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Full Length Research Paper

Antimicrobial, insecticidal, and antioxidant activity of essential oil and extracts of *Guarea kunthiana* A. Juss

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The goal of this study was to assess the antimicrobial activity of the essential oil and aqueous, alcoholic, and ethyl acetate extracts of *Guarea kunthiana* A. Juss against ten *Salmonella* serotypes from poultry origin (Enteritidis, Infantis, Typhimurium, Heidelberg, Mbandaka, Give, Saintpaul, Ohio, Gallinarum, and Agona); the insecticidal potential of the oil and extracts against larvae and adults of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) (Panzer, 1797); and also to determine the antioxidant activity of these compounds using the capture method of radical 2.2-diphenyl-2-picrylhydrazyl (DPPH). With respect to the antimicrobial activity of the essential oil of *G. kunthiana*, the most sensitive serotypes were Infantis, Typhimurium, and Give, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 54.6 µg/ml. Regarding the other serotypes tested, the action of the oil was classified as moderate, weak, or inactive. With respect to the extracts, the greatest microbial susceptibility was observed in the activity of the alcoholic extract, with MIC and MBC values of 0.39 mg/ml for the serotype Infantis, and MIC and MBC values of 0.78 mg/ml for the serotype Gallinarum. The results of the insecticidal activity of the essential oil and the extracts were found to be low, with 28% mortality of larvae and 12% of adults, at a concentration of 200 mg/ml. Regarding the extracts, the best results were observed using the alcoholic extract at concentrations of 10%, with 34 and 44% mortality of larvae and adults, respectively. The values of antioxidant activity showed that there were no significant differences between the synthetic antioxidant butylhydroxytoluene (BHT), the essential oil, and the alcoholic extract, revealing that both the essential oil and the alcoholic extract of *G. kunthiana* exhibited high antioxidant capacity.

Key words: Poultry farming, *Salmonella* species, *Alphitobius diaperinus*, mortality.

INTRODUCTION

The intense growth of aviculture sector has caused some problems, such as the emergence of enteric diseases caused mainly by the genus *Salmonella*, which can lead to loss of productivity due to increased mortality and contamination of products of poultry origin (Santos and Turnes, 2005).

Another major problem of the poultry sector is the proliferation of insects and pests, mainly *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) (Panzer, 1797), known in Brazil as "*cascudinho* of the aviaries." Besides

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damaging feed conversion and the weight gain of birds, larval and adult forms are supporters of viable pathogens on the external surface and in their digestive tract. Therefore, they are potential vectors of pathogens that cause diseases, mainly bacteria of the genus *Salmonella* species (Hazeleger et al., 2008).

Due to the constant concern of the population compared to synthetic insecticides, as toxicity to applicators, environmental pollution and the presence of residues in food, researchers have been encouraged to develop studies in order to insert new alternative control practices for pest control, especially the use of insecticides of plant origin (Almeida et al., 2004; Tavares and Vendramim, 2005; Pereira et al., 2008).

Members of Meliaceae family exhibit a wide variety of chemical compounds, including limonoids, triterpenes, steroids, diterpenes, sesquiterpenes and coumarins (Cortez et al., 2000; Garcez et al., 2004; Lago and Roque, 2009). The limonoids are the most abundant compounds, and probably the greatest representatives of the class of terpenes with insecticidal activity (Luo et al., 1999). In addition to these properties, these compounds may also act as antitumor, antifungal, antibacterial, antiviral (Champagne, 1992), antioxidants (Jayaprakasha and Patil, 2007), antileishmanial (Lima, 2006) and antimalarials (Kirandeep et al., 2009).

Studies conducted on the Brazilian native plant *Guarea kunthiana* A. Juss (Meliaceae) reported the insecticidal potential of its hexanic and ethanolic extracts obtained from the leaves (Mesquita et al., 2005; Coelho, 2006; Lima, 2006). However, the antimicrobial and antioxidant potential as well as the insecticidal activity against *A. diaperinus* have not been reported in the literature, thus the present study is the first one on the subject.

The goal of this study was to assess the antimicrobial activity of the essential oil and aqueous, ethyl acetate, and alcoholic extracts of *G. kunthiana* against ten *Salmonella* serotypes in order to determine the insecticidal activity of these compounds against *A. diaperinus* and their antioxidant activity.

MATERIALS AND METHODS

Collection and identification of plant

The leaves of *G. kunthiana* were collected from September to December, 2013 in a rural property of the western region of the State of Paraná, Brazil (Latitude 24°31' S, Longitude 53°44' W, altitude of 442 m). Drying of the leaves was held in an oven at 35°C for subsequent milling in a knife mill until obtaining the crushed plant material with particle size less than 0.42 mm. An exsiccate specimen was sent to the Herbarium of the State University of Oeste do Paraná (UNOP) for botanical identification, deposited under number 7843 Pandini, J. A.

Obtaining extracts

The preparation of extracts was carried out in accordance with the method proposed by Weber et al. (2014) and Ceyhan et al. (2012), with modifications. For the preparation of the aqueous extract, 10 g of crushed plant material were added to 100 ml of distilled water. This mixture was kept on a rotary shaker at 220 rpm for 24 h. After this period, the solution was filtered using Whatman No. 1 filter paper and centrifuged for 15 min at 5000 rpm. The supernatant was collected, thus obtaining the extract at the final concentration of 100 mg/ml. The extract was stored at 4°C. The organic extracts were prepared according to the same method of the aqueous extract. After collection of the supernatant, the extract was submitted to rota-evaporation. The crude extracts were diluted in 10% dimethylsulfoxide (DMSO) until a final concentration of 400 mg/ml and stored at 4°C.

Phytochemical prospection

The phytochemical tests for detecting the presence of steroids, triterpenoids, tannins, alkaloids, coumarins, saponins, anthocyanidins, and flavonoids were conducted according to the method proposed by Matos (1997).

Obtaining essential oil

The essential oil was obtained in accordance with the method proposed by Weber et al. (2014). To this end, about 60 g of crushed plant material were submitted to extraction by hydrodistillation in 700 ml of distilled water for a period of 3 to 4 h using a Clevenger-type apparatus. After extraction, the essential oil was stored at 4°C in the dark.

Antimicrobial activity

Microorganisms and test conditions

The microorganisms used were 10 *Salmonella* serotypes of greater occurrence in the western region of the State of Paraná, Brazil (Scur et al., 2014). These serotypes were isolated from feces (cloacal swabs), poultry litter (drag swabs), and poultry feed (flours/ingredients of poultry feed) from different poultry houses of the western region of the State of Paraná, Brazil, provided by a veterinary laboratory of Cascavel, Paraná, Brazil. The serotypes were Enteritidis, Infantis, Typhimurium, Heidelberg, Mbandaka, Give, Saintpaul, Orion, Gallinarum, and Agona.

For the test, the microorganisms were recovered from brain heart infusion broth (BHI) and incubated for 24 h at 36 ± 0.1°C. After this period, the microbial strains were standardized in saline solution (0.85%) until they reached the final concentration of 1×10^5 UFC/ml to serve as inoculum.

Determination of the minimum inhibitory concentration (MIC)

Essential oil and plant extracts

The MIC of the essential oil and plant extracts was determined according to the broth microdilution method proposed by Santúrio et al. (2007) and Weber et al. (2014). The essential oil was diluted in methyl alcohol and Mueller-Hinton broth (MHB) at a proportion of 1:10 until it reached the concentration of 7,000 µg/ml. A total of 150 µl of MHB were distributed from the second column in 96-well microtiter plates. The first columns received 300 µl of oil solution of *G. kunthiana* and, thereafter, dilutions of 7,000 to 3.4 µg/ml were performed. Finally, 10 µl of inoculum were added in each well and the plates were incubated for 18 to 24 h at 36 ± 0.1°C. After this time interval, 10 µl of 10% triphenyltetrazolium chloride (TTC) were

added and the plates were again incubated for 3 h at 36 ± 0.1°C. The presence of red coloring was interpreted as negative evidence of inhibitory effect of the essential oil.

Determination of the minimum bactericidal concentration (MBC)

The MBC of the extracts and the essential oil was performed in accordance with the method proposed by Weber et al. (2014). Before the addition of the TTC into all wells, an aliquot of 10 µl was withdrawn and inoculated on MH agar surface. The plates were incubated for 24 h at 36 ± 0.1°C and, after this period, the MBC was defined as the lowest concentration of essential oil able to cause the death of the inoculum.

An amount of 200 mg/ml of gentamicin was used as positive control and methyl alcohol as negative control to test the essential oil, and 10% DMSO was used to test the extracts.

Classification of antimicrobial activity

The MIC and MBC of the essential oil and the extracts were classified according to the criteria proposed by Sartoratto et al. (2004) for the essential oil, and according to Araújo (2010) for the plant extracts. For the essential oil, the activity was classified as high (<100 µg/ml), moderate (between 100 and 500 µg/ml), low (between 500 and 1000 µg/ml), and very low (above 1000 µg/ml). For the extracts, the classification was high (≤12.5 mg/ml), moderate (12.5 to 25 mg/ml), low (50 to 100 mg/mL), and very low (above 100 mg/ml).

Insecticidal activity

The assessment of the insecticidal potential of the essential oil and extracts of *G. kunthiana* was conducted according to the method proposed by Marcomini et al. (2009), with modifications. To this end, larvae and adults of *A. diaperinus* from a commercial poultry house of Maripá, Paraná, Brazil were used.

Application of plant extracts

Aqueous, alcoholic, and ethyl acetate extracts of *G. kunthiana* were diluted in 10% DMSO until they reached concentrations of 10 and 5%. The application of the extracts was performed directly on the insects, arranged in a Petri dish, and 1 ml was sprayed on each plate using a Potter tower calibrated with pressure of 0.70 kgf/cm^2. After application, the insects were transferred to another Petri dish and kept in a BOD camera (26 ± 1°C and 14-h photophase), and fed with about 2 g of poultry feed. Five repetitions with 30 insects each were prepared, with a total of 150 insects per treatment. The control procedure consisted of the application of sterile distilled water (general control) on the insects and also a control procedure with 10% DMSO.

Application of essential oil

The essential oil of *G. kunthiana* was diluted in 10% acetone until it reached concentrations of 100 and 200 mg/ml. The application on the larvae and adults of *A. diaperinus* was carried out following the same procedures of the extracts application. The control procedure consisted of the application of sterile distilled water and acetone at a concentration of 10%, following the same procedure performed in the other treatments. The assessment of insects' mortality was conducted after 10 days, considering dead those insects that did not respond to the touch of a clamp.

Analysis of data

The data were assessed to determine normality and homogeneity using Kolmogorov-Smirnov test and Levene's test (Central Limit Theorem), respectively. From this information, the variance was assessed using the F test and the averages were compared using Tukey's test at 5% significance employing the Statistica® software, version 7.0 (StatSoft Inc, Tulsa, USA).

Antioxidant activity

The antioxidant activity was determined using the method of reducing the free radical 2.2-diphenyl-1-picrylhydrazyl (DPPH) based on the method proposed by Scherer and Godoy (2009), Rufino et al. (2007), and Weber (2014). To this end, aliquots of 0.1 ml of the oil at a concentration of 6,000 µg/ml and aqueous of 100 mg/ml, ethyl acetate and alcoholic of 400 mg/ml extracts were treated with 3.9 ml of DPPH methanolic solution (0.2 mM) and slightly homogenized in a tube agitator. After agitation, the tubes were left to stand for 30 min in the dark. After the reaction time, the absorbance of samples was measured at 515 nm. An aliquot of 0.1 ml of the control solution (methyl alcohol, acetone, and water) was used for the negative control and the synthetic antioxidant butylhydroxytoluene (BHT) was used for the positive control under the same conditions of the negative control. Methyl alcohol was used as blank for the calibration of the spectrophotometer. The ability of free radical sequestration was expressed by the equation:

$$I\% = [(Abs_0 - Abs_1) / Abs_0] \times 100,$$

where Abs_0 is the absorbance of the control and Abs_1 is the absorbance of the sample. The IC_{50} (amount of antioxidant substance required to reduce by 50% the initial DPPH concentration) was calculated on the basis of the equation of the line obtained from the calibration curve. The tests were carried out in triplicate.

Analysis of data

The data obtained by calculations of DPPH radical sequestration capacity and the IC_{50} were assessed using Tukey's test, at 5% significance, employing the Sisvar software (Ferreira, 2011).

RESULTS AND DISCUSSION

Phytochemical prospecting

The phytochemical profile of extracts of *G. kunthiana* revealed the occurrence of triterpenoids in the aqueous and ethyl acetate extracts, and tannins and flavonoids in the aqueous and alcoholic extracts. The presence of alkaloids, coumarins, saponins, anthocyanins, and anthocyanidins was not observed (Table 1).

Antimicrobial activity

Considering the MIC and MBC values, it was possible to confirm that the essential oil exhibited high activity

Table 1. Classes of secondary metabolites present in the aqueous, alcoholic, and ethyl acetate extracts of *G. kunthiana*.

Classes of metabolite	Extracts		
	Aqueous	Alcoholic	Ethyl acetate
Pyrogallic tannins	+	+	-
Alkaloids	-	-	-
Coumarins	-	-	-
Saponins	-	-	-
Anthocyanins	-	-	-
Flavonoids	+	+	-
Triterpenoids	+	+	+
Steroids	-	-	-

+: Presence of the compound; -: Absence of the compound.

against *Salmonella* Infantis, *Salmonella* Typhimurium, and *Salmonella* Give, with MIC and MBC values of 54.6 µg/ml. Regarding *Salmonella* Saintpaul and *Salmonella* Agona, the values were moderate, with MIC and MBC of 218.7 µg/ml for the serotype Saintpaul, and MIC and MBC of 437 µg/ml for serotype Agona. For the serotypes Ohio and Gallinarum, the values showed low activity, with MIC and MBC of 875 µg/ml. With respect to *Salmonella* Heidelberg, the MIC and MBC values of 1750 µg/ml were considered very low. The essential oil had no activity against *Salmonella* Enteritidis and *Salmonella* Mbandaka (Table 2).

With respect to the classification of antimicrobial activity of extracts, it was possible to confirm that the alcoholic extract exhibited activity against the serotypes Infant and Gallinarum, with MIC and MBC values of 0.39 and 0.78 mg/ml, respectively. On the other hand, the ethyl acetate extract showed low antimicrobial activity against the serotypes tested, with MIC and MBC values ranging from 100 to 200 mg/ml. Lastly, the aqueous extract did not exhibit antimicrobial activity (Table 3).

Insecticidal activity

The 10% alcoholic extract exhibited the highest percentage of larval mortality (34.0%), whereas the ethyl acetate extract exhibited 21.3 and 26.0% at concentrations of 5 and 10%, respectively. On the other hand, the water extract exhibited the lowest mortality values, with 12.5 and 14.6% at concentrations of 5 and 10%, respectively, therefore showing low activity (Table 4).

With respect to the essential oil, it was observed that the concentration of 200 mg/ml exhibited higher mortality percentage (28.6%), followed by the concentration of 100 mg/ml, which exhibited lower mortality value (14.0%) (Table 5).

With respect to adult mortality values resulting from the application of the essential oil, it was observed that the mortality values were low, with 10.0% for the concentration of 100 mg/ml and 12.0% for the concentration of 200 mg/ml, since the two concentrations did not differ statistically (Table 5).

Antioxidant activity

Considering the results of the antioxidant activity, it should be noted that the IC_{50} values are inversely related to the DPPH sequestration percentage, since the higher the sequestration rate, the lower the IC_{50} value. It is observed that there were no significant differences between the positive control (BHT), the essential oil, and the alcoholic extract, this way demonstrating that they exhibited good antioxidant activity. Comparing the aqueous extract with the synthetic antioxidant, it was possible to observe that there were significant differences, thus demonstrating a moderate antioxidant capacity. On the other hand, the ethyl acetate extract exhibited low capacity of radical sequestration (Table 6).

The results of phytochemical screening were in line with data from the literature. Lago and Roque (2009) assessed the compounds present in the ethanolic extract of *Guarea macrophylla* and observed the presence of triterpenoids. Pereira et al. (2012) assessed the compounds present in the methanolic extract and observed the presence of flavonoids. Hernes and Hedges (2004) found tannins and triterpenoids in extractions with ethyl acetate solvents and water in *Guarea rubriflora* and *Guarea trichiliodes*.

Flavonoids are phenolic compounds which have broad biological and therapeutic functions and distributed in the leaves, seeds, flowers and roots of plants, and their biological activity depends on its chemical structure and relative orientation of groups in the molecule (Agati et al., 2012). Tannins are compounds responsible for the astringency of many fruits and other plant products. Having the ability to complex with proteins, this factor is responsible for the action in the control of insects, fungi and bacteria (Mello and Santos, 2002).

Regarding the results of antimicrobial activity, it was observed that there was variation of antimicrobial susceptibility among the serotypes tested. This variation was also reported by Carramiñana et al. (2004) and Scur et al. (2013), who explained the differences in some serotypes tested against different antimicrobials. The authors attributed this variation to the origin of the serotypes and the selective pressures that they may suffer as a result of the use of different antimicrobials, leading to the selection of resistant serotypes.

The mechanism of antimicrobial action of essential oils involve different actions, such as the cytoplasmic membrane disruption, interruption of proton motive force and the flow of electrons, active transport, and cellular content coagulation (Burt, 2004). One of the most important aspects is hydrophobicity of the chemical components, which allows a partition of lipids of the bacterial

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oil of *G. kunthiana* against the microorganisms tested.

Microorganism	MIC/MBC [a]		
	Essential oil[b]	Methanol[c]	Gentamicin[d]
S. Enteritidis	Na/Na	Na/Na	0.78/0.78*
S. Infantis	54.6/54.6	Na/Na	0.78/0.78*
S. Typhimurium	54.6/54.6	Na/Na	0.78/0.78*
S. Heildelberg	1750/1750	Na/Na	0.78/0.78*
S. Mbandaka	Na/Na	Na/Na	0.78/0.78*
S. Give	54.6/54.6	Na/Na	0.78/0.78*
S. Saintpaul	218.7/218.7	Na/Na	0.78/0.78*
S. Ohio	875/875	Na/Na	0.78/0.78*
S. Gallinarum	875/875	Na/Na	0.78/0.78*
S. Agona	437/437	Na/Na	0.78/0.78

[a]Minimum inhibitory concentration (MIC)/Minimum bactericidal concentration (MBC); [b]Tested at a concentration of 7,000-3.4 µg/ml; [c]Tested at a concentration of 7,000 µg/ml; [d]Tested at a concentration of 100-0.78 mg/ml; Na = No activity; Nt = Not tested* mg/ml.

Table 3. Minimum inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentration (MFC) of different extracts of *G. kunthiana* against the *Salmonella* serotypes tested.

Microorganism	MIC/MBC (mg/ml)[a]				
	Aqueous[b]	Ethyl acetate[c]	Alcoholic[d]	DMSO[e] 10%	Gentamicin[f] (200 mg/ml)
S. Enteritidis	Na/Na	200/200	200/200	Na/Na	0.78/0.78
S. Infantis	Na/Na	200/200	0.39/0.39	Na/Na	0.78/0.78
S. Typhimurium	Na/Na	200/200	200/200	Na/Na	0.78/0.78
S. Heildelberg	Na/Na	200/200	200/200	Na/Na	0.78/0.78
S. Mbandaka	Na/Na	200/200	200/200	Na/Na	0.78/0.78
S. Give	Na/Na	100/200	100/200	Na/Na	0.78/0.78
S. Saintpaul	Na/Na	200/200	200/200	Na/Na	0.78/0.78
S. Ohio	Na/Na	100/200	Na/ Na	Na/Na	0.78/0.78
S. Gallinarum	Na/Na	200/200	0.78/0.78	Na/Na	0.78/0.78
S. Agona	Na/Na	200/200	200/200	Na/Na	0.78/0.78

[a]Minimum inhibitory concentration (MIC)/Minimum bactericidal concentration (MBC) mg/ml; [b]Tested at a concentration of 50-0.04 mg/ml; [c]Tested at a concentration of 200 to 0.09 mg/ml; [d]Tested at a concentration of 200 to 0.09 mg/ml; [e]Tested at a concentration of 10%; [f]Tested at a concentration of 100-0.78; Na = No activity.

cell membrane and the mitochondria, entailing a possible leakage of cellular content (Burt, 2004; Holley and Patel, 2005).

The antimicrobial potential of the alcoholic extract can be explained by the presence of some secondary metabolites, such as tannins, triterpenoids, and flavonoids (Table 1). The antimicrobial action of flavonoids is probably related to the capacity to complex extracellular and soluble proteins, as well as the structures of the bacterial cell wall (Sato et al., 1996). Cushnie and Lamb (2011) suggest that the antibacterial activity of flavonoids can be attributed to damages in the cytoplasmic membrane (perforation and/or reduction of membrane fluidity), inhibition of the synthesis of nucleic acids (caused by inhibition of topoisomerase), and inhibition of the energetic metabolism (caused by inhibition of NADH-cytochrome C reductase).

The antimicrobial action of tannins may be related to the fact that these compounds are able to complex macromolecules such as polysaccharides and proteins. This way, tannins may cause denaturation and, consequently, they may change the proteins of the bacterial cell membrane. This action occurs with proteins due to non-specific interactions, such as hydrogen bridges, hydrophobic effects, and through covalent bonds (Simões et al., 2007).

Triterpenes are very frequent in plants and they have many biological activities, mainly antimicrobial and insecticidal action (Chung et al., 2011; Garcez et al., 2013). Their mechanism of action in the bacterial cell is

Table 4. Mortality percentage of cascudinho larvae (*A. diaperinus*) ten days after being submitted to direct application of the extracts and the essential oil of *G. kunthiana*, under laboratory conditions (26 ± 1ºC, 14-h photophase).

Treatment (extracts)	Mortality (%)
Control (water)	3.3 ± 1.49^{e}
DMSO - 10%	4.0± 1.24^{e}
Aqueous extract - 5%	12.5 ± 1.59^{d}
Aqueous extract - 10 %	14.6 ± 1.69^{cd}
Acetate extract - 5 %	21.3 ± 1.33^{bc}
Acetate extract - 10 %	26.0 ± 2.21^{b}
Alcoholic extract - 5 %	24.6 ± 2.26^{b}
Alcoholic extract - 10 %	34 ± 1.63^{a}
Treatment (essential oil)	**Mortality (%)**
Control (water)	2.6 ± 1.49^{c}
Acetone - 10%	3.3 ± 1.24^{c}
Oil - 100 mg/ml	14 ± 1.24^{b}
Oil - 200 mg/ml	28.6 ±1.69^{a}

Averages followed by the same letter in the column do not differ from each other according to Tukey's test (p<0.05).

Table 5. Mortality percentage of cascudinho adults (*A. diaperinus*) ten days after being submitted to direct application of the plant extracts and the essential oil of *G. kunthiana*, under laboratory conditions (26 ± 1ºC, 14-h photophase).

Treatment (plant extracts)	Mortality (%)
Control (water)	2.0 ± 1.33^{c}
DMSO - 10%	2.0 ± 0.81^{c}
Aqueous extract - 5%	16.6 ± 2.35^{b}
Aqueous extract - 10%	19.3 ± 2.44^{b}
Acetate extract - 5%	23.3 ± 1.82^{b}
Acetate extract - 10%	28.0 ± 2.26^{b}
Alcoholic extract - 5%	26.6 ± 3.49^{b}
Alcoholic extract - 10%	44.6 ± 4.42^{a}
Treatment (essential oil)	**Mortality (%)**
Control (water)	1.3 ± 1.33^{b}
Acetone - 10%	2.0 ± 0.81^{b}
Oil - 100 mg/ml	10 ± 1.49^{a}
Oil - 200 mg/ml	12 ± 1.33^{a}

Averages followed by the same letter in the column do not differ from each other according to Tukey's test (p<0.05).

related to the disruption of lipophilic compounds (Bagamboula et al., 2004).

Even though the presence of steroids, tannins, flavonoids, and triterpenoids in the aqueous extract was detected (Table 1), it did not exhibit antimicrobial activity. This fact can be explained by the assumption that these compounds are present in very small quantities or due to the amount of extract applied, which may not have been enough to promote inhibitory action (Degáspari et al., 2005). This finding can also be reported for the ethyl acetate extract, which had triterpenoids in its composition (Table 1) and exhibited low antimicrobial activity.

With respect to the effect on adults of *A. diaperinus*, the 10% alcoholic extract exhibited the greatest mortality value (44.6%), followed by ethyl acetate extract (23.3 and 28.0%) at concentrations of 5 and 10%, respectively, and lastly, the aqueous extract exhibited the lowest mortality values at the same concentrations (16.6 and 19.3%) (Table 5). It was possible to observe that there were differences in the activity of the different solvents used, which confirms the issue of affinity difference of solvents with respect to the different plant compounds. According to Ferri (1996), extreme polar extracts (aqueous) exhibit less activity when compared with extracts of intermediate polarity (ethanolic).

It is observed that for both larvae and adults the alcoholic extract exhibited the highest efficiency. Compounds such as tannins, present in the composition of this extract (Table 1), may explain the activity found, since they act against pests. They have the ability to bind with digestive proteins of insects acting as digestive reducers, significantly reducing the growth and survival of insects by turning off digestive enzymes and creating a tannin-protein complex of difficult digestion (Mello and Filho, 2002; Cavalcante et al., 2006).

Studies on the use of extracts of Meliaceae family against larvae of *A. diaperinus* are scarce on the literature, specifically G. *kunthiana* are not found. Similar results were reported for Zorzetti et al. (2012), evaluating the action of ethanol and aqueous extracts of some plants of Meliaceae family against the beetle *Hypothenemus hampei* results obtained in 44% mortality for the ethanol extract and 12% for the aqueous extract of *Azadirachta indica*, both in the concentration of 10%. Cosme et al. (2007), assessing the mortality of the larvae of coleopteran *Cycloneda sanguinea* with compound azadirachtina isolated from *A. indica*, had an index of 83.3 and 65.3% mortality at a concentration of 100 mg/ml for the first and second larval instar, respectively.

The essential oil exhibited greater activity against the larvae than against adults. The insecticidal action of essential oils can occur in different ways and may cause mortality, deformities at different stages of development, repellency, and deterrence. Through the contact, essential oils can interact with the tegument of insects, besides acting in digestive and neurological enzymes (Isman, 2006; Knaak and Fiuza, 2010).

The high antioxidant capacity of the alcoholic extract can be associated with the presence of phenolic compounds detected in the phytochemical prospection, such as flavonoids and tannins (Table 1). The antioxidant potential of phenolic compounds is due mainly to their reducing properties and chemical structure. These

Table 6. Index of 2.2-diphenyl-2-picrylhydrazyl (DPPH) (% of sequestration) and IC_{50} of the essential oil and the different extracts tested.

Test solution	DPPH sequestration (%)	IC_{50}
Positive control (BHT)	95.85±0.04[a]	9.27 ± 0.08[a]
Essential oil	91.52±0.09[a]	17.54 ± 0.18[a]
Alcoholic	92.60± 0.86[a]	15.33 ± 1.62[a]
Aqueous	76.54± 2.00[b]	45.30 ± 3.75[b]
Ethyl acetate	6.61 ±1.04[c]	176.84 ± 1.96[c]

The values correspond to the average and standard deviation of the triplicates. Values followed by the same letter in the column do not differ from each other according to Tukey's test ($p<0.05$).

characteristics are responsible for playing an important role in the neutralization or sequestration of free radicals and chelation of transition metals, acting both in the initiation step and in the propagation of the oxidative process (Chun et al., 2005; Souza et al., 2007).

Flavonoids can act as reducing agents, sequestrators of free radicals, metal chelators, or deactivators of singlet oxygen (Melo and Guerra, 2002; Canterle, 2005). Various tannins act as sequestrators of free radicals, which intercept the active oxygen forming stable radicals (Mello and Santos, 2007).

According to the results obtained, the essential oil and the extracts of *G. kunthiana* tested exhibited antimicrobial, insecticidal, and antioxidant activity. This study is the first report in the literature on antimicrobial activity and insecticidal potential against *A. diaperinus*, and antioxidant activity of the essential oil and the aqueous, alcoholic, and ethyl acetate extracts of *G. kunthiana*. At the same time, this study can serve as the basis for conducting further research on plants that have unknown biological potential. It is important to stress the importance of further studies in order to determine the action of the compounds present in the essential oil and extracts tested in isolation and in synergism, and even toxicity tests, which can contribute to the use of these products in the poultry sector.

Conclusion

The essential oil of *G. kunthiana* exhibited greater antimicrobial activity against the serotypes Infantis, Typhimurium, and Give. With respect to the extracts, the highest antimicrobial susceptibility was observed in the alcoholic extract against the serotypes Infantis and Gallinarum. The insecticidal activity values were considered low for the test with the essential oil and the plant extracts. The results of the antioxidant activity revealed that the essential oil and the alcoholic extract did not exhibit significant differences when compared with the synthetic antioxidant. The importance of further studies were highlighted to determine the action of the compounds present in the essential oil and extracts tested alone and in synergism and even toxicity tests, which may contribute to the application of these products in the poultry sector.

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Conflicts of interest

The authors declare have not declare any conflicts of interest.

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Full Length Research Paper

Gas chromatography mass spectrometry (GC-MS) analysis of ethanolic extracts of kolanut (*Cola nitida*) (vent) and its toxicity studies in rats

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In this study, gas chromatography-mass spectrometry (GC/MS) was used to analyse the isolated caffeine from kolanut and deternine the acute and chronic toxicity of the extract and the isolated caffeine. In chronic toxicity test, rats were divided into five groups (10 rats per group). Each rat was administered with normal saline (control group), crude kolanut extract (11.9 mg/kg), isolated caffeine (7.5 mg/kg), synthetic caffeine (6 mg/kg) or (6 mg/kg) decaffeinated kolanut extract orally for 90 days. Biochemical assessment and body weight of the rats were determined. In acute test, the limit test dose of 2000 mg/kg was administered to the rat and observed for 48 h post treatment. This dose caused behavioural changes but did not cause mortality in the rats tested. The results of the chronic administration showed that caffeine significantly ($P < 0.05$) decreased body weight. Liver enzymes were significantly ($P < 0.05$) increase, total plasma protein levels, creatinine, bilirubin, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and total serum cholesterol levels were also significantly ($P < 0.05$) higher. However, urea was significantly ($P < 0.05$) lower in the caffeine treated groups. The results of the GC-MS analysis showed that the isolated caffeine from kolanut extract contains 82.69% pure caffeine with 96% in quality. Our results showed that the kolanut extract is rich in high quality caffeine and chronic consumption of it is associated with significant toxic effects as shown by elevated biochemical parameters, and reduction in body weight.

Key words: Acute and chronic toxicity, kolanut extract, *Cola nitida,* caffeine, decaffeinated, caffeine extraction, gas chromatography-mass spectrometry (GC-MS), biochemical parameters.

INTRODUCTION

Cola nitida (vent.) Schott Endl., a member of the tropical family sterculiaceae, is indigenous to West Africa (Russel, 1955). Its fruits contain seeds known as kolanuts. The nuts are consumed by humans in different part of the world because of its stimulatory properties (Jayeola, 2001). Kolanuts are used as gesture of peace, friendship, hospitality and it is important in various social ceremonies and religious activities (Purgesleve, 1977; Hatasaka et al., 2001). It has been used also in folk medicine as an aphrodisiac and an appetite suppressant (Esimone et al., 2007).

Previous reports have shown that administration of kolanut extract stimulates the central nervous system activities (Scotto et al., 1987), increases the cardiac muscle

contraction (Chukwu et al., 2006), increases gastric acid secretion (Osim et al., 1991), increases glucose uptake in skeletal muscle in dogs (Salahdeen and Alada, 2009) and causes relaxation of smooth muscle (Salahdeen et al., 2014). The biological effects of the kolanut extract have been attributed to its caffeine content (Osim et al., 1991) even when the caffeine content in the kolanut extract has not been characterized.

The use of a natural caffeine source, such as guarana, coffee and kolanut, has increased in recent years for many purposes including athletic performance enhancement and weight reduction (Olsen, 2005). The pharmacological consequence of caffeine intake includes anorexia, agitation, nausea, tachycardia, psychomotor symptoms, and hypokalemia coupled with possible hypotension associated with excessive vasodilatation (American Psychological Association, 2007; Olsen, 2005; Hoffman et al., 2006). Some studies have shown that caffeine consumption during pregnancy is associated with an increased risk of foetal growth restriction (Grosso et al., 2001; Bicalho et al.,2002; Chiaffarino et al., 2006). Also excessive intake of caffeine can increase the risk of miscarriage (Barr and Sheissguth, 1991; Vik et al., 2003; Weng et al., 2008).

In another study, higher risk of ovarian cancer has been reported among women who drink five or more cups of caffeinated coffee per day compared to non-consumers of coffee (Lueth et al., 2008). Previous reports also indicated that caffeine enhances the formation of pancreatic tumors (Nishikawa et al, 1992) and mammary gland tumors (Welsch and Aylsworth, 1983; Nagasawa and Konishi, 1988). Inspite of the several studies that have been reported on different extracts of kolanut, there has not been any extensive analysis and characterization of the active compounds in the seed.

The present study therefore attempts to determine the nature and quantity of the active compounds in *Cola nitida*. Secondly, after an extensive search of the literature, only one report (Ikegwuonu et al., 1981) which was not detailed enough was found on the toxicity of an extract of kolanut. We therefore investigated further on the acute and chronic toxicities of the kolanut extract and the isolated caffeine compounds in the extract.

MATERIALS AND METHODS

Ethical considerations

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the Lagos State University College of Medicine and conform to the 1985 guidelines for laboratory animal care of the National Institute of Health (NIH).

Plant materials

Seeds of C. nitida used in this study were purchased from a market in Ibadan, Nigeria. Identification of the plant was carried out by the taxonomist of the Forestry Research Institute, Mr. K. A. Adeniji. Following identification, a specimen voucher number FHI 1008881 of the plant was deposited in the herbarium of the Forestry Research Institute, Ibadan, Nigeria.

Preparation of extracts

The seeds were dried under shade for two weeks and thereafter reduced to powdered form. Five hundred grams of the powdered seeds were obtained and exhaustively extracted with ethanol. Powdered kolanut was extracted twice with ethanol and water (80:20 v/v) for 72 h at room temperature. The solvent was evaporated at 40°C under vacuum (Rotavapor), and final ethanolic extract lyophilized (kolanut extract yield 15.7%). The stock solution was prepared as suspension with 4 g/100 ml of saline for this study (Salahdeen and Alada, 2009).

Extraction of caffeine and decaffeinated from kolanut

Five hundred grams (500 g) of the dried and ground sample kolanut was extracted three successive times with hot (100°C) water in a dark place (flask covered with aluminium foil) at room temperature (25°C). The collected extracts were filtered using N° 1 Whatman filter paper and evaporated to eliminate the solvent using rotary evaporator (at 45°C), and the obtained residues (crude extracts) kept in the refrigerator until use (Murray and Hansen, 1995; Hampp, 1996). Crude kolanut extract and sodium bicarbonate (Na2CO3) solution were added in a clean Erlenmeyer flask, swirl the mixture until all the sodium bicarbonate dissolves. Methylene chloride was also added to this mixture and vigorously swirled for about 20 min. This was allowed to stand until two separate layers were formed, that is, dark aqueous top layer and a clear methylene chloride bottom layer. The upper layer was an organic layer which contained the caffeine while the bottom layer contains decaffeinated. The two layers were evaporated separately with methylene chloride in the hood on a warm hot plate and the melting point of the caffeine recovered was determined (Murray and Hansen, 1995; Hampp, 1996).

Gas chromatography-mass spectrometry analysis

The GC-MS analysis was carried out using a Hewlett Packard Gas Chromatograph (Model 6890 series) equipped with a flame ionization detector and a Hewlett Packard 7683 series injectors, MS transfer line temperature of 250°C. The GC was equipped with a fused silica capillary column-HP-5MS (30 × 0.25 mm), film thickness 1.0 µm. The oven temperature was held at 50°C for 5 min holding times and raised from 50 to 250°C at a rate of 2°C/min,

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employing helium gas (99.999%) as a carrier gas at a constant flow rate of 22 cm/s. One microns of extract (1 mg dissolved in 1 mL absolute alcohol), at a split ratio of 1:30. MS analysis was carried out on Agilent Technology Network Mass Spectrometer (Model 5973 series) coupled to Hewlett Packard Gas Chromatograph (Model 6890 series) equipped with NIST08 Library software database. A mass spectrum was taken at 70 EV/200°C; a scanning rate of 1 scan/s. Identification of compounds was conducted using the database of NIST08 Library. The mass spectrum of the individual unknown compound was compared with the known compounds stored in the software database Library.

Animals

Healthy, young adult, Wistar albino rats of both sex, weighing 200-230 g, were obtained from the Animal House of the Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria, after obtaining approval from the ad hoc Ethical Committee of the College. The rats were fed standard rat chow (Livestock Feeds, Ikeja, Lagos State, Nigeria) and water *ad libitum*. The animals were maintained at standard laboratory conditions (12/12 h dark/light cycle, 20 ± 2°C temperatures, and 65 ± 5% humidity). The animals were fasted for 12 to 16 h before the commencement of the experiment.

Acute toxicity studies

The acute oral toxicity studies for the natural caffeine extracted from kolanut were carried out using a preliminary limit dose test of the up and down procedure statistical program-AOT 425statPgm – according to the World Health Organization (WHO) guideline (OEGD, 2002) and the Organization of Economic Co-operation (OECD, 2008) guideline for testing of chemicals. Five rats (100 to 2000 mg/kg) were used to determine the LD_{50}. The animals were fasted for 24 h following which different doses of the extract were administered orally and then observed for a period of 48 h for any signs of toxicity such as posture, reactive activities, obvious physiological signs and death.

Chronic toxicity studies

According to OECD guideline (OECD, 2008) rats were grouped into five groups of ten per grouping. Group I: the control received 1.0 mL of normal saline. Group II rats treated with crude ethanolic kolanut extract (11.9 mg/kg) (KNTE). Group III rats received (7.5 mg/kg) natural caffeine extracted from kolanut (NKNCAF). Group IV rats received (6 mg/kg) synthetic caffeine (SYCAF), and group V rats were given de-caffeinated kolanut extract (6 mg/kg) (DEKNTE). Each group received the treatment orally, daily for 90 days.

Measurement of body weight

Body weights of the treated rats were measured on the 1st, 45th and 90th day of the experiment with a mettle weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland). The weight difference on the 45 and 90 day in reference to the initial weight per group was calculated.

Collection of blood samples from rats

At the end of the 90-day experimental period, all animals were fasted for 16 to 18 h and then anaesthetized with intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg on the day 91. Blood samples for blood chemical analysis were taken from common carotid artery. All rats were sacrificed after the blood collection.

Serum biochemical parameters determination

Serum lipid profile including total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL-C) and low density lipoprotein (LDL-C) were determined according to the method of Meiathnin et al. (1978). Total protein was measured using Biuret reaction (Lanzatot et al., 2005), while albumin levels were measured by spectrophotometric estimation using the Sigma Diagnostic Kit (Sigma Diagnostics, UK). Globulin was obtained from the difference of total protein and albumin. Serum enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined by the method of Duncan et al. (1994) using enzyme kits prepared by Randox Laboratories Ltd, UK. Serum urea and creatinine levels were determined using spectrophotometric methods described by Coles (1986). The total bilirubin and conjugated bilirubin concentrations were determined as described by Balistreri and Shaw (1987). The unconjugated bilirubin concentration was calculated as the difference between total and conjugated bilirubin. Serum sodium and potassium were estimated using the reagent titrimetric method. Serum chloride was determined by the method of Schales and Schales (1941).

Statistical analysis

Data are expressed as means ± SE, where *n* equals the number of animals. The data were analyzed using two-way ANOVA. The Student-Newman-Keuls post hoc test was used to identify differences between individual means. The confidence interval was set at 95%, so that in all cases, results with a value of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

The results of the gas chromatography-mass spectrometry (GC-MS) analysis identified the various compounds present in the crude ethanolic extract of kolanut (Figure 1 and Table 1). In Figure 1, gas chromatogram analysis of the ethanolic extract of kolanut revealed 39 distinct peaks were identified by GC-MS while the compounds identified through the NIST08 database are listed in Table 1. The major compounds present in the ethanolic crude extract of kolanut identified by GC-MS were caffeine with RT: 19.601 and 19.761 of Total: 50.569% and quality: 96 (Table 1). The mass spectrum of caffeine was shown in Figure 1. Other components also identified in the seed of crude ethanolic extract of kolanut were hexadecanoic acid, ethyl ester (RT: 20.43), 9, 12-Octadecadienoic acid, ethyl ester (RT: 22.353), 9-Octadecadienoic acid, ethyl ester, ethyl oleate (RT:22.422), cyclohexanone, 2-methyl-5-(1-methylethenyl) Octadec-9-enoic acid decanoic acid, 10-(2-hexylcyclopropyl).

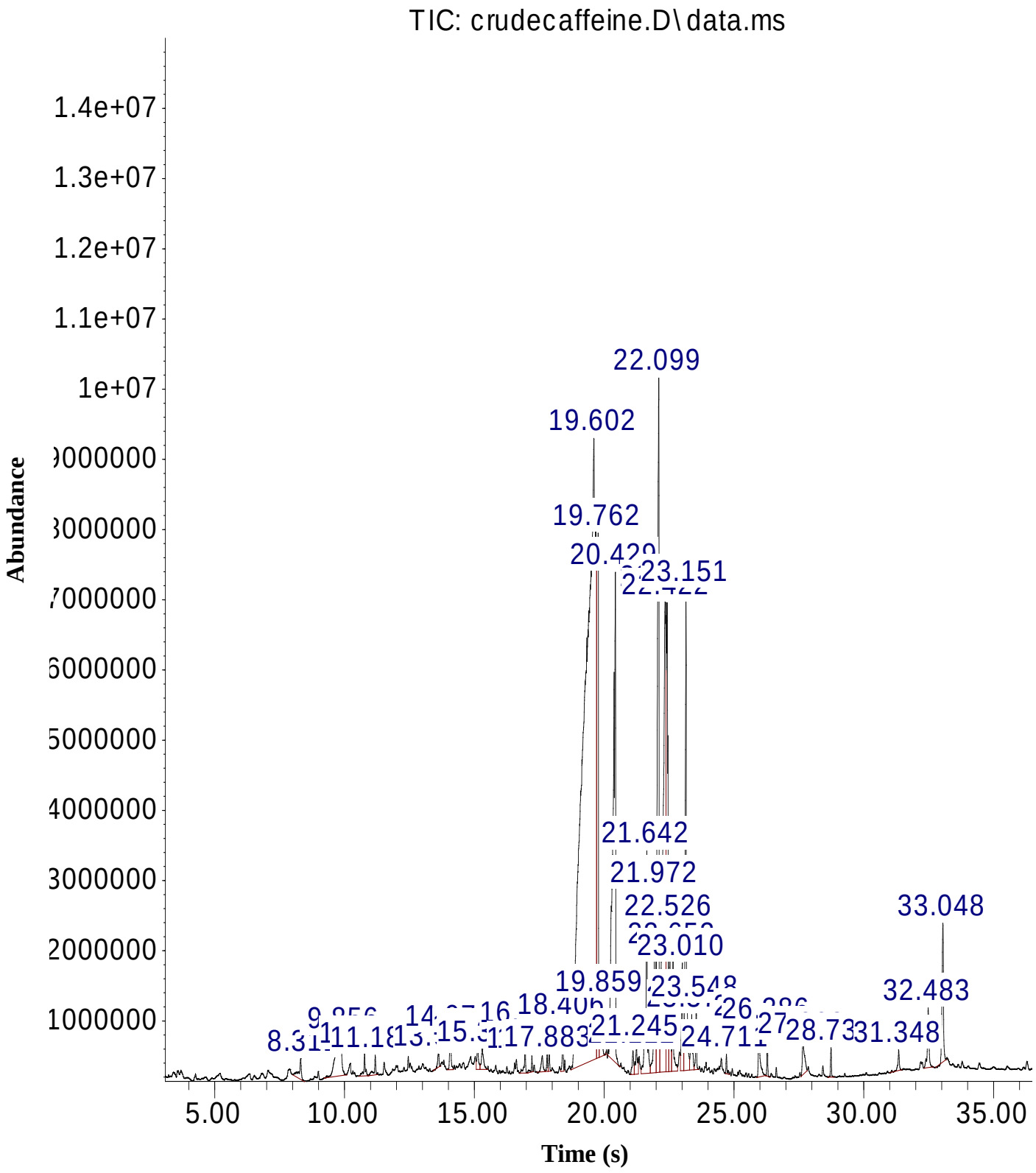

Figure 1. GC-MS chromatogram of ethanolic extract of kolanut peak 19.601 caffeine was identified as the major phyto-compound of the kola nut while other peaks were of the various phyto-compounds present.

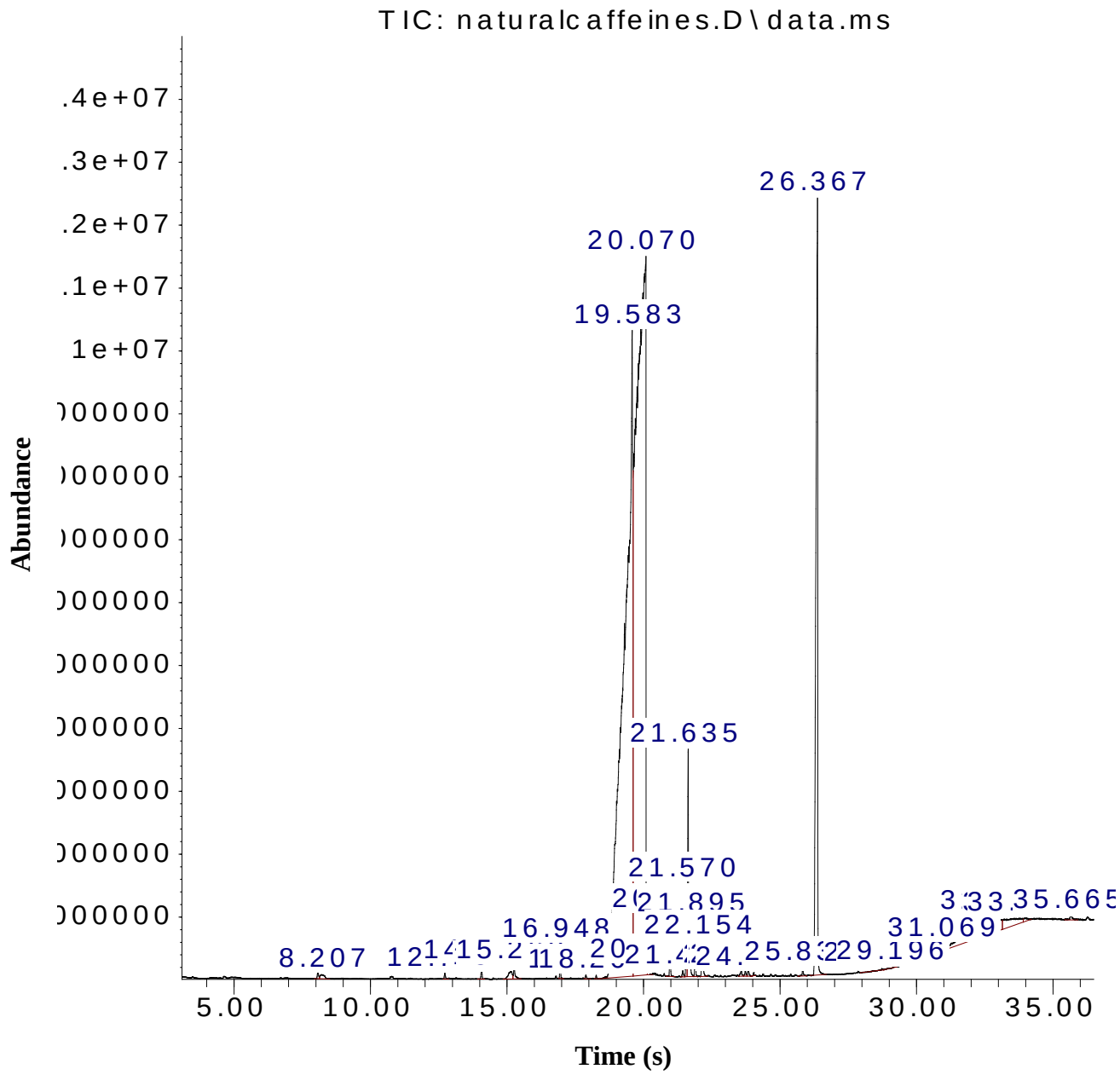

Figure 2. GC-MS chromatogram of caffeine extracted from kolanut Peak 19.583 caffeine was identified as the major phyto-compound of the kola nut while other peaks were of the various phyto- compounds present.

Also, the results of the GC-MS analysis of isolated caffeine from kolanut extract showed various compounds present in this extract (Figure 2 and Table 2). Figure 2 shows the gas chromatogram of the extract which shows 31 distinct peaks identified by GC-MS while the compounds were identified through the NIST08 L. The database is listed in Table 2. The major compound identified by GC-MS analysis was caffeine with RT: 19.583 and 20.07 with Total: 82.699% and quality: 97 (Table 2). Similarly, our results on the GC-MS analysis identified the various compounds present in the decaffeinated extract of kolanut (Figure 3 and Table 3). GC-MS analysis also shows that this extract contains caffeine of 4.449% and quality: 96. However, the major compound identified by GC-MS analysis was methyl 9, 10, methyllene-hexadecanoatwith RT: 20.59, Total: 29.736% and quality 89

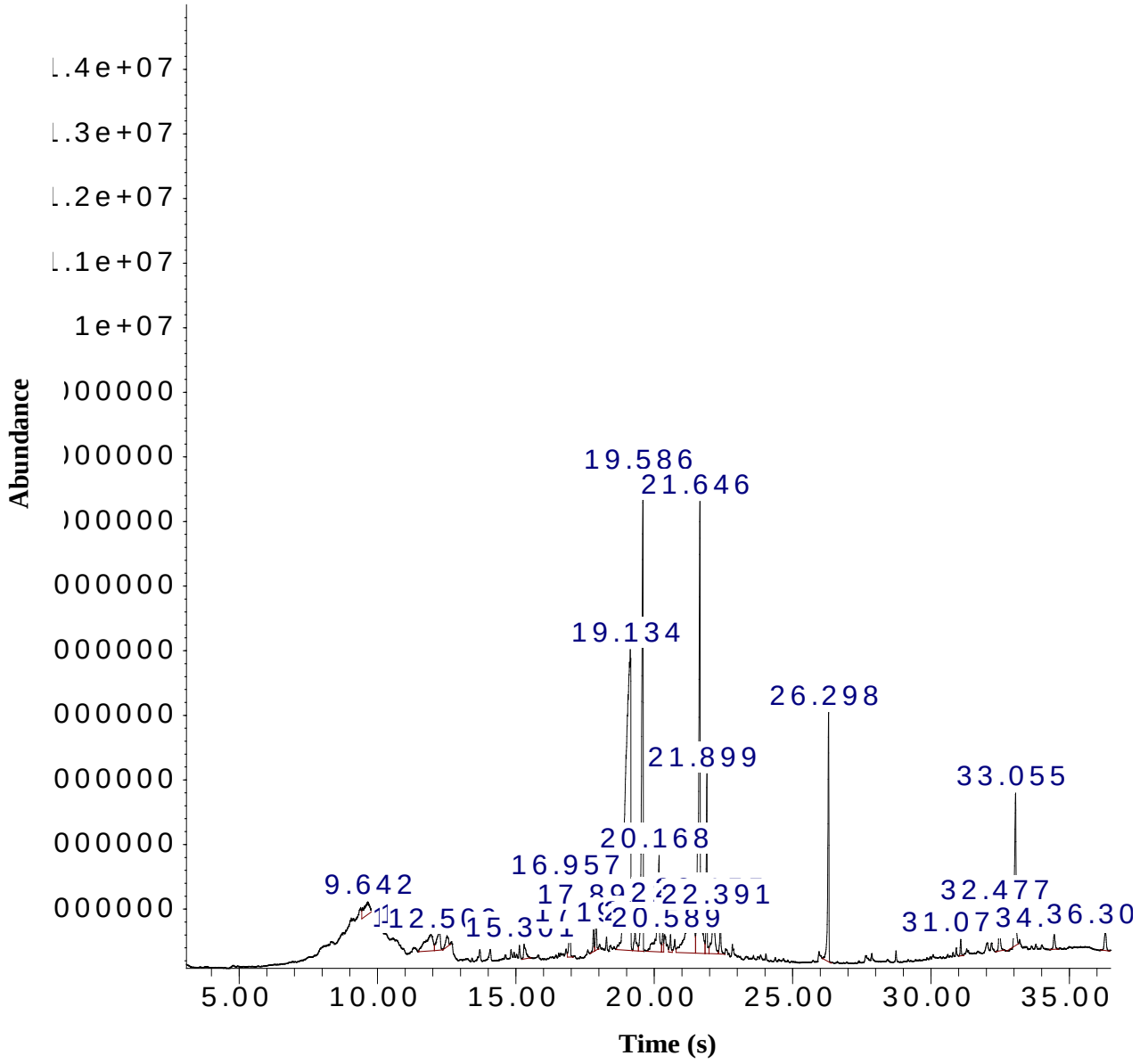

Figure 3. GC-MS chromatogram of decaffeinated kolanut extract peak 19.137 was identified as Methyl 9,10-methylene-hexadecanoat and as the major phyto-compound of the kola nut while other peaks were of the various phyto- compounds present.

(Table 3). Toxicity sign was observed in rats 48 h post oral treatment with 100 to 2000 mg/kg doses. Kolanut extract did not cause mortality but showed overt toxicity sign like restlessness, excitement and irritability for a short period. The oral median lethal dose (LD_{50}) of the crude kolanut extract in rats was therefore ≥ 200 mg/kg p.o. The rats treated with isolated caffeine from kolanut and synthetic caffeine (2000 mg/kg p.o) doses became recumbent and died within 72 h post treatment observation. However, an assessment based on 48 h post p.o treatment observation gave a calculated median lethal dose of 150 mg/kg p.o in rats.

During the period of chronic study, the rats started showing signs of toxicity such as restlessness, excitement and irritability and dieresis. These signs persist for few weeks during the experimental period. The control and decaffeinated groups did not show any of these signs.

At the end of the 90 days experimental period, the final body weights of the rats were determined in all the groups.

groups. Table 4 shows the percentage weight gain in each group. In all groups, there was an increase in body weight after the 90 days period of the experimentation. However, the percentage increase in weight in crude ethanolic kolanut extract, caffeine isolated from kolanut extract and synthetic caffeine were significantly lower ($p < 0.05$) when compared with the control. There were no significant changes in the percentage weight gain and final body weight of both the normal and decaffeinated kolanut extract when compared with their corresponding groups (data not shown). Table 4 shows the result of chronic consumption of crude ethanolic extract of kolanut, isolated caffeine from kolanut extract synthetic caffeine and decaffeinated on plasma electrolytes. There was no difference in the plasma level of all electrolytes measured when compared to control.

The result of chronic toxicity shows that the rat treated with kolanut extract showed a significant increase in AST serum level ($p < 0.05$) (Figure 4a). An ALP serum level also significantly increased in crude kolanut extract, natural caffeine isolated from kolanut and synthetic caffeine treated groups compared with the normal control group ($p < 0.05$) (Figure 4b). While serum levels of AST, ALT and ALP were significantly ($p < 0.05$) decreased in the decaffeinated treated group compared to control (Figure 4c).

Isolated caffeine, synthetic caffeine and decaffeinated groups significantly increased the serum total cholesterol ($p < 0.05$) (Figure 5a). Synthetic caffeine and decaffeinated groups also showed a significant ($p < 0.05$) increase in serum high density lipoprotein level (HDL) ($p < 0.05$) (Figure 5b). Serum level of very low density lipoprotein (VLDL) showed a significant increase in crude extract of kolanut ($p < 0.05$) and also significant increase in isolated caffeine, synthetic caffeine and decaffeinated groups compared to control group (Figure 5c). The serum levels of low density lipoprotein level (LDL) in isolated caffeine, synthetic caffeine and decaffeinated groups were significantly increased ($p < 0.05$) when compared with control (Figure 5d). Crude extract of kolanut, isolated caffeine from the kolanut, synthetic caffeine and decaffeinated kolanut groups showed significant ($p < 0.05$) increases in serum total glycerol levels compared to control group (Table 5). Although serum levels of both albumin and total proteins increased, these increases are not significant (Table 5). The serum level of urea decreased sinificantly in all test groups ($p < 0.05$) (Table 6). The caffeine treated group also showed significant increases in serum bilirubin and creatinine ($p < 0.5$) when compared with control (Table 6).

DISCUSSION

Kolanut seed under investigation has been widely consumed in both Western and central African because of its nerve stimulator property. Cola species have been cultivated in tropical South and Central America, the West Indies Sri Lanka and Malaysia (Arogba, 1999). In the present study, gas chromatography-mass spectrometry analysis of kolanut seed revealed that the crude ethanolic extract of kolanut contains 51.1% of caffeine with about 97% in quality and the isolated caffeine from kolanut extract was 82.9%. This is in agreement with the recent report (Salahdeen et al., 2014), in contrast to the earlier report of Oguntuga (1975) who reported that kolanut contains 0.05% of caffeine. The discrepancies in this study may be due to method of preparation used and other factors like time and period of collection, geographical origin and climatic conditions which influence the concentration of the active constituents particularly alkaloids and phenolic compounds present in the kolanut. Sometimes, the influence of these factors may be dominating, leading to absence of active constituents in the same plant collected from different regions, as evidenced by several research reports (Hicks et al., 1996; Arogba, 1999). Therefore, the varying of caffeine contents in kolanut reported by various workers may imply that the caffeine constituents of kolanut vary with season, environment and/or condition or time of collection, geographical and climatic conditions. The present study showed that kolanut extract and caffeine caused overt toxicity sign and death in rats 48 h post oral treatment in all concentrations administered. The oral LD_{50} of the crude extract of kolanut and caffeine was estimated to be ≥ 200 and 150 mg/kg in rats. According to the Organization for Economic Cooperation and Development (OECD, Paris, France) recommended chemical labelling and classification of acute systemic toxicity based on oral LD_{50} values as: very toxic ≥ 5 mg/kg; toxic > 5 ≤ 50 mg/kg; harmful > 50 ≤ 500 mg/kg; and not toxic or harmful > 500 ≤ 2000 mg/kg (Umoren et al., 2009; Ikechukwu et al., 2011). Based on this classification, the oral LD_{50} up to 200 mg/kg established in this study indicated relative oral harmfulness of this extract. The observation of overt toxicity signs in these experimental animals also pointed to that fact. This may be an indication that long term oral administration of the kolanut extract within these low doses could be harmful. The lower concentration (11.9, 7.5, 6 mg/kg/body weight) of crude kolanut extract, caffeine isolated from kolanut and synthetic caffeine used in this study were equivalent to three cups of coffee per day in human when the conversion is based on the metabolic body weight (70 kg) and one cup is equivalent to drinking 227 g of regular coffee, which contains 137 mg of caffeine (Donovan and De Vane, 2001). The dose was based on our previous study (Salahdeen and Alada, 2009).

The psychoactive behaviours effects of caffeine observed in this study were consistence with earlier reports (Bolton, 1981). Caffeine is a central nervous system and

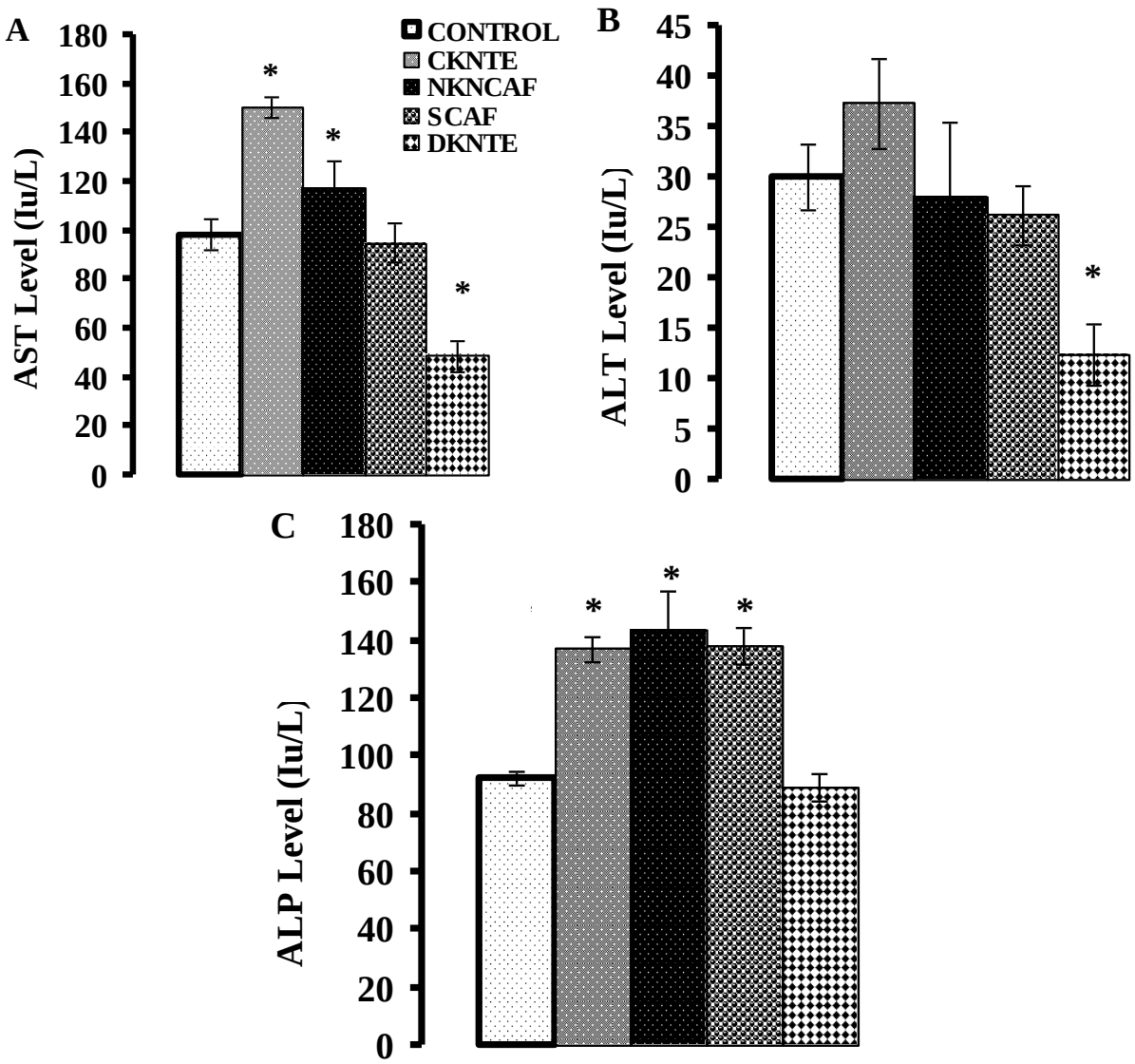

Figure 4. Effects of chronic consumption of normal saline (Control), crude extract of kolanut (CKNTE), caffeine isolated from kolanut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on blood plasma levels of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT) and (C) alkaline phosphatase (ALP). Values are means ± S.E. (N=10) ($P < 0.05$).

metabolic stimulant and is used both recreationally and medically to reduce physical fatigue and to restore alertness when drowsiness occurs (Conway et al., 2003). It also produces increased wakefulness, faster and clearer flow of thought, increased focus, and better general body coordination (Conway et al., 2003).

Another observation arising from this study is the effect of the extract on the average weight pattern in the treated rats. A significant reduction in body weight gain was recorded in caffeine groups. This observation is very important because the toxicity of chemical compounds in experimental animals is often associated with loss of body weight. This result is consistent with the previous studies (Zheng et al., 2004; Lopez-Garcia et al., 2006). Reduced body weight gain by caffeine may be attributed to increased thermogenesis (Greenberg et al., 2005), lipolysis and fat oxidation induced by caffeine (Conway et al., 2003). Also its may be related to an increase in body water loss through excessive urination observed during the experiment with the caffeine groups.

Another observation drawn from this study is the insignificant different plasma level of electrolytes in rats

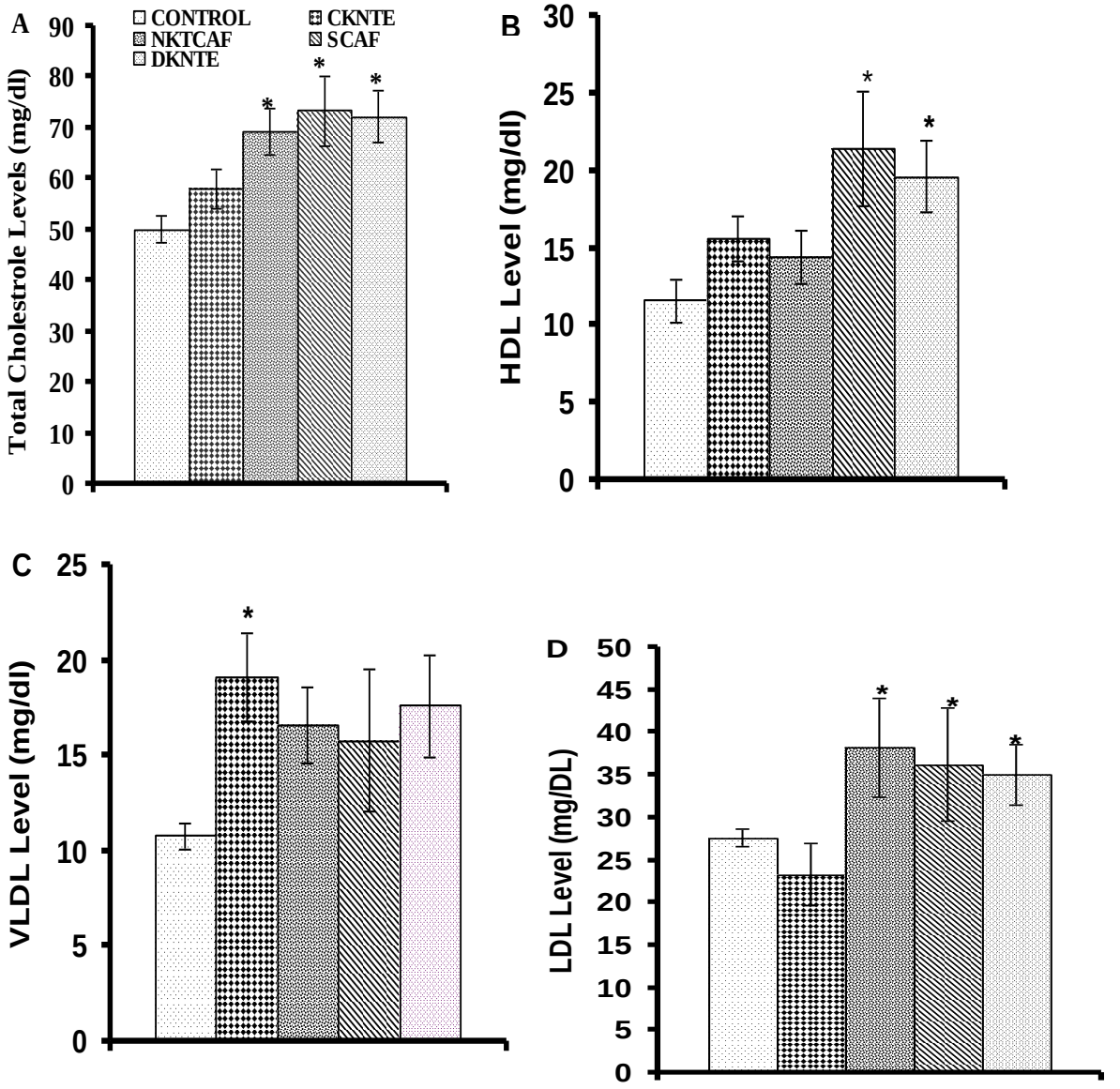

Figure 5. Effects of chronic consumption of normal saline (Control), crude extract of kolanut (CKNTE), caffeine isolated from kolanut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on blood plasma levels of (A) Total cholesterol, (B) high density lipoprotein (HDL), (C) very low density lipoprotein (VLDL),(D) and low density lipoprotein (LDL) Values are means ± S.E. (N=10) (P<0.05).

given caffeine or kolanut extract compared to controls. This observation was supported by the previous reports that chronic consumption of caffeine containingbeverages is as part of our normal lifestyle and these are not associated with electrolytes imbalance or dehydration (Nussberger et al., 1990; Neuhauser-Berthold et al., 1997). Although, acute studies have suggested that caffeine acts as a diuretic and studies have confirmed minor diuretic and natriuretic effects in experiments spanning a few hours (Robertson et al., 1978; Brater et al., 1983). To the best of our knowledge there is no information on the diuretic effects of caffeine at low doses. However, the only available report showed that 250 mg of caffeine increased urine volume and sodium excretion (Brater et al., 1983). The mechanism of the diuretic effect has been implied from work with theophyllines (Brater et al., 1983) which increased glomerular filtration rate (GFR) or renal blood flow (Cheul Do et al., 1997); this could

Table 1. GC-MS analysis of crude ethanolic kolanut extract showing the compounds identified by mass spectra, database, retention time, total percentage and relative qualitative of compounds.

PK no	Retention time	Mass spectral data	% total	Qual	Identified compound
1	8.311	11815 000091-20-3	0.305	97	Naphthalene
2	9.856	11004 000067-47-0	1.53	91	2-Furancarboxaldehyde, 5-(hydroxymethyl)-
3	10.772	10367 071932-97-3 35	0.224	35	4-Ethyl-2-hexynal, 4-Fluoro-2-methylphenol
4	11.184	12443 019550-10-8 59	0.182	59	2-Hexanone, 3,4-dimethyl-Hexanal, 2-ethyl-
5	13.615	172600 1000342-70-4	0.123	83	Octadecanesulphonyl chloride Tritetracontane
6	14.073	63985 000096-76-4	0.356	93	Phenol, 2,4-bis(1,1-dimethylethyl)
7	15.132	79877 000544-76-3	0.262	96	Hexadecane, Methoxyacetic acid, 2-tetradecyl ester
8	15.303	75930 000084-66-2	0.3	96	Diethyl Phthalate
9	16.957	91836 000124-10-7	0.28	99	Tridecanoic acid, 12-methyl-, methyl ester
10	17.22	46715 084820-13-3	0.103	64	Cyclohexene, 6-butyl-1-nitrobicyclo[10.1.0]tridec-1-ene
11	17.615	81212 000544-63-8	0.195	96	Tetradecanoic acid
12	17.804	126200 020294-76-2	0.102	87	1,2-Octadecanediol Dichloroacetic acid, heptadecyl ester
13	17.884	101149 000593-45-3	0.113	50	Octadecane
14	18.405	35634 1000186-25-5	0.292	38	11-Oxa-tricyclo[4.4.1.0(1,6)]undecan-2-ol Cyclohexanone
15	19.601	55120 000058-08-2	44.735	96	Caffeine
16	19.761	55118 000058-08-2	5.634	97	Caffeine
17	19.858	44940 000083-67-0	0.868	94	Theobromine
18	20.43	124589 000628-97-7	7.968	99	Hexadecanoic acid, ethyl ester
19	21.111	111881 029743-97-3	0.225	97	cis-10-Heptadecenoic acid, 9-Hexadecenoic acid
20	21.243	122785 054546-22-4	0.348	83	Ethyl 9-hexadecenoate, Z-11-Tetradecenoic acid
21	21.643	133716 000112-62-9	2.027	99	9-Octadecenoic acid (Z)-, methylster
22	21.972	67169 051937-00-9	2.009	92	9,12-Tetradecadien-1-ol, (Z,E)-
23	22.099	142891 003220-60-8	7.02	91	Methyl 2-octylcyclopropene-1-octanoate
24	22.353	142890 007619-08-1	8.636	99	9,12-Octadecadienoic acid, ethyl ester
25	22.422	144401 006512-99-8	4.91	98	9-Octadecenoic acid, ethyl ester, Ethyl Oleate
26	22.525	124556 000057-11-4	1.089	99	Octadecanoic acid
27	22.65	145979 000111-61-5	0.878	97	Octadecanoic acid, ethyl ester
28	23.011	67169 051937-00-9	0.96	90	9,12-Tetradecadien-1-ol, (Z,E)-1,2-Dioctylcyclopropene
29	23.148	24816 007764-50-3	3.794	83	Cyclohexanone, 2-methyl-5-(1-methylethenyl)
30	23.371	122782 1000190-13-7	0.548	87	Octadec-9-enoic acid Decanoic acid, 10-(2-hexylcyclopropyl)
31	23.549	122785 054546-22-4	0.379	90	Ethyl 9xadecenoate, cis-10-Nonadecenoic acid,
32	24.71	124592 000628-97-7	0.11	97	Hexadecanoic acid, ethyl ester, Octadecanoic acid
33	25.963	158684 023470-00-0	0.567	94	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)
34	26.284	192055 027554-26-3	0.239	94	1,2-Benzenedicarboxylic acid, diisooctyl ester
35	27.663	108922 056554-35-9	0.48	96	9,12, 13, 17-Octadecadien-1-ol, (Z,Z)
36	28.739	198715 000111-02-4	0.165	95	2,6,10,14,18,22-Tetracosahexaene2,,23-hexamethyl-E,
37	31.348	203745 000059-02-9	0.18	97	Vitamin E, dl-.alpha.-Tocopherol
38	32.481	199251 000083-48-7	0.612	99	Stigmasterol
39	33.047	199879 000083-47-6	1.249	98	bata, gamma.-Sitosterol
	Total		99.997		

induce diminished tubular reabsorption of sodium which may lead to diuresis (Brater et al., 1983). Therefore, the effects of chronic caffeine consumption on water-electrolyte status need to be fully investigated. Repeated treatment with caffeine and kolanut extract significantly increased the level of serum AST and ALT, which was consonant with earlier observation (Adedapo et al., 2007). Serum ALT is known to increase when there is liver cell damage and it has been employed as a tool for measuring hepatic necrosis (Kaplan, 1986). However, AST is not a liver specific enzyme as high levels of the enzyme can also be found in skeletal and cardiac muscle

Table 2. GC-MS analysis of isolated caffeine from kolanut showing the compounds identified by mass spectra, database, retention time, total percentage and relative qualitative of compounds.

PK no	Retention time	Mass spectral data	% total	Qual	Identified compound
1	8.071	11814 000091-20-3	0.057	97	Naphthalene
2	8.208	11814 000091-20-3	0.119	97	Naphthalene
3	12.723	48612 001931-63-1	0.041	91	Nonanoic acid, 9-oxo-, methyl este
4	14.067	70187 000111-82-0	0.086	96	Dodecanoic acid, methyl ester
5	15.137	75933 000084-66-2	0.19	97	Diethyl Phthalate
6	15.263	75933 000084-66-2	0.132	97	Diethyl Phthalate
7	16.946	91835 000124-10-7	0.172	97	Methyl tetradecanoate
8	17.89	102782 005129-66-8	0.031	87	Tetradecanoic acid, 12-methyl-, methyl ester
9	18.267	102770 007132-64-1	0.019	96	Pentadecanoic acid, methyl ester
10	19.583	55118 000058-08-2	36.551	97	Caffeine
11	20.07	55118 000058-08-2	46.148	98	Caffeine
12	20.161	119537 000084-74-2	0.08	46	Dibutyl phthalate
13	20.974	130506 056166-83-7	0.329	86	Methyl 2-ethylhexyl phthalate
14	21.437	99559 007206-25-9	0.049	93	9-Octadecene, (E)-
15	21.569	132259 002462-85-3	0.517	99	9,12-Octadecadienoic acid, methyl ester
16	21.638	133716 000112-62-9	1.362	99	9-Octadecenoic acid (Z)-, methyl ester
17	21.895	135381 000112-61-8	0.301	98	Octadecanoic acid, methyl ester
18	22.153	91910 016530-58-8	0.321	98	2-(4'-Hydroxyphenyl)-2-(4'-methoxy phenyl)propane
19	23.583	133702 013481-95-3	0.067	98	10-Octadecenoic acid, methyl ester
20	23.732	54765 003045-76-9	0.047	60	Cyclododecanone, 2-methylene- droxypropyl ester
21	23.841	132278 002566-97-4	0.063	93	9,12-Octadecadienoic acid, methyl ester, (E,E)
22	24.035	156020 1000352-20-6	0.017	76	Hexadecanoic acid, 14-methyl-, methyl ester
23	25.832	192055 027554-26-3	0.063	90	1,2-Benzenedicarboxylic acid, diisooctyl ester
24	26.37	192055 027554-26-3	9.41	91	1,2-Benzenedicarboxylic acid, diisooctyl ester
25	28.733	65539 122723-58-4	0.002	27	2-Oxabicyclo [4.4.0]dec-9-en-8-one 1,3,7,7-tetramethyl
26	29.196	64771 031897-93-5	0.008	22	N-Methyl-1-adamantaneacetamide
27	31.067	217228 019095-24-0	0.066	50	Octasiloxane -1-15-hexadecamethyl
28	32.996	217228 019095-24-0	2.791	68	Octasiloxane -1-15-hexadecamethyl
29	33.825	217228 019095-24-0	0.774	58	Octasiloxane -1-15-hexadecamethyl
30	33.991	217228 019095-24-0	0.084	58	Octasiloxane -1-15-hexadecamethyl
31	35.668	213584 019095-23-9	0.093	53	Heptasiloxane 1-13-tetradecamethyl-
		Total	99.99		

as well as red blood cells (Etuk and Muhammad, 2010). Transaminases play an important role in protein and amino acid metabolism which are found in the cells of almost all the body tissues and when diseases or injuries affected these tissues, they are released into the blood stream (Kuzminskaya and Bersan, 1975). Increase in serum ALP may be considered as an indicator of cholestasis, which may result from intracellular hepatic canaliculli obstruction associated with inflammation (Birkner et al., 2006).

Since we did not study the histology of the internal organ in this present study, it will not be unreasonable to conclude that cholestasis is responsible for the observed significant increases in the level of both ALT and ALP in the caffeine treated groups. This is particularly due to the fact that an increase in total bilirubin with a preponderance of the conjugated types was also observed in this study. However, ALP levels in the blood are also a good indicator of bone activities since osteoblasts secrete large quantities of this enzyme (Rock et al., 1986). It can also be deduced from this study that exposed rats to caffeine may have led to the disruption in the activity of these osteoblasts, thus leading to increase in the mean ALP values.

Blood urea nitrogen is a part of urea, the waste product that is left over from the breakdown of protein. Urea

Table 3. GC-MS analysis of decaffeinated kolanut extracts showing the compounds identified by mass spectra, database, retention time, total percentage and relative qualitative of compounds present in caffeine isolated from kolanut extract.

PK no	Retention time	Mass spectral data	% total	Qual	Identified compound
1	9.644	75930 000084-66-2	1.445	97	Diethyl Phthalate, Phthalic acid, cyclobutyl ethyl ester
2	11.933	55118 000058-08-2	2.498	96	Caffeine
3	12.225	55120 000058-08-2	1.367	96	Caffeine
4	12.511	55120 000058-08-2	0.584	96	Caffeine
5	15.303	75932 000084-66-2	0.670	97	Diethyl Phthalate
6	16.957	91836 000124-10-7	1.700	99	Methyl tetradecanoate
7	17.804	78201 000295-65-8	0.468	93	Cyclohexadecane, Hexadecyl heptafluorobutyrate
8	17.901	102766 007132-64-1	0.835	60	Pentadecanoic acid, methyl ester,Tetradecanoic acid,
9	19.137	55118 000058-08-2	0.445	98	Hexadecanoic acid, methyl ester
10	19.309	133727 1000333-61-3	0.829	58	trans-13-Octadecenoic acid, methyl ester
11	19.583	113705 005129-60-2	12.897	98	Pentadecanoic acid, 14-methyl-, methyl ester
12	20.167	102726 000057-10-3	4.616	99	n-Hexadecanoic acid
13	20.31	121344 074685-33-9	0.510	99	3-Eicosene, (E)-
14	20.59	122810 1000336-38-0	29.736	89	Methyl 9,10-methylene-hexadecanoat
15	21.288	133727 1000333-61-3	6.618	98	trans-13-Octadecenoic acid, methyl ester
16	21.643	133716 000112-62-9	15.574	99	7-, 9-, 10- Octadecenoic acid (Z)-, methyl ester
17	21.901	135390 000112-61-8	3.129	98	Octadecanoic acid, methyl ester
18	22.152	122781 000506-17-2	2.560	98	cis-Vaccenic acid, cis- trans-13-Octadecenoic acid
19	22.393	124558 000057-11-4	0.992	96	Octadecanoic acid
20	26.301	119596 004376-20-9	5.325	87	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester
21	31.079	208064 004651-48-3	0.330	35	Stigmasta-5,22-dien-3-ol, acetate(3.beta.)-
22	32.475	199250 000083-48-7	1.212	99	Stigmasterol
23	33.053	199878 000083-46-5	4.463	97	beta.-Sitosterol, gama.-Sitosterol
24	34.455	199256 001058-61-3	0.485	87	Stigmast-4-en-3-one, Stigmast-4-en-3-one methy
25	36.303	64708 056619-93-3	0.705	22	Propanamide, N-(3-methoxyphenyl)-2 ,2-dimethyl-
	Total		99.993		

Table 4. The effects of chronic consumption of normal saline (Control), crude extract of kola nut (CKNTE), caffeine isolated from kola nut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kola nut extract (DKNTE) on electrolytes.

Parameter	Control (NS)	Crude kola nut extract (CKNTE)	Isolated caffeine from kola nut (NCKNT)	Synthetic caffeine (SYCAF)	Decaffeinated kolanut (DCAKNT)
Na (mmol/L)	144±1.2	137.8±2.4	138.2±1.1	144±1.6	139.6±1.1
K+ (mmol/L)	6.9±0.5	7.7±0.4	6.7±0.9	6.06±0.2	4.9±0.06
Cl^- (mmol/L)	101.2±0.9	97.8 ±1.07	98.6±1.1	100.4±1.5	100.4±1.1
HCO3 (mmol/L)	21.0±0.4	21.8±0.5	23.2±0.8	26.4±2.4	21.0±1.04

Values are expressed as mean ± SE. (N=10).

Table 5. The effects of chronic consumption of normal saline (Control), crude extract of kola nut (CKNTE), caffeine isolated from kola nut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on serum level total proteins, albumin and trigylcerol.

Parameter	Control (NS)	Crude kolanut extract (CKNTE)	Isolated caffeine from kolanut (NKNCAF)	Synthetic caffeine (SYCAF)	Decaffeinated Kolanut (DKNTE)
T. Protein	5.8±0.1	7.2±0.1	7.3±0.3	6.9±0.09	5.8±0.2
Albumin	2.8±0.08	3.2±0.07	3.2±0.06	3.1±0.1	3.2±0.05
Triglycerol	54±3.4	94.8±1.5*	14.4±1.7*	21.4±3.7*	19.6±2.3*

Values are expressed as mean ± SE. (N=10). *(p<0.05).

Table 6. The effects of chronic consumption of normal saline (Control), crude extract of kolanut (CKNTE), caffeine isolated from kolanut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on serum level of urea, creatinine and total bilirubin.

Parameter	Control (NS)	Crude kola nut extract (CKNTE)	Isolated caffeine from kolanut (NCKNT)	Synthetic caffeine (SYCAF)	Decaffeinated kolanut (DCAKNT)
Urea	56.6±5.1	45.4±5.1	40.6±2.6	42.6±2.2*	27.6±0.8*
Creatin	52.7±3.6	41.3±7.8	62.3±12.2*	71.1±9.0*	63.3±7.8*
Bilirubin	0.2±0.02	0.7±0.04	0.6±0.1	0.5±0.07	0.4±0.05*

Values are expressed as mean ± SE. (N=10). *(p<0.05).

circulates in the blood until it is filtered out by the kidneys and excreted in the urine. If the kidneys are not functioning properly, there will be excess urea levels in the blood stream. It has been reported (Ikegwuonu et al., 2006) that chronic administration of caffeine increased serum urea while others (Jossa et al., 1993) showed that there is no relationship between caffeine consumption and the concentration of urea in serum of rats. The observed significant decrease in serum urea in the present study pointed out that therapeutic advantage can be taken of the kolanut extract because of its ability to reduce uric acid in hyperuricemia, a condition that can pre-dispose to gouty arthritis, intense inflammation of soft tissues on which uriate crystals are deposited when taken at lower doses (Jossa et al., 1993).

Creatinine is a compound that is produced by the body and excreted in the urine. Compounds that leave the body in the urine are processed by the kidney, therefore creatinine may be used to monitor the kidney function. The observed increased serum creatinine in the caffeine and kolanut treated groups indicates that chronic consumption of caffeine or kolanut may result in renal dysfunctions and development of nephritis (Ikegwuonu et al., 2006). The significant decrease in serum lipid profile in the this study is in agreement with previous study which showed that triglycerol level was decreased after treatment with caffeine (Ikegwuonu et al., 1981; Birkner et al., 2006). Also, the observed significant increase in glycerol, total cholesterol, HDL, LDL, VLDL and triglycerides in caffeine groups and decaffeinated kolanut extracts treated animals in this study agree with other reports (Curb et al., 1986). Several lines of evidence favour the presence of a causal relation between coffee drinking and higher levels of total cholesterol and LDL cholesterol serum lipids (Curb et al., 1986; Jossa et al., 1993). Similarly, the importance of this relationship was replicated in studies conducted in different populations and with different study designs (Thelle et al., 1987; Jansen et al., 1995; Wei et al., 1995). However, the increase in serum cholesterol in decaffeinated kolanut extract group in this study is of interest. This suggests that the cholesterol-raising effect of the kolanut extract is probably not due to the caffeine itself but to other ingredients of kolanut.

The phytochemical screening of the kolanut extract revealed the presence of phenolic compounds; catechin, quinic acid, tannic acid, and chlorogenic acid in large quantities as compared with fruits such as grapes, pears, peaches and apples (Davies et al., 1988; Williams et al., 1985). It is therefore possible that increased HDL-cholesterol concentrations caused by polyphenolic substances derived from kolanut extract may contribute to the suppression of VLDL concentration observed in this study. The mechanisms by which polyphenolic compounds elevate plasma HDL-cholesterol concentrations are not known. Epidemiological studies showed an association between coffee consumption and elevated levels of total and low-density lipoprotein (LDL) cholesterol, which persists even after adjustment for age, ethnicity, obesity and cigarette smoking (Jansen et al., 1995; Lee et al., 1987). In addition to total and LDL cholesterol, coffee intake exceeding two to three cups per day has been correlated with higher levels of apolipoprotein B when adjusted for various confounders including anaerobic capacity, nutritional intake and stress (Jansen et al., 1995). However, kolanut consumption mechanisms of action on lipids metabolisms need to be fully investigated.

Conclusion

Cola nitida (Kolanut) is generally recognized for its enriched caffeine constituents which are the main contributors of their biological activity or therapeutic effect. This study provides evidence that caffeine contents of *C. nitida* is higher than previously reported, and chronic consumption of kolanut is associated with adverse effects such as general toxicity which suggest that its prolong usage must be avoided. Further toxicity studies including developmental and genetic toxicity studies as well as mutagenicity and carcinogenicity tests

still need to be done for the complete elucidation of the toxic effects of kolanut extract.

Competing interests

We have no conflicting or competing financial interests.

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Full Length Research Paper

Erectile dysfunction: Definition and materia medica of Bapedi traditional healers in Limpopo province, South Africa

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Erectile dysfunction (ED) is a neurovascular event modulated by various factors impacting on the physiological functioning of the penile tissue. Interest in therapeutic substances of plant origin, used to treat ED, has progressively increased in the last decades. This ethnobotanical study on Bapedi aphrodisiacs was undertaken during 2010/2011 to document the floral diversity, species utilization, extract preparation and administration. The emphasis was on the customs of traditional healers residing in 17 municipalities, in three districts in the Limpopo province of South Africa. Data was obtained, from 34 healers, using a semi-structured questionnaire. According to these traditional healers, ED entails the inability to sustain an erection during coitus as well as a decreased libido. Findings indicated the use of 12 species, 10 of them with new documentations. Among these species, *Zanthoxylum humile* was the most frequently used species, and only *Osyris lanceolata* and *Securidaca longepedunculata* were previously recorded in the treatment of ED. There was a definite selection for underground parts. Preparation was uncomplicated; with cooking and pounding of the preferred methods. Administration was mostly attained via oral administration; however, the vehicle for administration varied. This manuscript validated the application of two species as aphrodisiacs. It is concluded that the major contribution is the 10 species that have not been documented earlier.

Key words: Bapedi, aphrodisiacs, erectile dysfunction, Limpopo province, *Zanthoxylum humile*.

INTRODUCTION

The dependence on aphrodisiacs by cultures such as the Chinese, Romans and Greeks, to enhance sexual performance, has been demonstrated for millennia (Elferink, 2000). Guirguis (1998), quite eloquently stated that “the erect penis has always been a symbol of power, virility and fertility”. It is therefore not surprising that in the presence of observed sexual enhancing properties, and the lack of scientific support, the use of such “love drugs”

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(Drewes et al., 2003) remain in high demand.

These drugs are almost exclusively used to enhance male sexual performance, more specifically the ability to perform optimally during coitus. Worldwide, approximately 152 million males suffer from erectile dysfunction (ED) (McKinlay, 2000). ED is broadly defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance (Lue et al., 2004). Drewes et al. (2003) noted that the introduction of phosphodiesterase type 5 inhibitors ($PDE5_i$), such as the designer drug VIAGRA® (Pfizer, 235 East 42nd Street, NY 10017) captivated the public's imagination. It also sparked a reassessment of older natural products for possible value in the treatment of ED, as well as the search for novel natural products to compete effectively against such synthetic drugs. The pursuit for more cost-effective herbal alternatives to compete against designer drugs is of the utmost importance as it will grant those with socio-economic constraints, access to treatment. Efficacy of these herbal substitutes depends on their potential to alter phosphodiesterase activity in the penile tissue.

Phosphodiesterases are a class of enzymes with the capability to cleave the phosphodiester bond in either cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), to yield 5'-cyclic nucleotides. They are therefore responsible for the cellular regulation of cAMP and cGMP levels (Omori and Kotera, 2007). The human genome encode 21 PDE genes; categorised into 11 families based on their protein sequence, structure, substrate specificity, enzymatic properties, tissue distribution, and sensitivity to selective inhibitors (Kotera et al., 2005). Of interest in the treatment of ED is PDE5, a highly specific cGMP enzyme. It is predominantly distributed in the smooth muscles located in the blood vessels and corpora cavernosa of the penis. This enzyme forms part of a cascade of mechanisms, where its inhibition leads to the accumulation of cGMP (Drewes et al., 2003). Therefore, any therapeutic intervention, from either natural products or designer drugs, targeting cGMP-binding will promote the cellular elevation of cGMP. These elevated levels induce a low Ca^{2+} state, which supports muscle relaxation (Williams and Melman, 2012). The resultant relaxation of the trabecular smooth muscle causes vasodilation, which increases blood flow into the corpora cavernosa via the pudendal artery. Consequently, the penis becomes engorged with blood and becomes erect (Andersson and Wagner, 1995); a purely hemodynamic event.

Regardless of the advances in modern medicine, traditional medicine is still the mainstay of primary health care for many people (Meyer et al., 2008); where herbal remedies are used to treat various diseases and disorders, including ED. In South Africa, a number of *in vitro* studies on species used to treat ED have been conducted. Drewes et al. (2002) evaluated pyrano-isoflavones isolated from *Eriosema kraussianum*, a species often used by the Zulu, in KwaZulu-Natal, to treat ED. Rakuambo et al. (2006) and Meyer et al. (2008) concentrated on species used by the Vha-Venda, residing in the Limpopo province.

The latter two studies predominantly focussed on *Securidaca longepedunculata*, and concluded that extracts showed potential as a therapeutic lead. In contrast to this, the Bapedi, the most dominant ethnic group in the Limpopo province (Monning, 1967) has received no attention with regard to their herbal remedies used to treat ED. It is well documented that male sexual performance and prowess is influenced by a multitude of factors. Amongst these factors, social and cultural norms play an important role in male sexual behaviour and performance; highlighting the fact that sexual performance is well correlated with self-esteem (Silberschmidt, 2001; Ahmed and Bhugra, 2007). The question thus remains whether the traditional definition of ED, among the Bapedi, is in line with its clinical definition. This study will also address to what extent they depend on herbal remedies to alleviate the symptoms of ED. Therefore, this study was conducted to investigate the species diversity, plant parts used, and specific preparation and administration of extracts used by this group to treat ED.

METHODOLOGY

Study area and study population

The study area is situated in the Limpopo province, in the far North of South Africa (Figure 1). Data was collected from three districts (Capricorn, Sekhukhune and Waterberg) covering 17 local municipalities (Table 1). These districts were selected due to their sizeable population of Bapedi; a cultural group that resides primarily in the central, Southern and Western parts of the Limpopo province. A total of 34 traditional healers (2 per local municipality) were selected from the listed local municipalities (Table 1).

Survey

This study was conducted from July 2010 to February 2011. Traditional healers were identified by convenience sampling, that is, with the assistance of colleagues and recommendations from villagers. Prior informed consent was obtained, and a semi-structured questionnaire was used to obtain information regarding the diversity of species used, plant parts used, as well as how these remedies were prepared and administered. Traditional healers were interviewed individually, in the confines of their consultation rooms. The primary researcher accompanied traditional healers to the collection sites to confirm the identity of species and to collect samples. At the Larry Leach Herbarium (UNIN) these samples were taxonomically identified and voucher numbers allocated.

RESULTS

Among the participants, there was broad consensus regarding the definition of ED. According to them, ED is

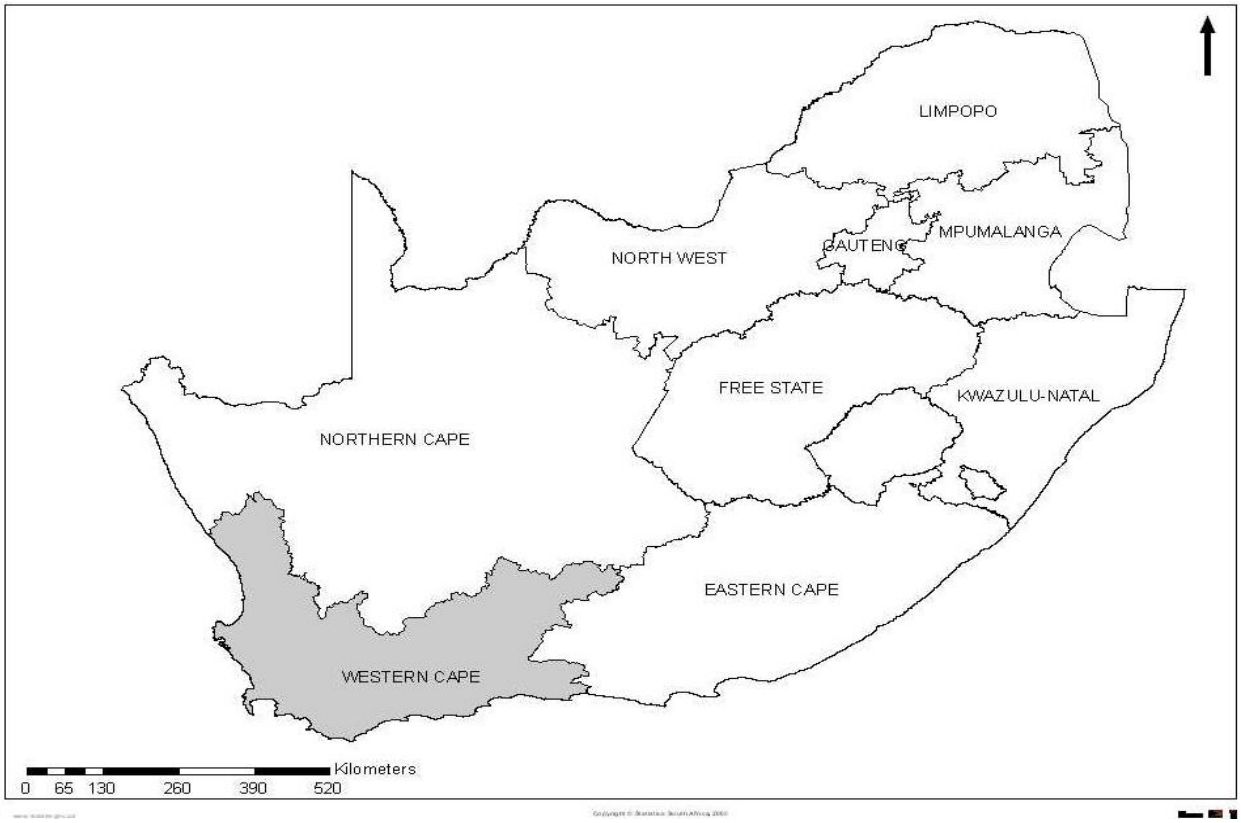

Figure 1. Map depicting the study area (Limpopo Province, South Africa), excluding the districts.
Source: http://geoinfo.statssa.gov.za

Table 1. Districts and local municipalities included in this study.

Capricorn district	Sekhukhune district	Waterberg district
Aganang	Elias Motsoaledi	Bela Bela
Blouberg	Fetakgomo	Lephalale
Lepelle-Nkumpi	Groblersdal	Modimolle
Molemole	Makhuduthamaga	Mogalakwena
Polokwane	Marble Hall	Mookgophong
-	Tubatse	Thabazimbi

an inability to complete coitus due to a gradual loss of tumescence and a decreased libido. This study recorded 12 plant species (12 families), which was used by the interviewees in the treatment of ED (Table 2). Only three species, *Hypoxis obtusa* (2), *Ozoroa sphaerocarpa* (2) and *Zanthoxylum humile* (6) were used more than once. Majority of the species featured as single extracts, and just three multi extracts were noted; with *Z. humile*, the most prominent species (67%) among the multi extract preparations.

Traditional healers from 12 (71%) of the 17 municipalities indicated that they treated ED (Table 3). There was a definite selection for underground parts, with only two occasions where the bark (*O. sphaerocarpa*), and the entire plant (*Myrothamnus flabellifolius*) were used. Most of the traditional healers preferred to use pounded material (65%), which was then either dissolved in a cup of hot water or mixed with soft porridge prior to oral consumption. In the Tubatse and Marble Hall municipalities (Sekhukune district), the pounded material was consumed with Mageu, a locally well-known non-dairy, non-alcoholic energy beverage prepared from *Zea mays* L. In the Fetakgomo municipality, pounded material was added to a cup of warm water and administered rectally, using a bulb syringe. The oral administration procedure was consistent throughout; one tin cup (± 340

Table 2. Diversity and usage frequency of species employed in the treatment of erectile dysfunction.

Scientific name and family	Frequency	
	Single extract	Multi extract with
Ammocharis coranica (Ker Gawl.) Herb. Amaryllidaceae	1	-
Artemesia annua L. Asteraceae	1	-
Asclepias fruticosa L. Apocynaceae	1	-
Carica papaya L. Caricaceae	1	-
Eucomis pallidiflora Baker Hyacinthaceae	0	*Hypoxis obtusa*
Hypoxis obtusa Burch. ex Ker Gawl. Hypoxidaceae	1	-
Myrothamnus flabellifolius Welw. Myrothamnaceae	1	*Zanthoxylum humile*
Osyris lanceolata Hochst. & Steud. Santalaceae	1	-
Ozoroa sphaerocarpa R.Fern. & A. Fern. Anacardiaceae	2	-
Prunus persica (L.) Batsch var. *persica* Rosaceae	1	-
Securidaca longepedunculata Fresen. var. *longepedunculata* Polygalaceae	0	*Zanthoxylum humile*
Zanthoxylum humile (E.A.Bruce) P.G.Waterman Rutaceae	4	-

ml) consumed three times per day for a period of one week.

Slightly more than a third (35%, n=5) cooked their plant material. Most of them (n=4) using a cooking time of 20 min; followed by a single healer favouring 10 min.

DISCUSSION

Ethnopharmacology follows a utilitarian approach focussing on the experimental investigation and validation of species with potential medicinal value (Balick and Cox, 1996). According to Etkin and Elisabetsky (2005), the broad perspective is to contextualize ecology and address the perception of plants; their utilization, pharmacology and physiology in human communities. Currently, the human-plant interface, incorporating the pharmacological basis of plant constituent interactions, compliments this view. An important indicator regarding the medicinal value of the species, preceding the utilitarian investigation into its medicinal significance, relates to their traditional application. The South African floral diversity makes it virtually impossible to test the bio-active profile of all species, hence the trend to follow-up on traditional usage.

The clinical definition of ED focus on the inability to attain an erection or when it is attained, to last long enough to successfully complete coitus. Among the Bapedi traditional healers, these concepts were well understood and applied in their practices. Their symptomatic diagnosis include: (i) a lack of sexual desire, which can easily represent an inability to attain an erection and (ii) an inability to have coitus for an extended time. In the latter, even with the consideration that “extended time” is difficult to pin point, it is evident that the male partner either reaches orgasm too soon or that he becomes flaccid before reaching an orgasm.

The fact that not all traditional healers use herbal remedies to treat ED was unexpected. Within the socio-cultural confines, where virility and sexual prowess is highly valued, a possible explanation might be that males feel incapacitated, and therefore only a limited number of those affected consult, leading to an underutilisation of traditional healers.

This study found that Bapedi traditional healers used 12 species to treat ED. A number of these species, or at best some genera from the current study, are known for medicinal uses other than for the treatment of ED. Among the Swazi people (Swaziland), bark from *Amorpha fruticosa* is used to treat asthma (Amusan et al., 2007). The Vha-Venda use *O. lanceolata* to treat sexually transmitted diseases (STDs) and infertility (Mulaudzi et al., 2011). Prozesky et al. (2001) indicated the use of *Ozoroa* species (*Ozoroa engleri* R.A. Fernandes) in the treatment of STDs. However, after a comprehensive literature search, this study was unable to find information regarding the use of the following species in the treatment of ED; *Ammocharis coranica*, *A. fruticosa*, *Artemisia annua*, *Carica papaya*, *Eucomis pallidiflora*, *Holospora obtusa*, *M. flabellifolius*, *O. sphaerocarpa*, *Prunus persica* var. *persica* and *Z. humile*. To the best of our knowledge, this is a first report describing their species-specific use in the treatment of impotence. However, a recent study (Van Andel et al., 2012) did indicate the use of the genus *Zanthoxylum* as an aphrodisiac. A number of these species had limited use among the healers interviewed, as most of them were used in a single municipality (Table 2). This seemingly insignificant application of a species should be approached with caution, as it cannot be discarded in the light of its perceived limited usefulness. As a matter of fact to comprehend the value of such a species, it is important to understand that thousands of people reside in those municipalities and many of them might be using

Table 3. District and municipality distribution of species used to treat erectile dysfunction; including extract preparation and administration.

District	Municipality	Species and part used	Preparation	Administration
Capricorn	Aganang	*Carica papaya* (root) *Prunus persica* (root)	Pounded material	Five teaspoons of material taken with soft porridge.
	Blouberg	*Securidaca longependuculata* (root) and *Zanthoxylem humile* (root)	Pounded material	Five teaspoons of material, from each species, taken with soft porridge.
	Lepelle-Nkumpi	*Myrothamnus flabellifolius* (root) and *Zanthoxylem humile* (root) *Osyris lanceolata* (root)	Pounded material A piece of root cooked for 20 min	Five teaspoons in a cup of warm water. -
	Molemole	*Zanthoxylem humile* (root)	Pounded	Five teaspoons in a cup of warm water.
Sekhukhune	Fetakgomo	*Eucomis pallidiflora* (bulb) and *Hypoxis obtusa* (tuber) *Zanthoxylem humile* (root)	Pounded	Equal amounts added to one cup of warm water and taken rectally with a bulb syringe. Pounded material taken with soft porridge.
	Makhuduthamaga	*Ozoroa sphaerocarpa* (bark)	Pounded	Pounded material taken with soft porridge.
	Marble Hall	*Myrothamnus flabellifolius* (entire plant) *Hypoxis obtusa* (tuber)	Pounded A piece of tuber is cooked for 20 min	Five teaspoons of material in a bowl of Mageu. -
	Tubatse	*Zanthoxylem humile* (root)	Pounded	Four teaspoons in a cup of Mageu.
Waterberg	Bela Bela	*Ammocharis coranica* (fleshy tuber)	A piece of tuber is cooked for 10 min	-
	Lephalale	*1. Asclepias fruticosa* (root)	A piece of root is cooked for 20 min	-
	Modimolle	*1. Zanthoxylem humile* (root)	Pounded	Five teaspoons of material taken with soft porridge.
	Mogalakwena	*1. Artemesia annua* (root) *2. Ozoroa sphaerocarpa* (root)	In both cases a piece of root is cooked for 20 min	-

these aphrodisiacs to improve their sexual performance. Therefore, comparing the value of a medicinal species to the number of healers using it, this might be a short-sighted and sub-optimal approach.

In this study, only a few number of the species had scientific support for their use as sexual performance enhancers. In the case of *S. longepedunculata*, species-specific proof was found of its possible value in the treatment of impotence. Two studies investigated this species and concluded that it active compounds causes smooth muscle relaxation in the corpora cavernosum (Meyer et al., 2008; Rakuambo et al., 2006). Rakuambo et al. (2006) reported its use among the Vha-Venda as a general remedy to treat various disorders, including ED. However, its use by the Bapedi is not as extensive and is restricted to the Blouberg municipality (Capricorn district), a geographical area in close proximity to Venda, which might explain this specific usage pattern. *In vitro* studies by Rakuambo et al. (2006) and Meyer et al. (2008) confirm the potential of this species in the treatment of ED, subsequently validating its use by the Bapedi and Vha-Venda.

Evidence supporting the use of *O. lanceolata* by South African ethnic groups to treat ED, could not be located. However, this cannot be considered a first description, as Muthee et al. (2011) indicated its use to treat ED in Kenya.

The sexual performance enhancing properties of two other more frequently used species, *Z. humile* and *H. obtusa* could not be validated, but other species from these genera had reported pharmacological value in the treatment of ED. This is a first report of *Z. humile* being used to treat ED; however, roots of *Z. capense* are used by the Zulu to treat ED (Corrigan et al., 2011). This observation reflects positively on the potential value of *Z. humile* in the treatment of ED. Furthermore, combining this species with *S. longepedunculata* in the Blouberg municipality, highlights the fact that ethnic groups, such as the Bapedi and Vha-Venda, residing in close proximity to one another, often employ similar species or combinations, as distribution and availability of species are relevant predictors of usage. This is significant when it is considered that *Z. humile* has a very limited distribution (Germishuizen and Meyer, 2003).

The report by Drewes et al. (2008) shed light on the use of *Hypoxis* species, however, within the context of their study, it can be accepted that specific reference was made to the use of *H. hemerocallidea*, and not *H. obtusa*. Therefore, this study seems to be a first species-specific report of the therapeutic application of *H. obtusa* in the treatment of ED.

In this study ED was almost exclusively treated using underground parts such as bulbs, tubers and roots. The most feasible explanation for this is the cultural belief that the underground parts, due to their close contact with the soil, contain the highest concentration of bio-active compounds. The nearly exclusive use of *O. sphaerocarpa* bark, instead of the expected use of its root, needs further investigation. Rankoana (2000), however, reported that the bark of this species is used by the Bapedi to treat gall-sickness among cattle. Thus it would seem that among the Bapedi there is a propensity for the use of bark from this species. The exclusive use of *Ozoroa* (*O. engleri*) bark is also reported by Mabogo (1990) for the Vha-Venda, where it is used as a remedy for STDs.

When herbal remedies are used, the dosage form, as well as the method of preparation and administration is very important (Steenkamp, 2003), as it will determine bio-availability. This availability is dependent on geographical distribution patterns (Van Wyk and Albrecht, 2008), which supports findings from this study where no trends of consistency within and among municipalities was recorded. Arnold and Gulumian (1984) reported that the Vha-Venda prefer to prepare a decoction of the plant part in the form of a soft porridge. Similarly, evidence of this practice was noted among Bapedi traditional healers who mixed pounded plant material with soft porridge. In some cases, Mageu was used to substitute the soft porridge. The use of Mageu in only two of the municipalities, coincidentally from the same district, is a custom that is currently difficult to explain as this beverage is provincially readily available. It is therefore, reasonable to argue that the use of Mageu, is not necessarily based on its availability, but rather on its other advantages. Its unique sour taste can easily mask the taste of plant material, and similar to the use of soft porridge it can delay the absorption of bio-active compounds in the digestive tract, thereby prolonging the bio-availability of compounds. With the exception of the multi-extract preparation involving *E. pallidiflora* and *H. obtusa,* which is administered as an enema, all other administrations in this study were done orally.

A thorough literature search could not reflect on the presence of PDE inhibitors in majority of species being used by Bapedi to treat ED. However, *S. longepedunculata* has been linked to smooth muscle relaxation. Meyer et al. (2008) noted that an isolated xanthone from this species stimulated smooth muscle relaxation in the corpus cavernosa in a frequency dependent manner. Vasodilation of blood vessels results in engorgement of the corpus cavernosa, leading to an erection.

Although not species-specific, the genus *Zanthoxylum* was noted to exhibit cAMP PDEI activity (Borges et al., 2005). The unique Bapedi custom to combine *S. longepedunculata* and *Z. humile* allude to a possible synergistic action between these two species. This warrants further investigation.

Results from this study confirm that, among the Bapedi, plant resources are widely used to treat ED. The absence of a definite usage pattern, excluding *Z. humile*, among the municipalities and districts reveals that the Bapedi does not have a universal species used to treat impotence. The relative, yet not overwhelming, consistent use of *Z. humile* warrants further investigation. Similarly, 10 new species records have been disclosed, and can be important leading to the discovery of natural products with PDE5 activity, and thus the ability to compete effectively against designer drugs.

This study has a number of invariant limitations. These include: (i) sample size and (ii) data collection was limited to three of the five districts, even though these districts represent the vast majority of Bapedi in the Limpopo province, South Africa.

In conclusion, certainty exists regarding the degree to which the Bapedi definition of ED corresponds to its clinical definition. A wide range of species and applications are employed to treat this debilitating male reproductive disorder.

Conflict of Interest

Authors declare no conflict of interest.

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