginal swabs	3	1.0
Semen sample	3	1.0
Total	**302**	**100**

Table 2: Phenotyphic characteristics of the staphylococcal isolates.

Tests	No. of Isolates examined	No. Positive	% Positive
Catalase	302	302	100
Slide coagulase	302	215	71.2
Tube coagulase	302	215	71.2
Mannitol	302	218	72.2
Lactose	302	302	100
Beta heamolysis	302	48	15.9
Gamma heamolysis	302	254	84.1

Table 3: Methicillin resistance among staphylococcal isolates using conventional techniques

Technique	Resistance (%)	Intermediate (%)	Sensitive (%)
Oxacillin disc (1µg)	82 (27.2)	18 (6.0)	202 (66.9)
Cefoxitin disc (30µg)	65 (21.5)	0 (0.0)	237 (78.5)

Table 4: Distribution of Staphylococcal isolates (MSSA and MRSA) based on their ability to coagulate plasma

		Coagulase Test	
		Positive (%)	**Negative (%)**
Oxacillin 1µg	Resistant	58 (19.2)	24 (7.90)
	Intermediate	18 (6.0)	0 (0.0)
	Sensitive	63 (20.9)	139 (46.0)
Cefoxitin 30µg	Resistant	50 (16.6)	15 (5.0)
	Intermediate	0 (0.0%)	0 (0.0%)
	Sensitive	165 (54.7)	50 (16.6)

Table 5: Distribution of MRSA based on oxacillin and cefoxitin disc diffusion method

	OX (S)	**OX (R)**
FOX (S)	0 (0.0)	46 (42.6%)
FOX (R)	26 (24.1%)	36
(33.3%)		

Key: *OX (S) = Oxacillin Sensitive, OX (R) = Oxacillin Resistant*
FOX (S) = Cefoxitin Sensitive, FOX (R) = Cefoxitin Resistant

Table 6: Distribution of oxacillin/cefoxitin resistant isolates based on latex agglutination test

Pattern	**Positive (%)**	**Indeterminate (%)**	**Negative (%)**
OX (S)/ FOX (R)	20 (40.0)	0(0.0)	0 (0.0)
OX (R)/ FOX (S)	20 (40.0)	0(0.0)	0 (0.0)
OX (R)/ FOX (R)	9 (18.0)	1(2.0)	0 (0.0)

Discussion

More than half of the total number of isolates (60.9%) studied were recovered from blood culture followed by wound swabs with 21.9%, with the least being from semen and high vaginal swabs with 0.99% respectively, which is in agreement with the work done in Kano Norhtwestern Nigeria. [17] The staphylococcal isolates in this study varied in their relation to some biochemical tests and fermentation reaction carried out. All the isolates were shown to be catalase positive which agree with the work done by Kumurya. [16] The study showed that coagulase positive *S. aureus* had the highest prevalence with 71.2% isolates, which further agree with the report [18, 19] that recorded 69.0% and 80.8% respectively. All the 302 (100%) isolates ferment lactose when cultured on MacConkey agar which is in agreement with the work done [16] while 72.2% isolates ferment mannitol on growing on mannitol salt agar which disagree with the finding [16] that recorded 100% isolates ferment mannintol in same study site. This study revealed that only 15.9% isolates were beta heamolytic staphylococci which further disagree with the finding [16, 20] that recorded 94.0% and 100% respectively.

This study showed that prevalence rate of MRSA was 21.5% with cefoxitin (30µg) and 27.2% with oxacillin (1µg) using disc diffusion method. This prevalence is similar with the report[25] which recoded 21 – 30% in 8 African countries including Nigeria and also in agreement with the finding[16] which recorded the prevalence of 25.0% in Northwestern Nigeria. The prevalence of MRSA in this study was lower than the previous study in Nigeria where they reported 34.7%. [26] This may be due to different methods for detection of MRSA phenotypically with oxacillin disk diffusion test being the least reliable test for detection of MRSA. [28] This study also revealed that oxacillin disk diffusion method as the least reliable method (Table 5).

The present study revealed that PBP2a latex agglutination test is an alternative method with high sensitivity for detection of MRSA. There have been

many recent evaluations of the MRSA screen latex agglutination test, that reported the sensitivity for detection of MRSA as more than 99%. [32, 33, 34, 35, 36, 37, 38, 39, 40] In the present study, the sensitivity of PBP2a latex agglutination test (Oxoid, UK), from our data was 98 % which is in agreement with the finding of the above authors. while other MRSA evaluations of this test system ranged from 95·4 to 100 %. [41.42.43] Further study done by Kumurya in Northwestern Nigeria recorded the sensitivity 100%. [16]

Conclusion and Recommendations

The prevalence of 21.5% and 27.2% for MRSA using cefoxitin and oxacillin respectively were obtained in the study which is quite alarming. This study also provided baseline information in assisting physicians, clinical microbiologists and public health officials on critical issues regarding empirical and pathogen specific therapy. The PBP2a latex agglutination for MRSA screen test in rapid, easy to perform and highly reliable test for the detection of methicillin resistance in *S. aureus*. Therefore the MRSA screen test (latex agglutination test) for detection of PBP2a is an alternative that could be used in clinical laboratories. Which is practically useful for urgent confirmation of methicillin resistance.

Routine screening of MRSA should always include both oxacillin and cefoxitin discs. PBP2a/PBP2' latex agglutination test, can also be used as an alternative method to molecular for the detection of MRSA due to its high sensitivity and specificity. Lastly, Physiciants, clinical microbiologists and public health official should work together to implement antibiotics stewardship in their health institutions to prevent spread of MRSA.

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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 indirect method by milk yield estimation of pup weight and weight gain. The result showed that acute toxicity LD_{50} 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* protocatechuic 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 40ºC – 45 ºC 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 liters 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 ± standard of error mean (Mean ± 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, cardenoledes, 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	+
Cardenoledes	+
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 LD_{50} 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

* = P<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*

* = P<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 µg/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.

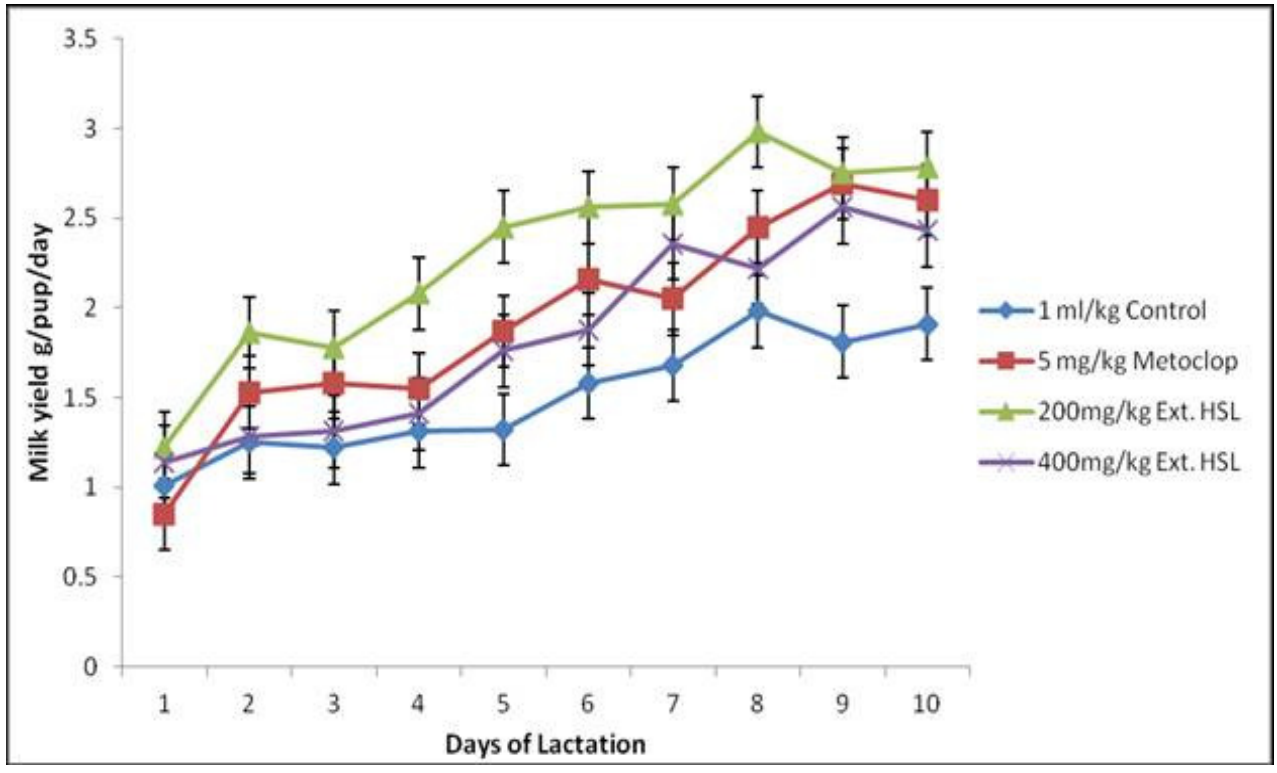

Figure 1a: Effect of aqueous extract of *Hibiscus sabdariffa l.* on milk production 18 hours after administration.

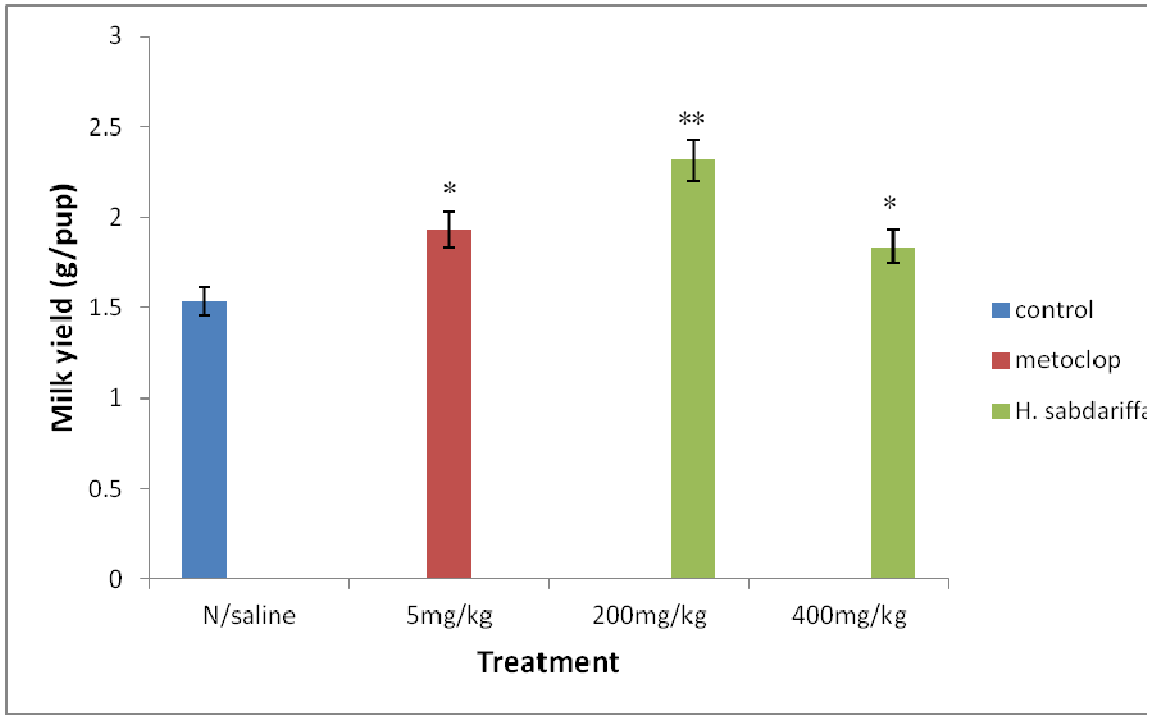

Figure 1b: Mean milk production per day for the effect of aqueous extract of *Hibiscus sabdariffa* L.

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**

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

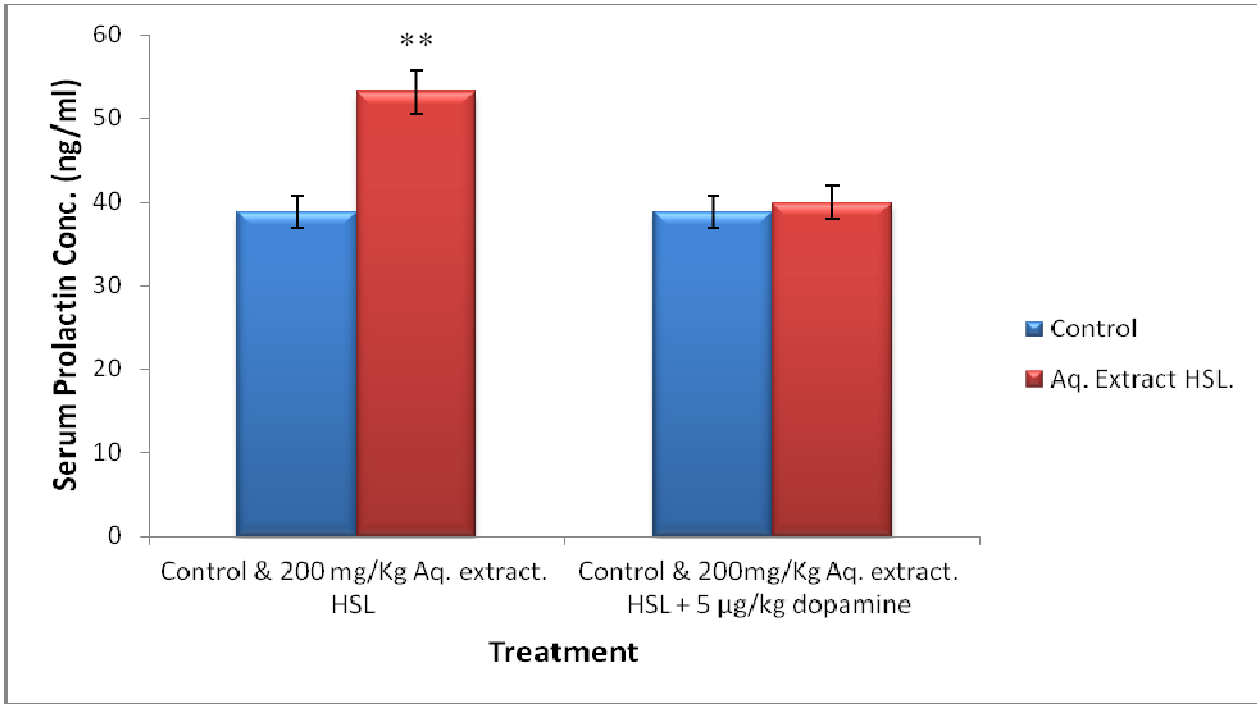

Figure 2: Effect of dopamine and aqueous extract of *Hibiscus sabdariffa* L. seed on serum prolactin level in lactating Wistar albino rats.

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 LD_{50} 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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Competing Interests The author has declared that no competing interests exist.

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Psychrophilic Bacteria as Alternative Sources of Unsaturated Fatty Acids

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Abstract

Unsaturated fatty acids (UFAs) have several biological roles, which include regulation of the membrane fluidity and nutritional and pharmaceutical values. Vegetable oils and fish have been described as the main sources of essential fatty acids, the body requires for regular physiological activities. However, the rising demands for these fatty acids necessitate the need to search for cheaper, flexible and reliable alternative sources. Psychrophilic bacteria produce abundant amounts of unsaturated fatty acids, which can be scaled up through biotechnological processes. In this study, two psychrophilic bacteria identified as *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB, based on cultural, morphological, biochemical and molecular examinations were grown at a temperature of 15 ^{o}C. Fatty acid contents of these bacteria were analysed via Gas Chromatography-Mass Spectrometry (GC-MS). Both bacteria were found to contain appreciable unsaturated fatty acid contents. However, higher amount was detected in *Arthrobacter* sp. PB (45.06%) relative to *Arthrobacter* sp. 3B (37.96%). This suggests that the bacteria might be ideal candidates for biotechnological manipulations, as alternative source of essential fatty acids.

***Keywords*:** Psychrophilic bacteria, Unsaturated fatty acids, Membrane fluidity, *Arthrobacter* spp, Gas chromatography-mass spectrometry.

Introduction

Fatty acids are organic compounds that are made up of carbon chains with a carboxylic group at one end and a methyl group at the other end. They are grouped based on saturation into saturated (double bond free-fatty acids) and unsaturated (fatty acids with one or more double bonds) fatty acids. The unsaturated fatty acids may be in the form of monounsaturated or polyunsaturated.[1] Unsaturated fatty acids are very crucial for the normal physiological activities of all organisms. They constitute the major components of membrane phospholipids for constant membrane fluidity at low temperatures [2,3] and serve as precursors for the synthesis of eicosanoids such as leukotrienes, prostaglandins and thromboxanes which are known to function as signalling molecules.[3] Several reports confirmed the nutritional and pharmaceutical importance of polyunsaturated fatty acids. They have proven antitumor effect, [3,4,5] protection against cardiac diseases, [3] antibacterial [3,6,7,8] and anti-inflammatory. [3,9,10]

Psychrophilic bacteria are bacteria that are able to survive cold temperatures of 15 ^{o}C or below and 20 ^{o}C as the optima and maxima, respectively. However, the term psychrotolerant (psychrotroph) is generally used to describe an organism that is able to grow at low temperatures but having optimum and maximum growth temperatures lower or higher than 15 ^{o}C and 20 ^{o}C, respectively.[11,12,13] Low temperature condition causes a serious damage to the overall structural integrity of cellular membrane of most microbial cells.[14] Psychrophilic bacteria respond to low temperature effects by synthesizing more unsaturated fatty acids.[15] Man and animals are unable to synthesis essential fatty acids required by the body which are only obtained from the diets. Fish has been the major source of essential fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[16,17] However, fish has become inadequate to meet the world rising demands for essential fatty acids due to overfishing activities. Therefore, the sought for alternative reliable sources of essential fatty acids has been of

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great research interest. Five psychrophilic bacteria, *Pseudomonas* sp. A3, *Pseudomonas* sp.A8, *Arthrobacter sp*. 1B, *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB were grown at 4 ^{o}C and analysed their fatty acids content by GCMS.[18] The results obtained showed higher unsaturation. In this study, the *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB were selected based on their higher unsaturation and incubated at 15 ^{o}C as the optimum growth temperature for psychrophilic bacteria and determined their overall fatty acids content in search for alternative sources of unsaturated fatty acids.

Materials and Methods

Collection of Psychrophilic Bacterial Isolates

The psychrophilic bacteria were collected from the Enzyme and Microbial Technology Research Centre, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia. The bacteria collected are *Arthrobacter sp*. 3B (GenBank accession no: KT072051) and *Arthrobacter sp*. PB (GenBank accession no: KT124224).

Molecular Identification of the Psychrophilic Bacterial Isolates

The bacterial isolates were cultured on nutrient agar plates at 15 ^{o}C for 3-5 days. A sterile wire loop was used to inoculate 10 mL of nutrient broth (Difco^{TM} USA) with the bacteria and cultivated at 200 rpm for 24 hours at 15 ^{o}C. Genomic DNA of each bacterium was extracted using DNeasy$^{(R)}$ genomic DNA extraction kits (Qiagen) following the manufacturer's instructions and analysed on 1% (w/v) agarose gel with LambdarhindIII DNA maker (Fermentas).[19]

The 16S rRNA genes of the bacteria were amplified by Polymerase Chain Reaction (PCR) on a PCR thermocycler (G-Storm) using the following set of primers: '5AGAGTTTGATCCTGGCTCAG3' and '5ACGGCTACCTTGTTACGACTT3' as forward and reverse primers, respectively. The reaction mixture, 50 µL contained 2 µL of Genomic DNA, 19 µL of distilled water (dH_2O), 2 µL each of forward and reverse universal primers, and 25 µL of 2x PCR master mix (Thermo scientific, 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of dNTP). The PCR incubation conditions are 94 ^{o}C for 4 min, then 35 cycles of 94 ^{o}C for 1 min, 58 ^{o}C for 30 seconds and 72 ^{o}C for 1 min followed by a final extension at 72 ^{o}C for 10 min. PCR product was gel purified (GeneAll® kits) and sequenced at 1st BASE (Sdn, Bhd, Malaysia). The 16S rRNA nucleotide sequences were used to perform Nucleotide blast search at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast).

Related sequences were selected, aligned and performed phylogenetic tree analysis. In addition to 16S identification, the bacteria were also identified using morphological, cultural and biochemical tests.

Preparation of Fatty Acid Methyl Esters

The psychrophilic bacteria were incubated on nutrient agar plates (Liofilchem, Italy) at 4 ^{o}C for 7 days. Preparation of fatty acid methyl esters was performed according to MIDI protocol (Microbial Identification System, Microbial ID Inc., Newark, DE, USA). Approximately 40 mg of the bacterial cells was suspended in 1 mL of NaOH (15% w/v) in Methanol/ water (1:1 v/v) and saponified by heating at 100 ^{o}C for 30 minutes. The saponified mixture was supplemented with 2 mL of 6N Hydrochloric acid in methyl alcohol (13:11 v/v) and methylated by heating at 80 ^{o}C for 10 seconds. To extract the FAMEs, 1.25 mL of hexane (SupraSolv®, Germany) in tert-butyl-methyl ether (1:1, v/v) (Sigma, Aldrich) was added and vortex mixed for 10 minutes. Sample wash step was performed by adding 3 mL of sodium hydroxide in distilled water (1.2% w/v), vortex mixed for 5 minutes and placed an aliquot of the upper organic layer in a GC vial for GC-MS analysis.

Analysis of Fatty acids methyl esters using GC-MS

The fatty acid methyl esters (FAMEs) were analysed using Gas Chromatography-Mass Spectrometry (GCMS)(QP 2010 GCMS, Shimadu, Japan) using 30-m HP-5 column (0.25 mm x 0.25 µm) with helium as a carrier gas and a linear velocity of 30 cm/s. The column was set with an initial temperature of 40 ^{o}C for 1 minute, ramping at 2 ^{o}C per minute and then raised to 240 ^{o}C. The heating temperature was increased to 300 ^{o}C, ramping at 30 ^{o}C per minute, and finally held at 300 ^{o}C for 6 minutes with an injector temperature of 250 ^{o}C [20].

Results and Discussion

Identification of the Psychrophilic Bacterial Isolates

The Psychrophilic bacteria were grown on nutrient agar plates and subjected to Gram staining reactions which differentiated them as Gram positive rods. Genomic DNA of each bacterium was extracted and used as a template to perform PCR amplification of the 16S rDNA genes, analysed on 1% (w/v) agarose gel and detected an approximate size of 1500 bp (Figure 1). The genes were purified using gel purification and sent to First Base Laboratories Sdn, Bhd (Malaysia) for sequencing. The sequences were submitted to National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) and performed nucleotide BLAST which showed a maximum identity of 99% to16S rRNA gene sequences of certain *Arthrobacter* species.

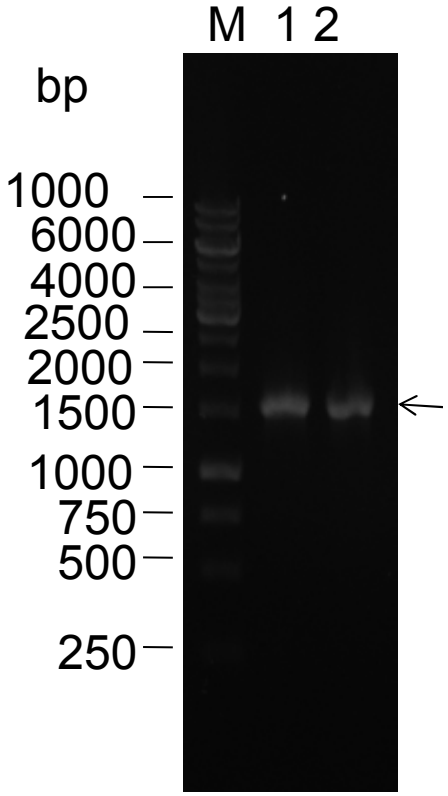

Figure 1: Analysis of 16S rRNA genes of the bacteria on 1% (w/v) agarose gel. M= Molecular marker (1kb DNA ladder), lane 1= 16S rRNA gene of *Arthrobacter* sp. 3B, lane 2= 16S rRNA gene of *Arthrobacter* sp. PB while the arrowhead indicates the estimated size of 1500 bp.

The genes were deposited at the GenBank to obtain gene accession numbers. Cultural characteristics and biochemical tests performed on the bacteria agreed with the 16S rRNA identification that the bacteria belonged to members of *Arthrobacter* species (Table 1). The bacteria were identified as Gram positive cocci, motile, non-spore forming, non-capsulated, catalase positive, citrate negative, indole negative, Methyl Red-Voges Proskauer negative, non-gas or hydrogen sulphide producers and are neither glucose nor lactose fermenters.[21]

Table 1: Morphological and biochemical characteristics of the bacteria

Psychrophilic bacterial isolate	Microscopic examinations/ Biochemical tests														
	G^1	S	C^1	M	O	C^2	I	H	C^3	U	L	G^2	G^3	MR	VP
***Arthrobacter* sp. 3B**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-
***Arthrobacter* sp. PB**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-

Key: G^1: Gram staining, S: Spore staining, C^1: Capsule staining, M: Motility test, O: Oxidase test, C^2: Catalase test, I: Indole test, H: Hydrogen sulphide production test, C^3: Citrate utilization test, U: Urease test, L: Lactose fermentation, G^2: Glucose fermentation, G^3: Gas production, MR: Methyl red, VP: Voges Proskauer.

Analysis of phylogenetic tree of the bacteria was conducted using Neighbour joining method in MEGA 6.0 with default parameters (Figure 2). The analysis revealed that the psychrophilic *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB share the same ancestral origin. However, the two bacteria have a slightly distant relationship with each other as they occupy entirely different clades. Moreover, the bacteria identified in this study appeared even far distantly related with members of their family already deposited in the public database (http://www.ncbi.nlm.nih.gov/blast). This suggests that the bacteria identified are of sufficient novelty and not previously identified conforming to the 99% identity of the Nucleotide Blast Search performed using the NCBI database

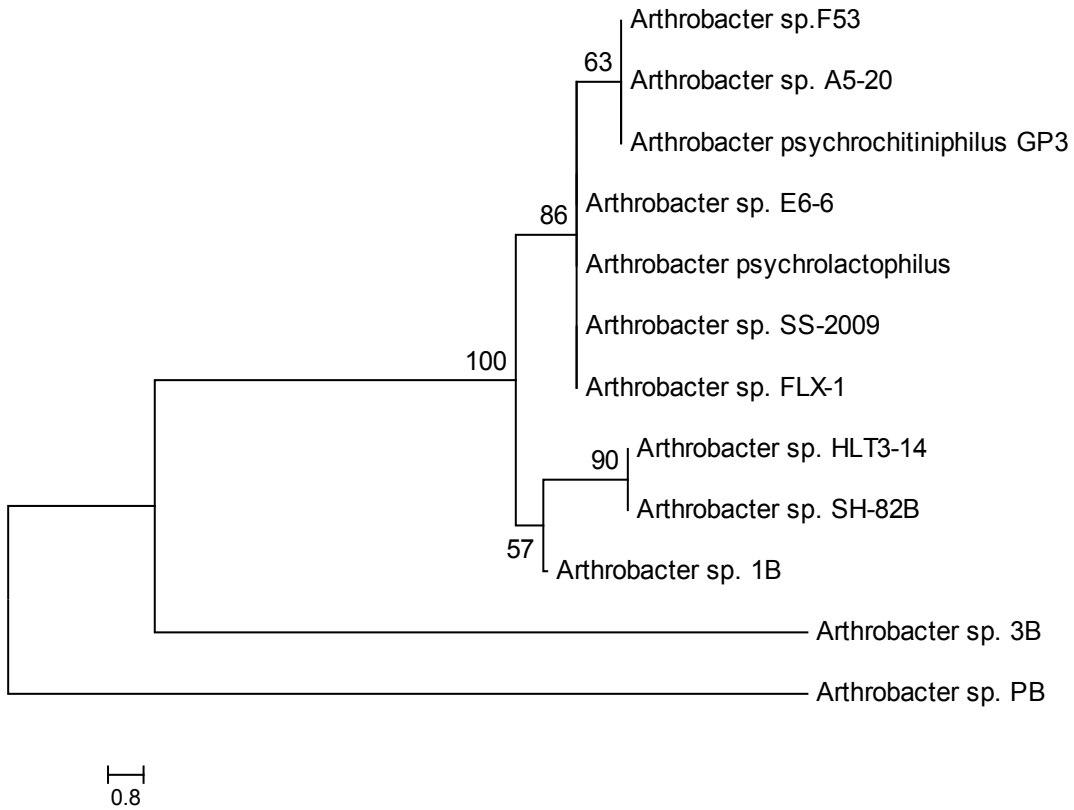

Figure 2: Phylogenetic analysis of the bacteria. The sequences of the 16S rRNA genes of the *Arthrobacter* sp. 3B, *Arthrobacter* sp. PB and selected members of their family were aligned and used to construct the phylogenetic tree using MEGA 6 software [22] using Neighbour-joining method [23] with default parameters.

The numbers on each clade stand for percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The psychrophilic *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB are indicated by asterisks * and ** respectively.

Fatty Acids Profile of the Bacteria Fatty acids of distinctive chain lengths and degrees of unsaturation detected in the psychrophilic *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB are shown in Table 2. Both saturated and unsaturated fatty acids were identified from the bacteria. Higher amount of overall saturated fatty acids were detected in *Arthrobacter* sp. 3B (62.04%) more than *Arthrobacter* sp. PB (54.94%). Both bacteria were found to contain palmitic (C16:0) and stearic (C18:0) acids. In addition, lauric acid (C12:10) was only detected in *Arthrobacter* sp. 3B while, myristic acid (C14:0) was detected only in *Arthrobacter* sp. PB. Fatty acid analysis showed higher overall unsaturation in *Arthrobacter* sp. PB (45.06%) compared with *Arthrobacter* sp. 3B (37.96%).

Table 2: Fatty acids profile of the bacteria

Fatty acids (%)	***Arthrobacter* sp. 3B**	***Arthrobacter* sp. PB**
C12:10	4.74±0.01	n.d
C14:0	n.d	16.45±0.01
C16:0	20.79±0.01	17.21±0.01
C18:0	36.51±0.02	21.28±0.01
∑SFA[(a)]	**62.04**	**54.94**
C16:1Δ9	26.46±0.01	28.80±0.04
C18:1Δ9	11.50±0.02	16.26±0.04
∑UFA[(b)]	**37.96**	**45.06**

Overall saturated (a) and unsaturated fatty acids (b) detected in each isolate. The bacteria were cultured at 15 ^{o}C for 7 days and analysed their fatty acids composition by GC-MS. The values are means of three replicates ±S.D experiments. n.d: not detected.

The only unsaturated fatty acids detected in *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB were Palmitoleic (C16:1Δ9) and oleic (C18:1Δ9) acids as indicated by the GCMS chromatograms shown in Figure 3. These fatty acids which represent the most commonly reported unsaturated fatty acids from several psychrophilic bacteria [13] are the precursors of polyunsaturated fatty acids such as omega 3, 6 and 7 fatty acids, which are known to be very essential for healthy growth and normal physiology.

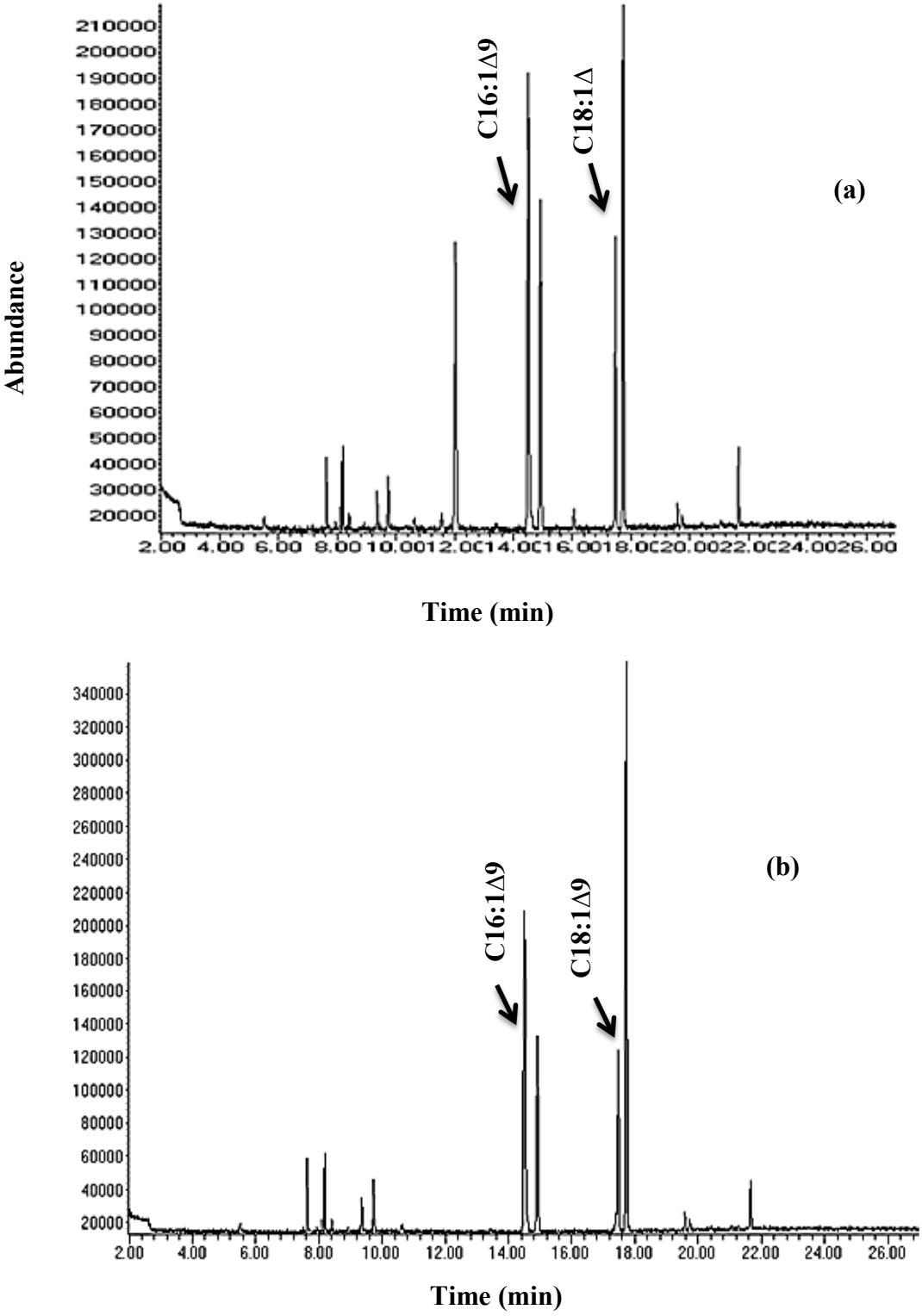

Figure 3: Major unsaturated fatty acids identified from the *Arthrobacter* sp. PB (a) and *Arthrobacter* sp. 3B (b). The bacteria were cultured at 15 ^{o}C for 7 days and analysed their fatty acids composition by GC-MS. The arrowheads indicate peaks for palmitoleic (C16:1Δ9) and oleic acids (C18:1Δ9). The results are means of three replicate experiments.

A number of reports showed that, monounsaturated fatty acids have been the main fatty acids detected in different psychrophilic bacteria including *Flavobacterium* and *Flectobacillus* species,[24] *Micrococcus cryophilus* [20], *Pseudoalteromonas* sp., MLY15,[25] *Halomonas* species [18,26] which are all in agreement with the findings of this study. In addition to unsaturated fatty acids, high amounts of saturated fatty acids were detected in *Arthrobacter* sp 3B (62.04%) and *Arthrobacter* sp PB (54.94%), and this is in agreement with a report on psychrophilic bacterial isolates BRI 7 (40-47%) and BRI1 (94.6%).[26]

Conclusion

In conclusion, monounsaturated fatty acids were detected in the psychrophilic bacteria which were identified as *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB. The results obtained may serve as stepping stones to isolating fatty acid desaturase enzymes (thought to be responsible for unsaturated fatty acids production in psychrophilic bacteria) for industrial applications.

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