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Full Length Research Paper

A simple and sensitive method for the detection of “Oxytetracycline” levels in ready-to-eat beef by liquid chromatography-mass spectrometry

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Antimicrobial drug residues have emerged as one of the public health problems worldwide. In this study, a modified sensitive liquid chromatography mass spectrometry (LC-MS) method to detect the “Oxytetracycline” (OTC) levels in ready-to-eat beef meat in Tanzania was evaluated. Beef samples were extracted in acetonitrile in ethylenediaminetetraacetic acid (EDTA) buffer (pH 4), followed by cleaning up with Supelclean ENVI-carb active coal and a stream of nitrogen gas. The wavelength of the diode array detector (DAD) was set at 275 and 355 nm. The detection limit of the method was calculated as 18.2 ng/g and the recovery rate of OTC was 78.6%. A total of 45 ready-to-eat beef meat samples were analyzed, with 16 (35.5%) and 29 (64.5%) barbequed and boiled samples, respectively. Of the 45 samples, 35 (77.8%) samples had OTC residues while 9 (25.7%) samples had violative residue levels above the maximum residue limits recommended by the Food and Agriculture Organization and the World Health Organization. The highest concentration was 545.2 ng/g. Therefore, withdrawal period and proper use of antibiotics for animal production should be of concern as consumers are at risk of adverse effects due to consumption of unacceptable levels of drug residues and a risk of developing microbial resistance. To the best knowledge of the authors, this is the first study to evaluate LC-MS method to detect the OTC levels in ready-to-eat beef meat in Tanzania.

Key words: Oxytetracycline, high performance liquid chromatography, mass spectrometry, ready-to-eat beef meat, residues.

INTRODUCTION

Antimicrobial drug residue in animal products is an increasing public health problem worldwide. One of the major areas of interest is investigating the proper use and

monitoring of antibiotics usage to prevent contamination (Alica et al., 2003). Questions have been raised about the drug label, discard times as several drugs are retained in

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animal bodies longer than indicated by the manufacturer (Seymour et al., 1988). Improper administration of antimicrobials by farmers and veterinarians without observing the withdrawal time for treated animals may not only result in antimicrobial residues in meat but may also contribute to the development of microbial drug resistance and spreading of drug resistant bacteria that may result in serious health consequences (Booth, 1988). Human health problems that could arise from the consumption of unacceptable levels of OTC residues in meat include gastrointestinal disturbances, hypersensitivity, bone and teeth problems in children and development of bacterial resistance (Larkin et al., 2004; Shankar et al., 2010).

The problem regarding tetracycline residues is very common and has to be addressed accordingly, since tetracyclines are the commonly used antimicrobial drugs. With this regard the Food and the Agriculture Organization (FAO) and the World Health Organization (WHO), 2004 recommended the maximum residue limits (MRLs) to be 200, 600 and 1200 µg/kg in muscles, livers and kidneys, respectively. For the analysis of tetracyclines levels, various methods have been reported in the literature mainly due to difficulties related to differences in physico-chemical properties between families of compounds (Kaufmann, 2009). Methods for the detection of tetracyclines are many but a more specific method such as HPLC is the efficient technique (Loksuwan 2002; Cinquina et al., 2003). The method efficiency is based on multi-detection on liquid chromatography coupled with tandem mass spectrometry (Bohm et al., 2009).

Residues are ordinarily measured on uncooked tissues. It is also important to monitor the levels of drug residues in both raw and ready-to-eat foodstuffs. Studies have shown that temperatures have effect on the levels of drug residues (Salah and Ali, 2013). It is even more important to analyse the levels of OTC residues and to evaluate if residues levels can be reduced by cooking procedures (Ibrahim and Moats, 1994). So far, there is limited literature about the effect of cooking on levels of residues and this creates a scientific gap of knowledge which needs to be addressed in Tanzania. Therefore, the objective of the present work was to modify and validate a simple and sensitive LC-MS method for analyzing Oxytetracycline (OTC) residues (Froehlich, 2013). The validated method was applied to determine the levels of OTC in ready-to-eat beef meat samples.

MATERIALS AND METHODS

Samples

A total of 45 ready-to-eat beef meat samples were randomly collected from different areas in Dodoma, Tanzania (Majengo Sokoni, Mnadani, Chakonichako, Rozi Garden and Bahama Mama). The samples collected were already prepared as barbecued "nyama choma" or boiled. These two methods of preparation

were selected because they are most practiced in Tanzania. Antibiotics-free meat samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The blank matrix samples were barbecued or boiled before extraction.

Sample pretreatment and extraction

The samples were kept at -20°C until analysis and were allowed to defrost at room temperature. A representative portion of the defrosted sample (10 g) was weighed and mixed with 25 mg of EDTA per gram sample. The sample and the EDTA were homogenized for 1 min using a blender. The blended sample was further ground using a mortar and pestle. One gram of homogenized sample was accurately weighed into 15 ml polypropylene centrifuge tubes. To the sample, 10 µl of 10 µg/ml carbamazepine D10 internal standard solution equivalent to 100 ng/g concentration was added.

Five milliliters acetonitrile were added to the sample and vortexed for 1 min. Each sample was centrifuged for 10 min at 7000 rpm and the supernatant was collected into a separate 15 ml centrifuge tube by decantation. 5 ml acetonitrile were again added to the residue and vortexed for 1 min. The samples were then centrifuged for 10 min at 7000 rpm. Both supernatants were combined in a 15 ml centrifuge tube bringing the total volume to 10 ml. All samples were briefly mixed using a vortex and dried under a stream of nitrogen gas to 2 ml, according to Froehlich's HPLC method (Froehlich, 2013).

Sample clean-up by Supelclean ENVI-carb active coal

After drying each sample to 2 ml, 0.5 ml of HPLC grade water and 30 µl of formic acid were added, making the mixture 1.2% acid. Then 15 mg of Supelclean ENVI-carb active coal was added to all the samples and mixed for 30 s using a vortex and centrifuged for 10 min at 7000 rpm. The supernatants were collected into separate 15 ml centrifuge tubes and dried to 0.5 ml. The dried samples were then transferred into HPLC vials washed with 0.02 mol/L EDTA solutions and injected into chromatographic system (Froehlich, 2013). The HPLC analysis was performed in 23 min.

Sample analysis by LC-MS method

The HPLC was equipped with DAD detector and mass spectroscopy (Model Agilent Technologies 6130 Quadrupole LC/MS) to target the flowing parent ions using Single Ion Monitoring (SIM) mode 461 mass per charge ratio (m/z) for OTC. The analytical column was reversed-phase Eclipse XDB C-18. 4.6 × 150 mm set at a flow rate of 0.5 ml/min. The column temperature was 25°C. Mobile phase A was HPLC water with 0.1% formic acid and solvent C was Acetonitrile with 0.1% formic acid. The starting mobile phase composition at 0 min was 85% Water: 15% Acetonitrile at 0.5 ml/min. The wavelength of the DAD detector was set at 275 and 355 nm, respectively. Internal calibration curves were prepared by spiking the blank matrix with pure chromatographic standard solutions in the range between 200 and 2500 ng/g injected for each compound and estimates of the amount of the analytes in samples were interpolated from these graphs.

Validation

To test the analytical method trueness, 14 samples were prepared. Each contained 1 g of homogenized muscle tissue of the negative control sample (blank matrix). Seven samples were spiked with 20

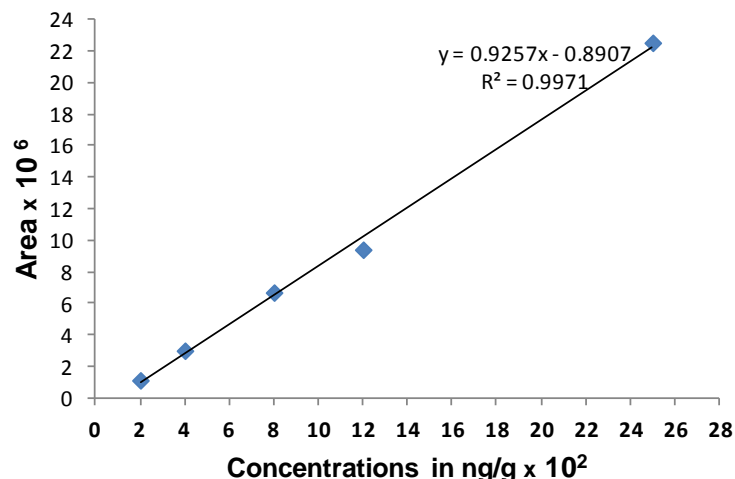


Figure 1. Calibration curve of oxtetracycline standard.

μ l of 10 ng/ml solutions, equivalent to 200 ng/g of analyte. Seven samples were spiked with 250 μ l equivalent to 2500 ng/g of the analyte. All samples were processed using the described LC-MS method.

Preparation of standard stock and working solution

A stock standard solution of OTC compound was prepared by dissolving 10 mg of the compound in 10 ml of methanol to obtain a final concentration of 1 mg/ml. The stock standard solution was then put in amber glasses to prevent photo-degradation and stored at -20°C and left to stabilize for at least 4 weeks. They were then diluted with 95% water: 5% acetonitrile to give a series of working standard solution of 200, 400, 800, 1200, and 2500 ng/g.

Recovery experiment

Samples recovery was determined with blank bovine muscle spiked at 200 ng/g. To test the recovery, 10 samples were prepared that contained 1 g of homogenized muscle tissue of the negative control. They were spiked with 20 μ l of 10 μ g/ml spiking solution equivalent to 200 ng/g of the analyte. Four samples were used to calculate the recovery mean and six samples were used to calculate the recovery-corrected content.

Data analysis

The data were analyzed using Epi Info (version 7) (Centre for Disease Control, Atlanta, USA). The association between different categorical and continuous variables was determined by the Fisher's exact test. One-way analysis of variance (ANOVA) test statistic was used to determine any significant differences in the mean residue levels of oxtetracycline; a probability of $P < 0.5$ was considered statistically significant.

RESULTS AND DISCUSSION

Calibration of OTC standard

OTC standard powder was accurately weighed and

dissolved in methanol to make the stock solution and several serial dilutions of the stock solution were made and injected to the LC-MS to plot the standard curve of linear R^2 value = 0.9971 within the range of 200 to 2500 ng/g (Figure 1).

Samples recovery

The recovery rate of OTC was 68% (Table 1), while the recovery-corrected rate for the samples were 78.6% ranging from 64.8 to 86.9% (Table 2). For repeatability and reproducibility, data were obtained by extracting 7 replicates on three successive days at two concentrations of 200 and 2500 ng/g; with coefficients of variation of 6.60 to 10.60% and 6.30 to 10.60% for OTC, respectively. Results of this study revealed that the repeatability and reproducibility were corresponding to the validation methods done by Biswas et al. (2007).

LC-MS technique was employed to determine the levels of OTC in ready-to-eat beef meat samples in Dodoma, Tanzania. In this method, carbamazepine D10 was used as internal standard to correct internal and external error. The detection of OTC residues levels was done by using LC method with MS detector. This is because OTC can be successfully determined using LC with MS detector in various matrices. Adequate treatment of samples during extraction was done in order to obtain maximum sensitivity of OTC and to reduce matrix interference. The samples were considered positive for OTC if their retention time and peak corresponded to that of the reference standard. The retention time of the standard was at 3.624 min. The chromatographic peak increased with increase in concentration of the standard

The limit of detection (LOD) is the lowest concentration which can be qualitatively measured, and is defined as the concentration at which the signal-to-noise ratio of the corresponding signal is 3-to-1. In this study, the LOD

Table 1. Certified reference materials for OTC in bovine muscle

Recovery of OTC from meat spiked at 200 ng/g of the analyte (ng/g)	
Recovery 1	129.0
Recovery 2	145.6
Recovery 3	134.6
Recovery 4	137.8
Mean recovery	136.8 (68%)

Table 2. Recovery-corrected contents

Analysis of certified reference material	Measured content (ng/g)	Recovery-corrected contents (%) $Y=B10/B15*100$
Replicate 1	184.6	74.1
Replicate 2	174.3	78.5
Replicate 3	163.4	83.7
Replicate 4	157.4	86.9
Replicate 5	198.9	64.8
Replicate 6	163.4	83.7
Mean	-	78.6±3.3
Standard deviation	-	8.1
Coefficients of variation	-	10.3
Recovery rate	-	78.6%

B10 is the mean recovery. B15 is the replicate 1.

Table 3. Number and percentage in parentheses of beef samples barbequed and boiled with and without oxytetracycline (OTC) residues

Cooking types	OTC residues (%)	No OTC residues (%)	Total (%)
Barbequed	12 (75)	4 (25)	16 (35.5)
Boiled	23 (79.3)	6 (20.7)	29 (64.5)
Total	35 (77.7)	10 (22.2)	45 (100)

Fisher exact test 0.73, P = 0.74

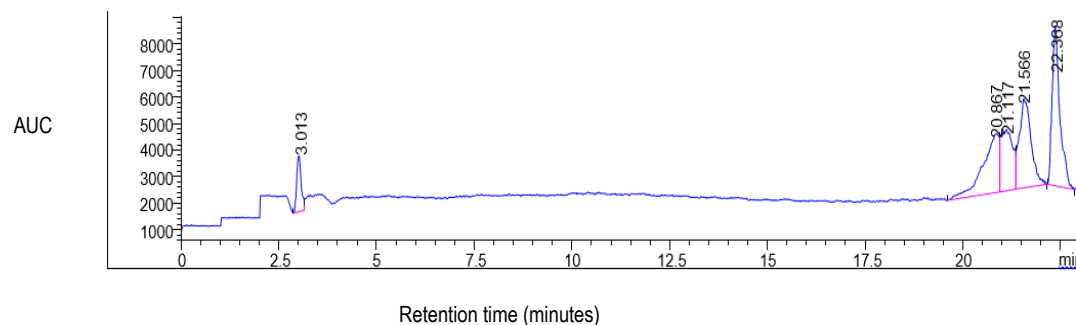
was 18.2 ng/g, corresponding to the LOD obtained by Hassani et al. (2008). The limit of quantification (LOQ) is the lowest concentration of analyte which can be quantitatively measured and was 54.6 ng/g.

Figure 2 shows LC-MS profiles of the OTC obtained from the blank beef meat samples, blank beef samples spiked with 400ng OTC, standard solution and spiked beef meat samples.

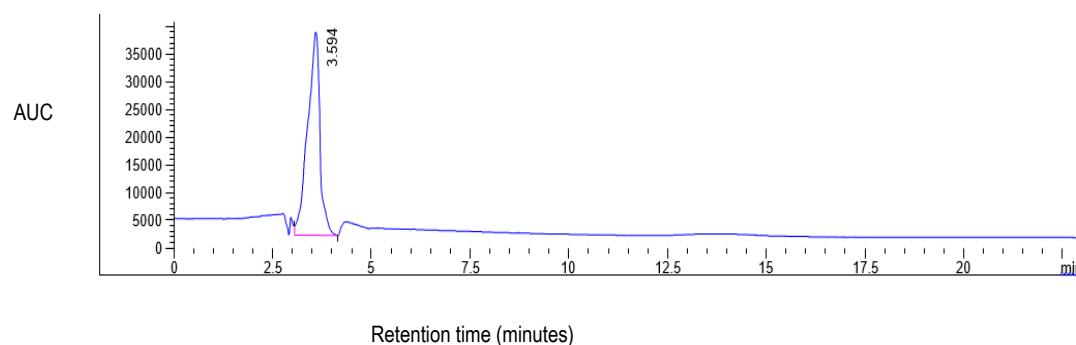
Results indicate that of the 45 beef meat samples analyzed 16 (35.5%) were barbequed samples and 29 (64.5%) boiled samples. The observed differences are statistically insignificant ($P > 0.05$) as shown in Table 3. Thirty five samples (77.8%) had OTC residues with 26 (74.3%) samples having residues below the FAO/WHO (2004) recommended MRLs. Nine (25.7%) samples had OTC at violative levels above the recommended MRLs.

Of the 9 samples with detectable violative OTC levels, 2 (22.2%) and 7 (77.8%) samples were barbequed and boiled meat samples, respectively. However, the observed differences were statistically insignificant ($P > 0.05$) as shown in Table 4. The study findings indicate the need for one health strategy to enhance the optimal health for humans, animals and the environment.

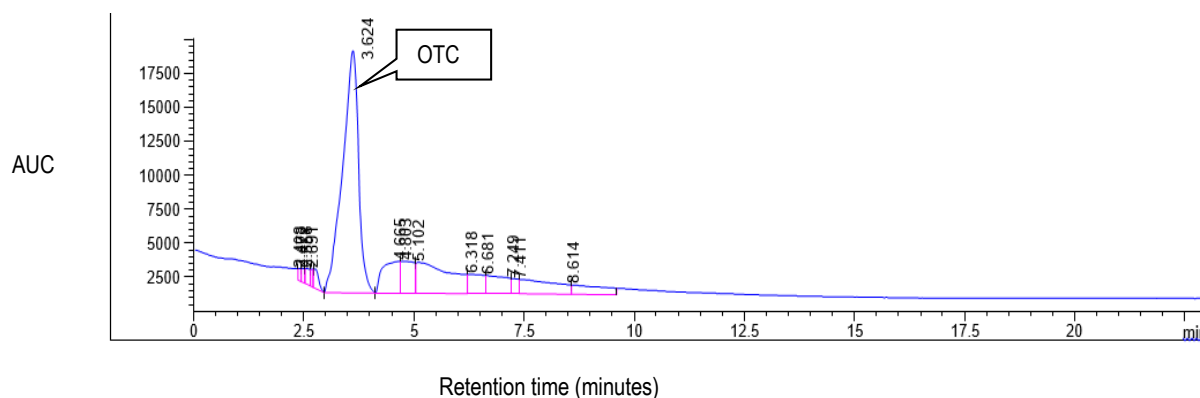
Mean concentration of OTC residues in barbequed and boiled samples were 130.67 ± 96.6 and 361.96 ± 69.40 $\mu\text{g}/\text{kg}$, respectively. The concentration of OTC residues from each sample is shown in Table 5. This study shows higher proportions of oxytetracycline-positive samples than those reported in other studies (Addisalem et al., 2012) and Bedada and Zewde (2012). Studies have reported varied drug residues in raw meat samples, 41.2% (Mmbando, 2004) and 76.4% (Nonga et al., 2013)



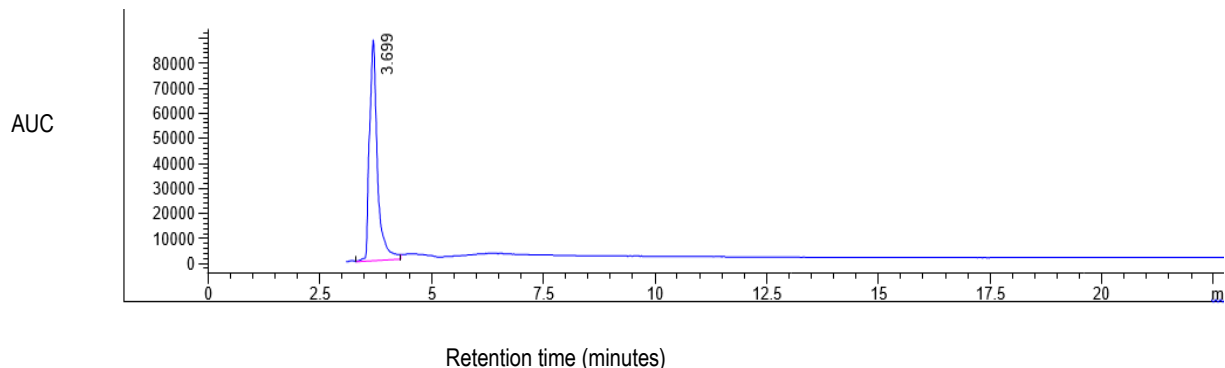
a) Chromatogram of blank beef meat sample



b) Chromatogram of blank beef samples spiked with 400ng OTC.



c) Chromatographic standard solution



d) Chromatogram of spiked beef meat sample of positive OTC thermally treated.

Figure 2. LC-MS profiles of OTC. (AUC = Area under the curve). (a) Chromatogram of blank beef meat sample. (b) Chromatogram of blank beef samples spiked with 400ng OTC. (c) Chromatographic standard solution. (d) Chromatogram of spiked beef meat sample of positive OTC thermally treated.

Table 4. Number and percentage in parentheses of beef samples with OTC residues

Cooking types	<MRLs of 200 µg/kg	>MRLs of 200 µg/kg	Total
Barbequed	10 (38.5%)	2 (22.2%)	12 (34.3%)
Boiled	16 (61.5%)	7 (77.8%)	23 (65.7%)
Total	26 (74.3%)	9 (25.7%)	35 (100%)

Fisher exact test 0.45, P =0.38

Table 5. OTC concentrations levels in ready-to-eat beef meat samples.

Cooking types	Sample code	Concentration OTC in ng/g			Total
		<MRL	>MRL	No-residues(ND)	
Boiled	SAMPLE 1C	-	-	0	-
	SAMPLE 2C	119.32	-	-	-
	SAMPLE 3C	184.11	-	-	-
	SAMPLE 4C	119.96	-	-	-
	SAMPLE 5C	-	-	0	-
	SAMPLE 6C	74.65	-	-	-
	SAMPLE 7C	-	440.11	-	-
	SAMPLE 8C	72.91	-	-	-
	SAMPLE 9C	25.92	-	-	-
	SAMPLE 10C	47.56	-	-	-
	SAMPLE 11C	103.28	-	-	-
	SAMPLE 12C	95.33	-	-	-
	SAMPLE 13C	-	288.75	-	-
	SAMPLE 14C	200.01	-	-	-
	SAMPLE 15C	-	-	0	-
	SAMPLE 16C	-	545.20	-	-
	SAMPLE 17C	183.74	-	-	-
	SAMPLE 18C	89.11	-	-	-
	SAMPLE 19C	-	326.46	-	-
	SAMPLE 20C	190.44	-	-	-
	SAMPLE 21C	-	295.36	-	-
	SAMPLE 22C	-	-	0	-
Boiled	SAMPLE 23C	-	417.54	-	-
	SAMPLE 24C	79.07	-	-	-
	SAMPLE 25C	190.96	-	-	-
	SAMPLE 26C	-	444.70	-	-
Barbequed	SAMPLE 27B	134.09	-	-	-
	SAMPLE 28B	-	-	0	-
	SAMPLE 29B	-	-	0	29
	SAMPLE 30B	142.54	-	-	-
	SAMPLE 31B	-	-	0	-
	SAMPLE 32B	-	-	0	-
	SAMPLE 33B	-	-	0	-
	SAMPLE 34B	77.95	-	-	-
	SAMPLE 35B	104.19	-	-	-
	SAMPLE 36B	81.53	-	-	-
	SAMPLE 37B	105.16	-	-	-
	SAMPLE 38B	-	-	0	-

Table 5. Contd

SAMPLE 39B	132.12	-	-	-
SAMPLE 40B	71.71		-	-
SAMPLE 41B	-	395.09	-	-
SAMPLE 42B	-	287.64	-	-
SAMPLE 43B	182.88	-	-	-
SAMPLE 44B	120.22	-	-	-
SAMPLE 45B	52.17	-	-	16
Total	26	9	10	45

in Tanzania. Nevertheless, the study conducted by Mmbando (2004) from muscle tissue in the Morogoro and Dodoma municipalities, Tanzania, indicate that only 41.2% of samples were positive for oxytetracycline residues. Drug residues in raw meat have also been reported in other countries, 44% in Nigeria (Stolker and Brinkman, 2005), 50% in Iraq (Tajick and shohreh, 2006), 21% in Ghana (Donkor et al., 2011) and 71.3% in Ethiopia (Addisalem et al., 2012). From Ghana, Donkor et al. (2011) and Mmbando (2004) reported 21 and 41.2% oxytetracycline residues in muscle tissue were relatively low compared to levels seen in the current study. These results reported here are consistent with those previously reported by Nonga et al. (2013) from Tanzania and those by Addisalem et al., (2012) from Ethiopia of 76.4 and 71.3%, respectively.

The presence of OTC residues in the ready-to-eat meat observed in the present study is a clear indication that drug residues are not destroyed by heating/cooking. The reasons might be due to the method used, time of cooking and type of tetracycline (TC) used. Several studies reported the effect of heat on foodstuffs. Nguyen et al. (2013) have reported that heat treatments were shown to reduce the concentration of drug residues level in foodstuffs, therefore decreasing the toxic effects to consumers. Javadi (2011) and Gratacós-Cubarsí et al. (2007) showed reductions in the concentration of doxycycline (DOC) and OTC residues level after different cooking processes. A study by Al-Ghamdi et al. (2000) also indicated that cooking by boiling decreased OTC, Chlortetracycline (CTC) and DOC levels in meat and liver.

Conclusion

A simple, rapid and sensitive LC-MS method for the detection of OTC levels in beef meat samples was evaluated. The method was capable of detecting residue and non-residue meat samples. A significant proportion of ready-to-eat beef meat samples (25.7%) had OTC level above the FAO/WHO MRLs of 200 µg/kg. This

indicates that animals are slaughtered without giving adequate withdrawal period or misuse of antibiotics for animal production in Dodoma region, Tanzania. The consumers of ready-to-eat beef meat are at risk of adverse effects due to consumption of unacceptable levels of drug residues and a risk of developing microbial resistance.

The study findings signify the need for the One Health approach for effective surveillance of drug residues in foodstuffs. Therefore, withdrawal period and proper use of antibiotics for animal production should be a public health concern given that the One Health approach aims to attain the optimal health for humans, animals and the environment. To the best knowledge of authors, this is the first study to evaluate LC-MS method to detect the OTC levels in ready-to-eat beef meat in Tanzania.

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Conflict of interests

The authors have declared that they have no conflict of interests.

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Full Length Research Paper

Antimicrobial potential evaluation of hydroethanolic extracts of the species *Anacardium occidentale* Linn.

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The species *Staphylococcus aureus* and *Escherichia coli* have shown increased antimicrobial resistance, which can be overcome with plant extracts, in view of their molecular complexity, for example extracts of *Anacardium occidentale* Linn (cashew). The objective was to evaluate the *in vitro* antimicrobial activity of cashew stem bark extract. Samples were collected in Santa Cruz / RN / Brazil, and after dried and crushed, the extract was prepared by maceration with ethanol: water solution (70:30 v / v) at a ratio (1:10 w / v) for 7 days. The filtrate was reduced by rotary evaporator and the resulting frozen. Extracts were characterized by Thin Layer Chromatography (TLC) and tested for antimicrobial activity in various concentrations (200 to 6.25 mg / ml) by diffusion disk. TLC testing indicated that the plant extract has phenolics, tannins mainly responsible for the pharmacological properties. The minimum inhibitory concentration was determined at 12.5 mg / ml only for *S. aureus*.

Key words: Cashew, antimicrobial activity, *S. aureus*.

INTRODUCTION

Humans are often affected by infections caused by microorganisms in the environment, especially bacterial species *Staphylococcus aureus* and *Escherichia coli*, which are showing increasing resistance to antibiotics (Tortora et al., 2005; Davis et al., 2005; Teixeira et al., 2008; Gomes, 2008; Howden et al., 2011; Sakunpak and Panichayupakranant, 2012).

An alternative to mitigate this problem refers to the use of medicinal plants whose products have higher molecular diversity than that derived from synthetic products (Davis

et al., 2005; Coutinho et al., 2008; Santos, 2011).

Plant species were always very important to humanity, as a food or for the treatment of diseases. Since ancient times there are reports of the use of medicinal plants to treat infections caused by microorganisms (Leite, 2009; Ansarullah et al., 2009; Van der kooy et al., 2009; Chandrasekaran et al., 2010; Brasil, 2012). Thus, there is the importance of scientifically analyze the antimicrobial activity of plant species, because these studies can contribute to a better understanding of its safety and

therapeutic efficacy. In Rio Grande do Norte / Brazil several plants are used for medicinal purposes (Rodrigues and Casali, 2002).

An example of this is the stem bark of *Anacardium occidentale* Linn, popularly known as cashew (Trevisan et al., 2006). *A. occidentale* Linn, belonging to the family Anacardiaceae, has the decoction extract of the stem bark used in traditional medicine, both for the oral or topical treatment for diarrhea and wound antiseptics (Lorenzi and Matos, 2002).

The literature reported several pharmacological activities (anti-inflammatory and antimicrobial) that have been proven from this plant (Trevisan et al., 2006; Konan and Bacchi, 2007; Kubo et al., 2011; Santos, 2011; Arekemase, 2011; de Abreu et al., 2013).

Therefore, this study aimed to evaluate the *in vitro* antimicrobial activity of the stem bark extract of the species *A. occidentale* Linn (cashew) against the bacteria *S. aureus* and *E. coli*.

METHODOLOGY

Sample collection and processing plant

The bark of the stem of the species *A. occidentale* Linn (cashew) has been previously cleaned with water before the procedures. The identification of plant species was based on the collection of the Herbarium of the Federal University of Rio Grande do Norte.

The samples were dried in an oven (BioPar -S150SD) at 33°C for 7 days. Later there was a size reduction of cashew samples by hand to then be grinded in a mill (Botini - F001643) previously sanitized. After grounding, the samples were stored in a desiccator for further realization of extraction (Gonçalves et al., 2005; Silva et al., 2007; Bertuccil et al., 2009; Palmeira et al., 2010).

Hydroethanol extraction

The extraction was performed by soaking in ethanol:water (70:30 v / v), 1:10 w / v, for 7 days under periodic agitation. The extract was filtered and the solution was reduced on a rotary evaporator (IKA - Control Model RV 10 CV) at 40°C. The crude extracts were obtained for each sample, which were stored in a freezer maintained at approximately -10°C.

Phytochemical characterization of plant samples

The qualitative phytochemical analysis of hydroethanolic extract was performed by Thin Layer Chromatography (TLC), as fixed phase silica gel F254 plates (MACHERY-NAGEL) and as a mobile phase, which was used twice, with polar and nonpolar solvents. The first mobile phase used was ethyl acetate: acetic acid: formic acid: distilled water (10: 1.1: 1.1: 2.6, v / v / v / v). The second mobile phase used was toluene: ethyl acetate: formic acid (5: 5: 0.5 v / v / v). Figure 1 The chromatographic developers used were the sulfuric vanillin (universal developer), ferric chloride (developer phenolic compounds), natural reagent A (specific reagent for the class of flavonoids) and the Dragendorff reagent (revealing specific to the class of alkaloids) (Alves et al., 2011).

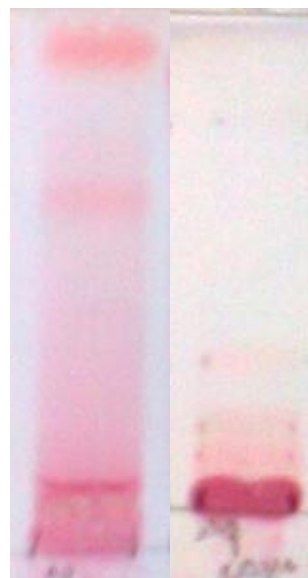


Figure 1. (a) Developer sulfuric vanillin. Fixed phase: silica gel. Polar mobile phase: ethyl acetate: acetic acid: formic acid: distilled water, 10:1.1:1.1:2.6 (v/v/v/v). (b) Developer sulfuric vanillin. Fixed phase: silica gel. Nonpolar mobile phase: toluene:ethyl acetate:formic acid 5:5:0.5 (v / v / v).

Bacteria

To achieve the standard *in vitro* tests we used strains of *S. aureus* and *E. coli* acquired in the Biosciences Center of the Federal University of Rio Grande do Norte - UFRN.

Determination of minimum inhibitory concentration (MIC)

The Hydroethanolic extracts were diluted in sterile distilled water and used in decreasing concentrations (200 to 6.25 mg/ml). The antimicrobial activity on plates was performed by antimicrobial susceptibility testing by disk diffusion, and used filter paper discs (Qualy) having 6 mm diameter, previously sterilized before, being impregnated with the extract of samples to be tested. As the culture medium, we used the Mueller Hinton Agar (DIFCO), being prepared according to the manufacturer's specifications (NCCLS, 2003). Strains of the species *S. aureus* and *E. coli* were grown in nutrient broth BHI (Brain Heart Infusion) and incubated at 37°C for 24 h. Then, it was diluted (1 mL of bacterial culture / 10 mL of sterile saline - 0.85% NaCl), and this solution was then used to seed the surface of plates containing culture medium (Catão et al., 2006; Silva et al., 2007).

After the seeding was introduced to the plates disks containing extracts to be tested, it then was incubated for 24 h at 37°C for measurement of the diameter of the inhibition zones (Nccls, 2003).

As a positive control we used disks containing the antibiotic gentamicin - 10 µg/disc (Poly Sensidisc DME 15 Gram Positive) for *S. aureus* and ciprofloxacin - 10 µg/disc (Poly Sensidisc DME 15 Gram negative) to *E. coli*, as a negative control we used on filter paper discs (Qualy) impregnated with sterile distilled sterile water



Figure 2. Antimicrobial activity of the hydroalcoholic extract of cashew against *S. aureus*: A: 200 to 125 mg/mL; B: 12.5 mg/mL.

(Nclls, 2003; Bastos et al, 2011.).

RESULTS AND DISCUSSION

Phytochemical characterization

Qualitative tests such as Thin Layer Chromatography (TLC) are able to demonstrate the presence or absence of chemical constituents (Costa, 2011). The plates eluted at more polar solvent system, showed the major components of the extract with the formation of well-defined bands. After processing with sulfuric vanillin, universal reagent, it was possible to observe the pink and red color bands. This staining is characteristic for phenolic compounds of the tannin class. These bands were also observed after the revelation of ferric chloride, with the development of blue color (presence of phenolic compounds). There was no formation of flavonoid characteristic bands after the revelation with natural reagent A. The revelation with Dragendorff reagent has not determined the observation characteristics of alkaloids bands.

Several studies have reported that the shell and leaves of the cashew have lots of phenolic compounds, mainly tannins, which are primarily responsible for the pharmacological properties (Hislan, 1966; Mota, 1982; Melo et al., 1997; Konan Ebacchi, 2007; Kubo et al., 2011). The tannins present in cashew shell are seen as responsible for their antimicrobial activity (Castillo-Juárez et al., 2007; Cui et al., 2008).

Minimum inhibitory concentration (MIC)

Microbiological studies showed that the hydroethanolic extract (cashew) was able to inhibit the *in vitro* growth of

S. aureus, with 12.5 mg/mL as a minimum inhibitory concentration (MIC) (Figure 2).

The disks containing 200 mg/ml hydroethanolic extract showed the greatest inhibition zone against *S. aureus*, while discs containing 12.5 mg/ml showed smaller inhibition zones. However, no inhibition of *E. coli* species. The negative controls (disks with sterile distilled water) showed no inhibition against bacterial species. Already in the positive controls were observed inhibition zones: ciprofloxacin (10 mg/disc) - 30 mm front of *E. coli* and gentamicin (10 mg / disc) -32 mm against *S. aureus*.

The results are similar to those presented by Silva et al. (2007), confirming its potential use as a medicinal plant in infectious processes. In the study of Dahake et al. (2009), the antibacterial activities of hydroethanolic extract and petroleum ether extract of cashew stem bark showed significant variations, and among the extracts tested the hydroethanol showed the highest antimicrobial activity. The better antimicrobial activity was against *S. aureus* and *Bacillus subtilis*.

The results observed in a study by Akinjogunlaa et al. (2012) showed that the hydroethanolic extract of cashew stem also has inhibitory effect against *Streptococcus mutans*. In other studies it was shown that the extract also presents antibacterial effect opposite to the species of *Streptococcus* (Melo et al., 2006). In addition, inhibitory actions against gram-negative bacteria are reported as *Proteus morgani*, *Pseudomonas aeruginosa*, *E. coli* and *Salmonella typhi*, with high concentrations of the hydroethanolic extract (Laurens et al., 1992). Therefore, it is appropriate to popularly use for the treatment of infection and inflammation of the gums, mouth and bronchitis for example (Silva et al., 2007).

Among the phenolic compounds, tannins are one of those which have received more attention due to its antimicrobial activity compared to other phenolic

compounds, and the fact that most of them are able to inhibit microorganisms and virulence factors. Furthermore, tannins may also exhibit synergism with antibiotics (Rodriguez Vaquero et al., 2010; Jayaraman et al., 2010; Saavedra et al., 2010).

Several mechanisms may explain the effect of tannins in inhibiting bacterial growth, such as destabilization of the plasma membrane, the inhibition of enzyme activity, actions that occur directly on the microbial metabolism and the deprivation of the substrate required for microbial growth, especially essential minerals such as iron and zinc (Dixon et al., 2005; Heinonen, 2007).

Conclusion

The hydroethanolic extract of the species *A. occidentale* (cashew) has antimicrobial activity on the species *Staphylococcus aureus*, and its antimicrobial potential is similar to that observed in the literature. The fact of the same species have not shown inhibitory effect on *E. coli* species and positive results presented in other studies may have been due to the extraction method used. However, despite this study along with other show the antimicrobial potential of the extract to *S. aureus*, other studies are needed to better characterize the potential use, including toxicological risks.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of antidiabetic effect and hematological profile of methanol extract of *Ceiba pentandra* G (Malvaceae) stem bark on alloxan-induced diabetic rats

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The anti-diabetic effect of methanol extract of stem bark of *Ceiba pentandra* and its beneficial/toxicological effect on hematological parameters in normal and alloxan-induced-diabetic rats were studied. The acute toxicity (LD₅₀) test and phytochemical analysis were also carried out. The diabetic rats were divided into five groups of 5 animals each given oral administration of the extract daily for 14 days. The antidiabetic study was carried out using 200, 400, and 800 mg/kg body weight of *C. pentandra* extract. The methanol extract of *C. pentandra* significantly ($p < 0.05$) reduced the blood glucose level in diabetic and norm glycemic rats in comparison with glibenclamide (standard drug). The effect of *C. pentandra* at 800 mg/kg (33.6%) was more effective compared to glibenclamide (23.0%) in lowering blood glucose with the added benefit of restoring reduced hematological parameters in diabetic rats to near normal level in norm glycemic rats. The acute toxicity (LD₅₀) test of the methanol extract was found to be greater than 5000 mg/kg. This showed the extract is relatively safe. The plant is also rich in flavonoids, saponins, resins, terpenoids, glycosides, and tannins. The result of this study shows that *C. pentandra* does possess anti-diabetic activity, beneficial effect and hence can ameliorate hyperglycemia and anemia in alloxan-induced diabetic rats establishing its potential as a source for isolation of new oral antihyperglycaemic agents.

Key words: *Ceiba pentandra*, hyperglycemia, hematology, diabetes.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by a high blood glucose concentration caused by insulin deficiency. It is a major cause of disability and hospitalization and results in significant financial burden (Vats et al., 2002). World Health

Organization has reported that about 171 million people worldwide were suffering from diabetes mellitus in the year 2000, while predicting that this figure will double come the year 2030 (WHO, 2002). This report was corroborated by Wild et al. (2004). Regions with

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approximately 140 million people worldwide suffer from diabetes (WHO, 1999) with the greatest interests in Asia and Africa where diabetes rate could rise to 2 to 3 folds than the present rates (ADA, 1997). Glucose plays a vital role in the regulation of β -cell insulin secretion (Hunt, 1995).

Among individuals with diabetes mellitus, three types are recognized; patients with insulin dependent or type 1 are ketosis prone and have increased or decreased frequency of certain histocompatibility antigens (HLA) on chromosome 6 and islet-cell antibodies (Silver and Loraine, 1986). This type has been termed juvenile onset diabetes. Type II patients are non-ketosis prone. Both type I and type II lead to hyperglycemia which largely causes the acute signs of diabetes; excessive urine (polyuria), resulting in compensatory thirst (Polydipsia) and increased hunger (polyphagia), blurred vision, unexplained weight loss, lethargy and changes in energy metabolism. Other examples of impaired glucose tolerance are insulinoma or reactive hyperglycemia (Goodman and Gilman, 2006).

Long-term hyperglycemic condition is associated with damage and failure of many organs such as eyes, kidney, nerves, heart and blood vessels (Kumar et al., 2008). Recently, it is suggested that formation of free radicals involved in the pathogenesis of diabetes and the development of diabetic complications, because a prolonged exposure to hyperglycemia increases the generation of free radicals and reduces capacities of the antioxidant defense system (Sanders et al., 2008). High levels of reactive oxygen have been found to play a role in the pathogenesis of several diseases including non-insulin dependent diabetes mellitus. Diabetic patients, both type I and type II exhibit abnormal antioxidant status, auto-oxidation of glucose and excess glycosylated proteins (Young et al., 1992). Oxidative stress in diabetes mellitus leads to tissue damage, with lipid peroxidation, inactivation of proteins, and protein glycosylation as intermediate mechanisms for complications including retinopathy, nephropathy and coronary heart diseases. The underlying causes of diabetic complications have been attributed to hyperglycemia, which results in oxidative stress, alterations in enzyme activities, protein glycosylation and several structural changes (Wolfe et al., 1991).

Many traditional plant treatments for diabetes mellitus are used throughout the world (Marles and Transworth, 1995). Alternative strategies to the current modern pharmacotherapy of diabetes mellitus are therefore urgently needed. Many herbs and plant products have been shown to have hypoglycemic action. Many traditional plant treatments for diabetes are used throughout the world.

The hypoglycemic effect of several plant extracts and herbal formulations have been confirmed which are being used as antidiabetic remedies and their therapeutic capabilities are investigated intensively (Sharma et al., 1992). Laboratory studies and ethnobotanical information

have shown that extracts of *Vernonia amygdalina*, *foxglove*, *pterocarpus*, *Allium cepa*, *Allium sativa*, *Anarcadium occidentale*, *Acalypha wilkeseria*, *Bridelia feruginea*, and *African mistoetle* have been found to lower blood sugar levels in experimental animals (Odoh et al., 2014).

Ceiba pentandra, commonly called the silk-cotton or kapok tree belongs to the Malvaceae family. It is an emergent tree of tropical rainforest predominant in South East Asia and often described as majestic, because of its wide application in traditional medicine (Ngounou et al., 1995). Various morphological parts of this plant have been reported to be useful and efficacious remedies against diabetes, hypertension and cardiac reflexology, headache, dizziness, constipation, mental disorder, pyrexia, peptic ulcer, rheumatoid arthritis and leprosy. It is also used as renal fluid mobilizer (Nounou et al., 1995). Folk medicine in Nigeria uses the root bark for the treatment of infections. In India and Malaysia, it is used for bowel complaints and also in the treatment of diarrheas in West Africa (Elumalai et al., 2012). It has been documented that *C. pentandra* possesses anti-inflammatory, antilipidemic, anthelmintic, angiogenesis and hepatoprotective activities (Elumalai et al., 2012).

The hypoglycemic activity of stem bark aqueous extract of *C. pentandra* at high doses (>800 mg/kg/day) on streptozotocin (STZ) induced type I diabetes has already been reported by Olusola et al. (2003). The present study aims at the evaluation of antidiabetic effect and hematological profile of methanol extract of *C. pentandra* stem bark on alloxan-induced diabetic rats.

MATERIALS AND METHODS

Collection and preparation of the plant

Fresh stem bark of *C. pentandra* was collected from Imo State, in the South Eastern part of Nigeria in July, 2014. The plant material was identified and authenticated by Mr. Alfred Ozioko, a plant taxonomist and staff of International Centre for Ethno medicine and Drug Development, Nsukka, Nigeria. The voucher specimen was deposited at the Herbarium of the Department of Pharmacognosy and Environmental Medicines, University of Nigeria, Nsukka. The stem bark was cleaned, air-dried and pulverized using Thomas Wiley Laboratory Mill Model 4.

The powdered plant material was then stored in an air-tight container to keep it moisture free until the time of use.

Extraction of the plant extract

A 1.10 kg of the powdered stem bark was macerated with 4.00 L of 80% methanol for 48 h and then filtered. The filtrate was concentrated in a vacuo. The brownish extract was weighed and stored in the refrigerator for further experiments.

Animal

White albino mice (20 to 30 g) and rats (140 to 170 g) bred in the Animal House of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used for the experiments after

permission had been obtained from the institutional Animal Ethics Committee. They were kept under standard conditions for seven days with access to food and water before the onset of the experiments.

Phytochemical analysis

Preliminary phytochemical analysis was carried out to detect the presence or absence of phytoconstituents using standard method (Harbourne, 1973; Evans, 2002).

Acute toxicity test

The LD₅₀ of the extract was determined in the mice intraperitoneal using Lorkes' method (1983).

Antidiabetic evaluation

Effect of methanol extract of C. pentandra on the blood sugar level of norm glycemic rats

Twenty-five animals divided into 5 groups of 5 animals each were used. The fasting blood sugar levels were taken before treatment with the extract. Group I, II and III received 200, 400 and 800 mg/kg of extract, respectively. Group IV received 5 mg/kg of glibenclamide (standard drug), while Group V received 5 ml/kg of normal saline. Blood samples were withdrawn from the animals at 0, 1, 2, and 3 h after treatment with extract and standard drug and blood sugar levels determined using Accu-check glucometer for acute study and at 7 and 14 days for sub-acute study.

Effect of methanol extract of C. pentandra on alloxan-induced diabetic rats

The basal blood glucose level of the rats was taken before induction of diabetes by collecting blood sample from the tail vein of the rats and the glucose concentration determined using Accu-check glucometer. Diabetes was induced by intraperitoneal administration of 150 mg/kg body weight of alloxan monohydrate freshly prepared in normal saline. On the 3rd day, the fasting blood sugar levels were determined and animals with fasting blood sugar levels of 200 mg/dl and above were considered diabetic and grouped into five of five animals each based on their blood sugar level range. Groups I, II and III animals were treated daily with 200, 400 and 800 mg/kg body weight of methanol extract of stem bark of *C. pentandra* respectively and Group IV animals received 5 mg/kg of glibenclamide only while group V received 5 ml/kg of normal saline. Blood samples were withdrawn from the animals at 0, 1, 2, and 3 h after treatment with extract and standard drug and blood sugar levels determined using Accu-check glucometer for acute study and at 7 and 14 days for sub-acute study.

Determination of hematological parameters

Blood samples were obtained from the optical plexus of the rats using a heparinized (plain) haematocrit capillary. The determination of the differential leucocyte count, packed cell volume, haemoglobin concentration, red blood cell count, and white blood cell count was done according to Akah et al. (2007).

Statistical analysis

Data obtained from the experiment were subjected to one-way

analysis of variance (ANOVA) using the SPSS program (Woodson, 1987). Results were expressed as mean standard error of mean (SEM) of triplicate value. P values of 0.05 were considered to be significant.

RESULTS

Phytochemical analysis

The stem bark extract shows the presence of the following phytoconstituents: alkaloids, carbohydrates, flavonoids, glycosides, saponins, steroids, tannins, reducing sugars, terpenoids.

Acute toxicity test

After intraperitoneal administration of the extract in mice at up to 5000 mg/kg dose, no death was observed. This shows that the extract is safe.

Antidiabetic evaluation

Intraperitoneal administration of alloxan monohydrate into the rats caused significant diabetogenic response in wistar albino rats with significant increase in the levels of blood sugar compared with non-induced rats. The blood glucose levels increased from 80 to 567 mg/dl. Following oral administration of the extract at studied doses, the blood glucose level was significantly reduced ($P < 0.05$). The doses of 400 and 800 mg/kg had significant effect on the blood glucose level as compared with diabetic untreated rats in both norm glycemics and alloxan-induced diabetic rats (Tables 1 to 4). Values obtained at 800 mg/kg compared favorably well with that of glibenclamide treated group.

Hematological evaluation

There were some levels of restoration of almost all the hematological parameters after 14 days of treatment with the extract and a standard anti-diabetic drug (glibenclamide) as shown in Table 5. The packed cell volume (PCV), Hb concentration, red blood cell (RBC) count and the mean cell volume (MCV) values were restored to near normal levels in diabetic rats. After treatment, leucopenia (reduced total white blood cell (WBC) count) associated with diabetes was significantly restored in rats that were given 200 mg/kg of the extract and glibenclamide. Similarly, RBC count in rats treated with 200 mg/kg extract was also lower ($P < 0.05$) than the RBC count obtained in rats treated with 800 mg/kg of the extract. At 400 and 800 mg/kg of the extract given, RBC, PCV and Hb were better restored while at 200 mg/kg the lymphocytes and neutrophils improved better than at 400 and 800 mg/kg.

Table 1. Results of phytochemical analysis of extract of the stem bark of *C. pentandra*.

Secondary metabolite	Inference
Alkaloids	+
Carbohydrates	+
Flavonoids	+
Glycosides	+
Saponins	+
Steroids	+
Tannins	+
Reducing sugars	+
Terpenoids	+
Fats and oils	-
Protein	-
Resins	+

-- Absent; += present.

DISCUSSION

Diabetes is the world's largest growing metabolic disorder, and as the knowledge on the heterogeneity of this disorder is advanced, the need for more appropriate therapy increases (Bailey et al., 1986). Recent available hypoglycemic agents produce some serious side effects like hypoglycemic coma (Larner, 1985) and hepatorenal disturbances (Amjad et al., 2013).

Apart from the side effects, their costs are high for management of diabetic patients and as such alternatives are needed for better management of diabetes. Hence, the search for safer and more effective anti-diabetic agents has continued. Also, there is WHO's recommendation for search on the beneficial use of medicinal plants in the management of diabetes mellitus (WHO, 1980). Investigation on hypoglycemic agents derived from medicinal plants also gained relevance. Compounds from plants at the other hand can provide an alternative treatment for diabetes (Rashid et al., 1989)

The phytochemical studies of the methanol extract revealed the presence of glycosides, saponins, terpenoids, reducing sugars, fats and oil, alkaloids, carbohydrates, flavonoids and proteins. These secondary metabolites have been reported to have anti-hyperglycaemic effect. Saponin extract of *Citrullus colocynthis* fruit has been reported to cause marked hypoglycemic effect in alloxan-induced diabetic rats (Abdel-Hassan et al., 2000). Steroidal saponins isolated from *Balanites aegyptiaca* Delile exhibited prominent hypoglycemic activity in streptozocin-induced diabetic mice (Kamel et al., 1991). Saponins isolated from the leaves of *Acanthopanax senticosus* injected to mice (100 and 200 mg/kg,) decreased experimental hyperglycemia induced by injecting of adrenaline, glucose, and alloxan, without affecting the levels of blood sugar in untreated mice (Maliehe et al., 2015). An unsaturated triterpene

acid isolated from an ethanolic extract of *Bumelia sartorum* root bark produced a hypoglycaemic effect in alloxan-induced diabetic rats (Naik et al., 1991). It increased glucose uptake and glycogen synthesis on isolated rat diaphragm and plasma insulin level (Naik, 1991). It appears that this effect was mediated by an insulin secretagogue effect in pancreatic β -cell. Senegin II, a triterpenoid glycoside isolated from rhizomes of *P. Senega* had been reported to have anti-diabetic effect on mice (Kako et al., 1996). Glycoside of leucopelargonidin isolated from the bark of *Ficus bengalensis* demonstrated significant hypoglycemic, hypolipidemic and serum insulin raising effects in moderately diabetic rats (Cherian et al., 1993). Also from phytochemical study of *C. pentandra* revealed the presence of epicatechin (Noreen et al., 1998) and flavonoids (Noreen et al., 1998; Ngounou et al., 2000).

Epicatechin, isolated from other plants had been found to stimulate β -cells regeneration, increased insulin secretion or possessed an insulin like effect (Marles and Farnsworth, 1995; Kameswara et al., 2001). Some flavonoids were reported to possess hypoglycaemic activity (Lamba et al., 2000; Cetto et al., 2000).

In both stages of acute toxicity studies, no animal died thus, the LD₅₀ is greater than 5000 mg/kg body weight of the extract. This shows it is within nontoxic range and that the extract is relatively safe (Loomis, 1996).

Alloxan-induced hyperglycemia has been a useful experimental model to study activity of hypoglycemic agents (Szkudelski, 2001). Alloxan is well known for its selective pancreatic islet β - cell cytotoxicity and has been used to induce diabetes mellitus in animals. It interferes with cellular metabolic oxidative mechanisms forming highly reactive superoxide radicals which destroy the insulin producing beta-cells in the pancreas. Intraperitoneal administration of alloxan (150 mg/kg) effectively induced diabetes in normal rats as reflected by glycosuria, hyperglycemia, polyphagia and polydipsia compared with normal rats.

The crude methanol extract of the stem bark of *C. pentandra* showed marked anti-diabetic activity on alloxan-induced diabetic rats and at doses of 200, 400 and 800 mg/kg body weight of the extract, it exhibited significant ($p < 0.05$) anti-diabetic activity after 14 days. When compared with 5 mg/kg of glibenclamide used as positive control. The dose of 800 mg/kg body weight of the extract was found to be more effective in giving higher percentage reduction of blood glucose level in alloxan-induced diabetic rats than 5 mg/kg glibenclamide. Also 400 mg/kg dose of the extract have similar anti-diabetic activity to 5 mg/kg doses of glibenclamide.

Glibenclamide is a standard anti-diabetic drug that stimulates insulin secretion from β -cell of isles of Langerhans. Also, glibenclamide is ineffective when there is no functional β -cells left in the pancreas and this therefore suggests that alloxan does not cause total destruction of the β -cells. The possible mode of action of the plant extract might be by potentiation of the insulin

Table 2. Effect of methanol extract of *C. pentandra* stem bark on alloxan-induced diabetic rats (acute study).

Treatment	Dose(mg/kg)	Mean fasting blood glucose level (mg/dl)		
		0 h	1 h	2 h
Control (normal saline 5 ml/kg)	-	283.40±0.32	294.90±0.35 (4)	307.90 ± 0.36 (- 8.65)
Extract	200	568.25±1.00	556.25±0.86* (2.1)	529.40±0.31 (6.84)
	400	480.75±0.64	435.20±1.20* (9.5)	426.80±0.20 (11.22)
	800	349.00±1.42	322.00±0.80* (7.7)	310.50±1.03 (11.03)
Glibenclamide	5	355.30±0.58	349.80±0.62* (1.7)	341.30±1.11 (3.94)

Values are ±SEM, n = 5, *P < 0.05, percentage reduction of blood glucose level in parenthesis.

Table 3. Effect of methanol extract of *C. pentandra* on plasma glucose level of normoglycemic rats (subacute study).

Days	Treatment				
	Extract (mg/kg)			Glibenclamide (mg/kg)	Normal saline (ml/kg)
	200	400	800	5	5
0	98.11±0.54	109.20±0.52	105.8±0.88	100.8±1.31	110.20±0.50
7	89.21±0.45 (9)	100.50±1.36 (7)	90.77±0.61 (14)	91.92±1.05 (8.8)	110.80±1.30
14	78±0.22 (20.5)	86.95±1.44* (21.2)	60.50±0.83* (33.6)	77.44±0.85* (23)	112.13±1.21

Values are ±S.E.M, n = 5, *p < 0.05, percentage reduction of blood glucose level in parenthesis.

Table 4. Effect of methanol extract of *C. pentandra* stem bark on plasma glucose level of alloxan-induced diabetic rats (subacute study).

Day	Treatment group				
	Extract (mg/kg)			Glibenclamide. (mg/kg)	Diabetic control (Normal saline (ml/kg)
	200	400	800	5	5
0	568.25±0.38	480.75±0.42	349.0±1.28	335.30±0.82	283.40±0.18
7	450±13±2.10* (20.82)	*340.14±07 (29.32)	288.01±1.50* (17)	299.666±040* (15.6)	377.80±0.18
14	402.15±0.66 (29.24)*	280.40±0.80* (41.67)	*182.01±0.41 (47.9)	180.90±0.01* (47.3)	483.90±0.52

Values are ±S.E.M, n = 5, *p < 0.05, percentage reduction of blood glucose level in parenthesis.

effect by increasing the pancreatic secretion of insulin from β -cells of islet of Langerhans or its release from the bound form.

The effect of *C. pentandra* could be related to a stimulation of remaining β cells or regeneration of β -cells. It had been reported that β -cells regeneration occurred through both increasing the replication of pre-existing β -cells and neogenesis from the precursor cells located in or by the pancreatic duct (Lei et al., 2004).

The assessment of haematological parameters could be used to reveal the deleterious effect of foreign compounds including plant extracts on the blood constituent of animals. They are used to determine possible alterations in the levels of biomolecules such as

enzymes, metabolic products, haematology, normal functioning and histomorphology of the organs (Megalhaes et al., 2008). The occurrence of anaemia in diabetes mellitus has been reported due to increased non-enzymatic glycosylation of RBC membrane proteins (Oyedemi et al., 2011). Oxidation of these proteins and hyperglycemia in diabetes mellitus causes an increase in the production of lipid peroxides that lead to hemolysis of RBC (Arun and Raesh, 2002).

In this study, the red blood cells parameters such as HB, PCV, MCH, MCHC were studied to investigate the beneficial/toxicity effect of *C. pentandra* extract on the anaemic status of the diabetic rats. The levels of RBC, Hb, hematocrit, and MCHC in the diabetic animals were

Table 5. Effect of methanol extract of *Ceiba pentandra* stem bark on the haematological parameters in alloxan-induced diabetic rats after two weeks.

Parameter	Experimental groups			Diabetic control	
	Extract (mg/kg)			Glibenclamide (mg/kg)	Normal saline (Mg/ml)
	200	400	800	5	5
RBC count per mm ³ blood (N × 10 ⁴ cells)	*4.41±0.06	*4.50±0.12	*4.73±1.35	*4.50±0.18	4.10±0.02
Hb (g/dl)	*12.45±0.50	*12.48±0.04	*13.60±1.12	*13.31±1.01	10.92±0.3
PCV (%)	50±1.36	43±1.16	44±2.13	38±0.06	51± 0.18
MCV (fl)	*83.90±0.01	*88.89±0.14	*93.02±0.11	*80.85±1.12	124.39±0.1
MCHC (pg)	33.65±0.6	31.20±0.18	30.91±0.08	35.02±1.20	21.41±0.13
MCH (pg)	28.23±1.10	27.73±0.04	28.75±1.11	28.32±0.13	26.63±0.71
(WBC count)per mm ³ blood (N × 10 ³ cells)	*7.55±0.07	7.14±0.12	7.02±1.16	8.31± 1.36	5.60±0.09
Neutrophils (%)	18±0.41	25.78±1.30	26.70±1.18	23.30±1.5	23±2.1
Lymphocytes (%)	*67.40±0.16	60.71±0.13	66.1±0.44	64.13±1.03	51.2 23±0.33

Values are ±S.E.M, n = 5, *p < 0.05.

reduced which may be due to the infections on the normal body system. This observation agrees with report of Baskar et al. (2006) who reported antihyperglycemic activity of aqueous root extract of *Rubia cordifolia* in streptozotocin-induced diabetic rats. The alterations of these parameters are well known to cause anaemic condition in man (Balasubramanian et al., 2009).

Following the administration of *C. pentandra* extract, the level of RBC and its differential were appreciably improved especially at 400 and 800 mg/kg doses of the extract. This gives an indication that the plant extract may contain some phytochemicals that can stimulate the formation or secretion of erythropoietin in the stem cells of the animals.

Erythropoietin is a glycoprotein hormone which stimulates stem cell in the bone marrow to produce red blood cells (Ollsson et al., 2006). The stimulation of this hormone enhances rapid synthesis of RBC which is supported by the improved level of MCH and mean corpuscular hemoglobin concentration (MCHC) (Abu-Zaition, 2010). These parameters are used mathematically to define the concentration of hemoglobin and to suggest the restoration of oxygen carrying capacity of the blood. Though the mechanism of action of this extract is not investigated in this study, but it may be attributed also to the ability of plant extract to lower lipid peroxidation level that causes hemolysis of erythrocytes (Ashafa et al., 2009). Therefore, it could inhibit peroxidation of polyunsaturated fatty acids in the cells membrane and hemolysis of red blood cells in the diabetic animals as reported by Torell et al. (1986) and Faure et al. (1991).

For normal glycemic rats that received the extracts, there was no significant change in hematological parameters. Anemia following administration of an agent can be because of lysis of blood cells and/or inhibition of blood cells synthesis by the active constituents of the

extract and decrease in hematological parameters in experimental animals has been associated with anemia (Lillie, 1965). Significant increase in differential lymphocytes and neutrophils count in the diabetic rat must have resulted from the stress induced by diabetes in accordance with stress induced lymphocytosis and neutrophilia in avian species (Forbes et al., 2002). There was restorative effect in the rats treated with methanol extract of *C. pentandra*. The 400 and 800 mg/kg of the extract produced the highest restorative effects in RBC count, Hb, and MCV that is significantly higher ($P < 0.01$) than the effect of 200 mg/kg of the extract. The 200 mg/kg had more restorative effect in WBC count and lymphocytes than 400 and 800 mg/kg of the extract.

In addition, there was no significant change in hematological parameters in the extracts treated animals compared to the control, which indicates that there is no lysis of blood cells and/or inhibition in blood cells synthesis by the active constituents of *C. pentandra* extract. The above statement suggests the non-toxicity of *C. pentandra* in rats.

Conclusion

The results obtained from this study shows that, the stem bark of *C. pentandra* was effective in reducing blood glucose level in normal and alloxan-induced diabetic animal with the added benefit of restoring reduced hematological parameters. The constituent(s) responsible for this effect requires further investigation.

Conflict of interest

The authors have not declared any conflict of interest.

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