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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).

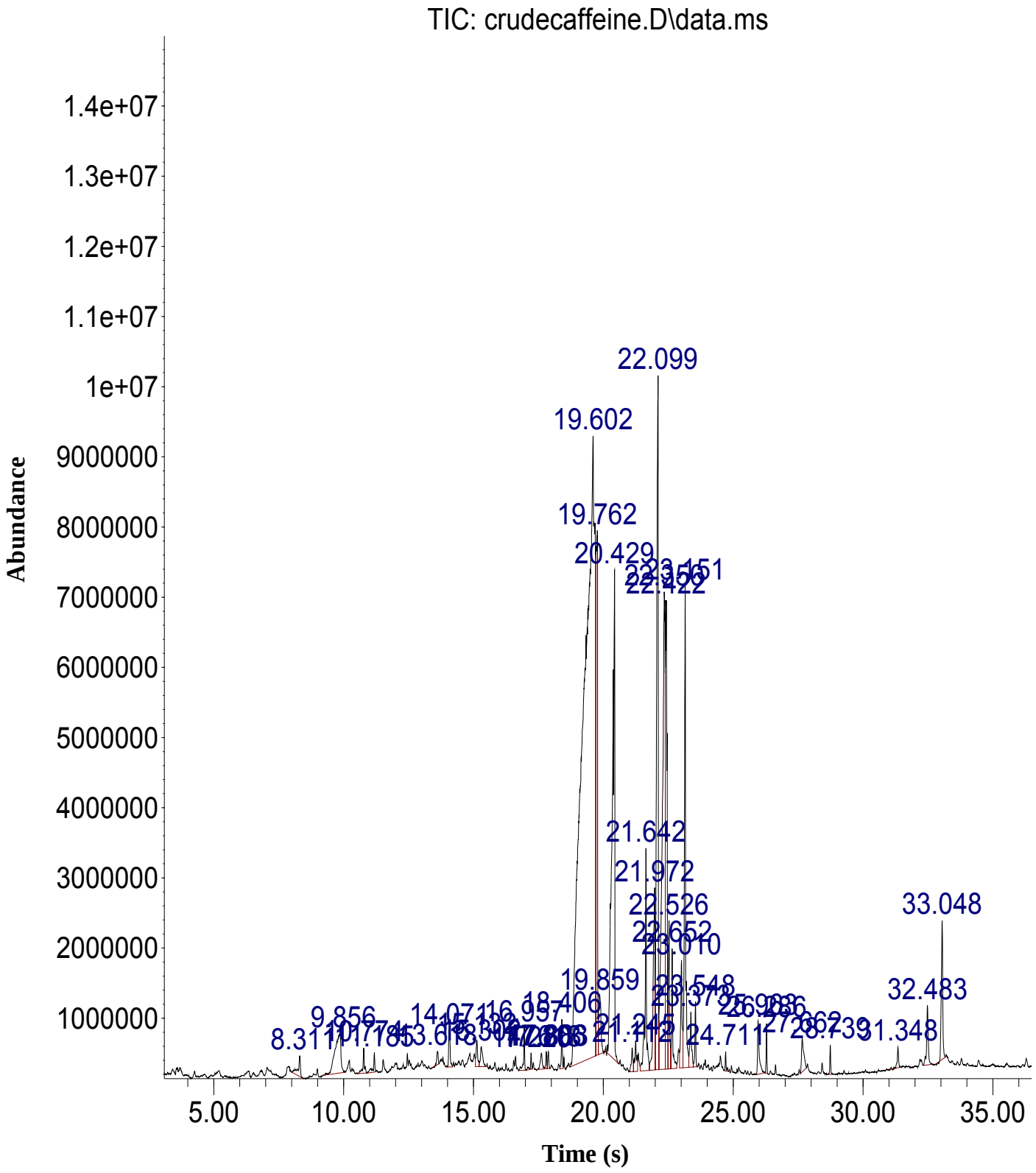

Figure 1. GC-MS chromatogram of ethanolic extract of kolanut peak 19.601 caffeine was identified as the major phyto-compound of the kola nut while other peaks were of the various phyto-compounds present.

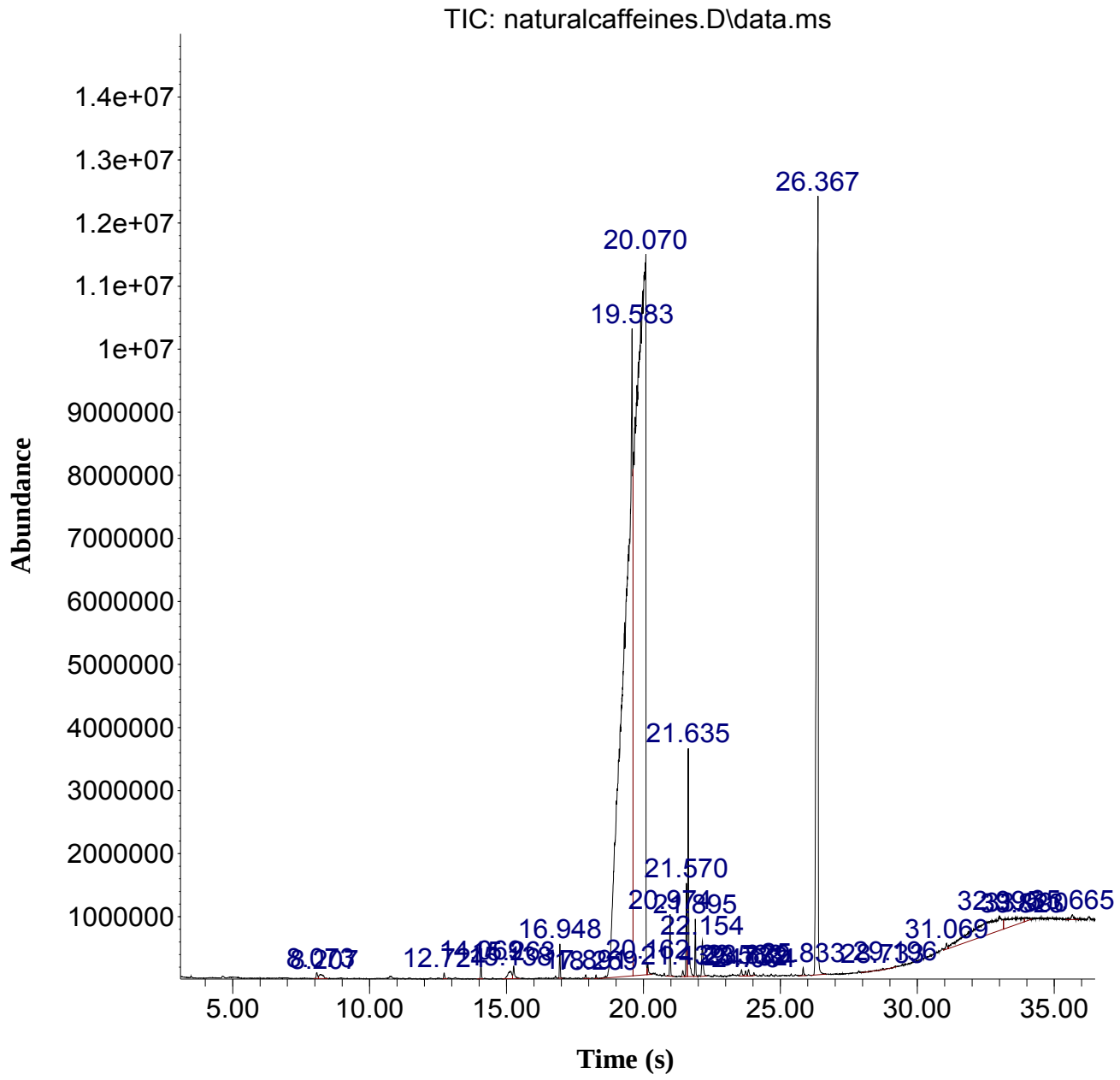

Figure 2. GC-MS chromatogram of caffeine extracted from kolanut Peak 19.583 caffeine was identified as the major phyto-compound of the kola nut while other peaks were of the various phyto- compounds present.

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

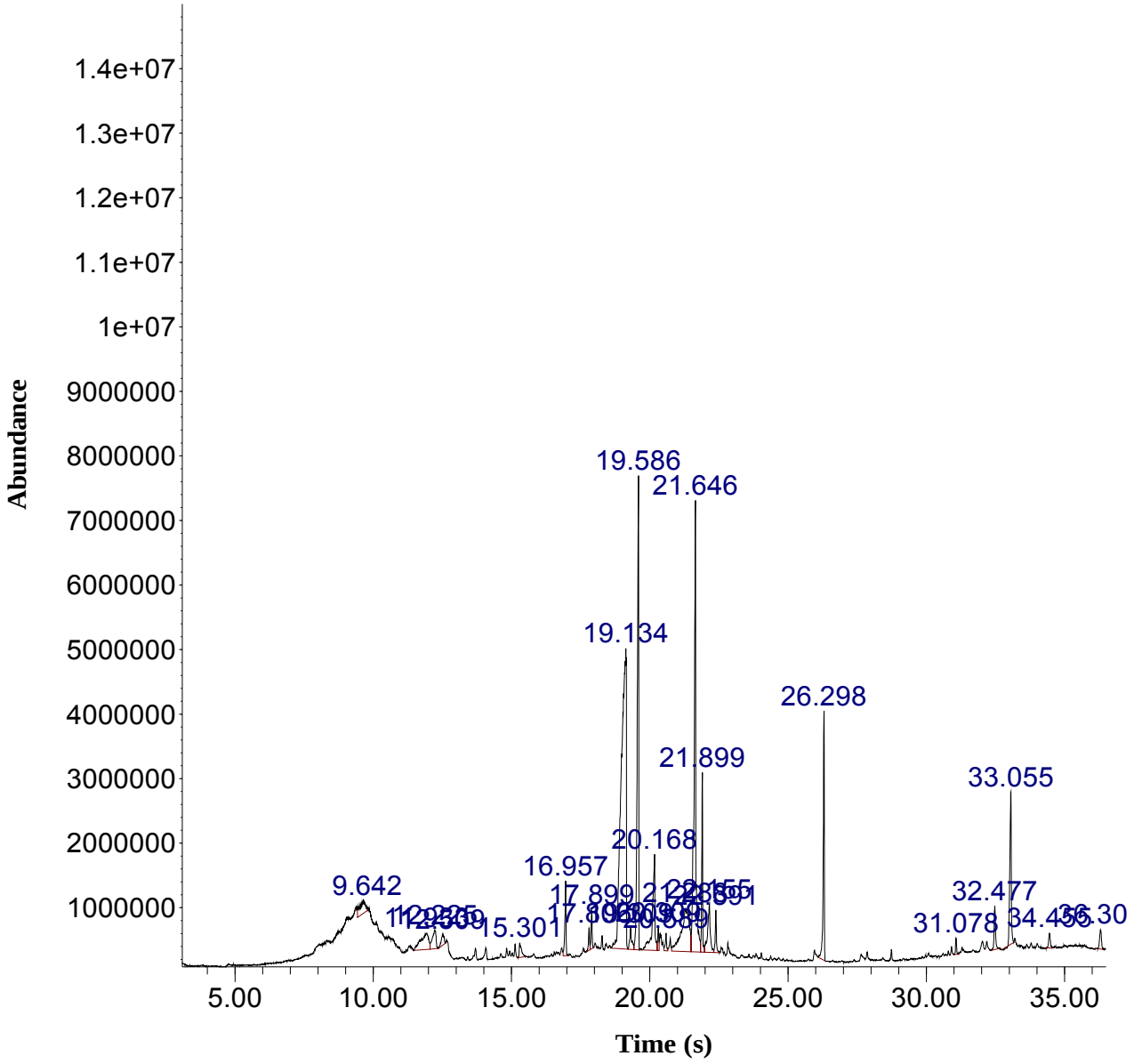

Figure 3. GC-MS chromatogram of decaffeinated kolanut extract peak 19.137 was identified as Methyl 9,10-methylene-hexadecanoat and as the major phyto-compound of the kola nut while other peaks were of the various phyto- compounds present.

(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

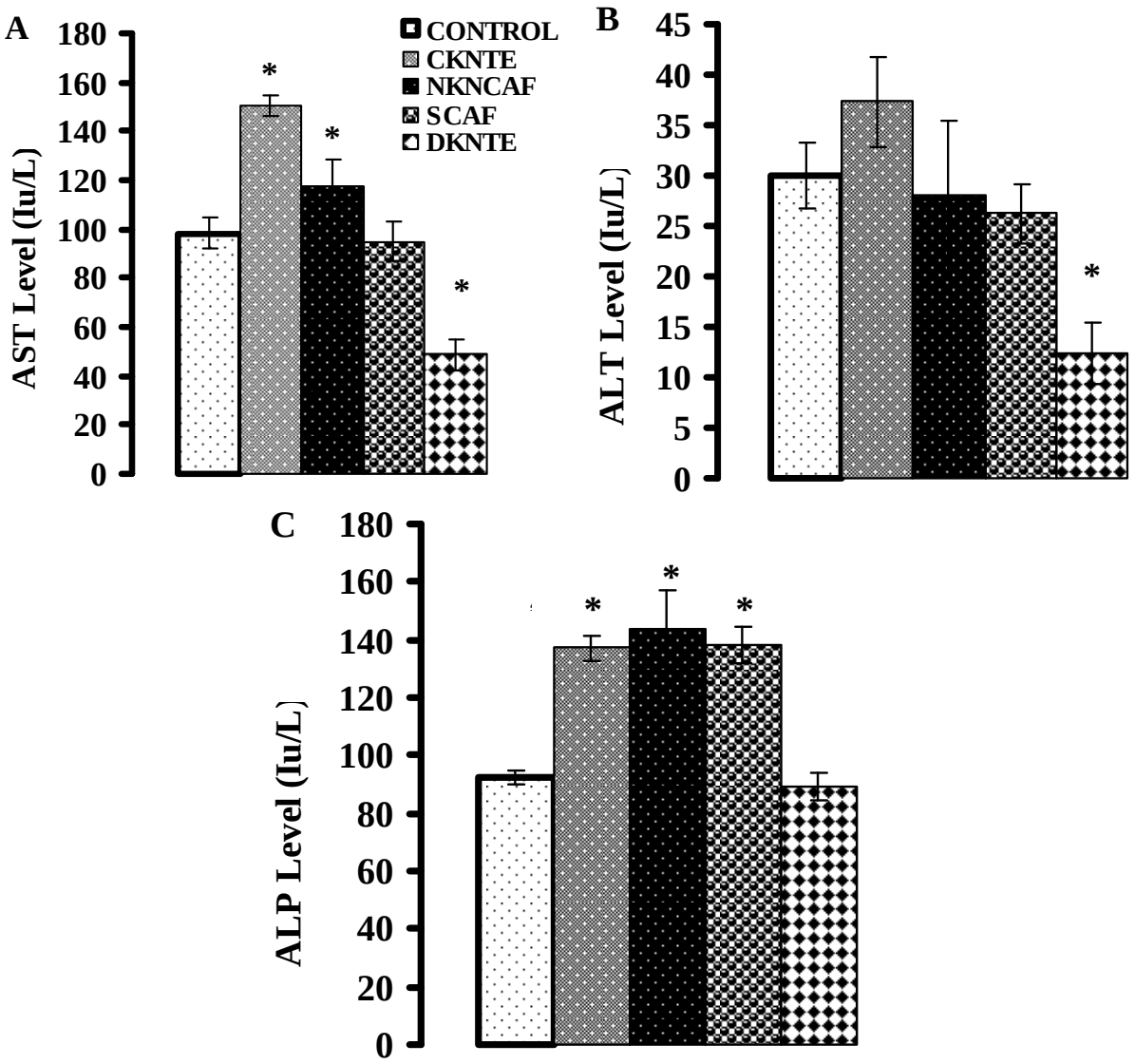

Figure 4. Effects of chronic consumption of normal saline (Control), crude extract of kolanut (CKNTE), caffeine isolated from kolanut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on blood plasma levels of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (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

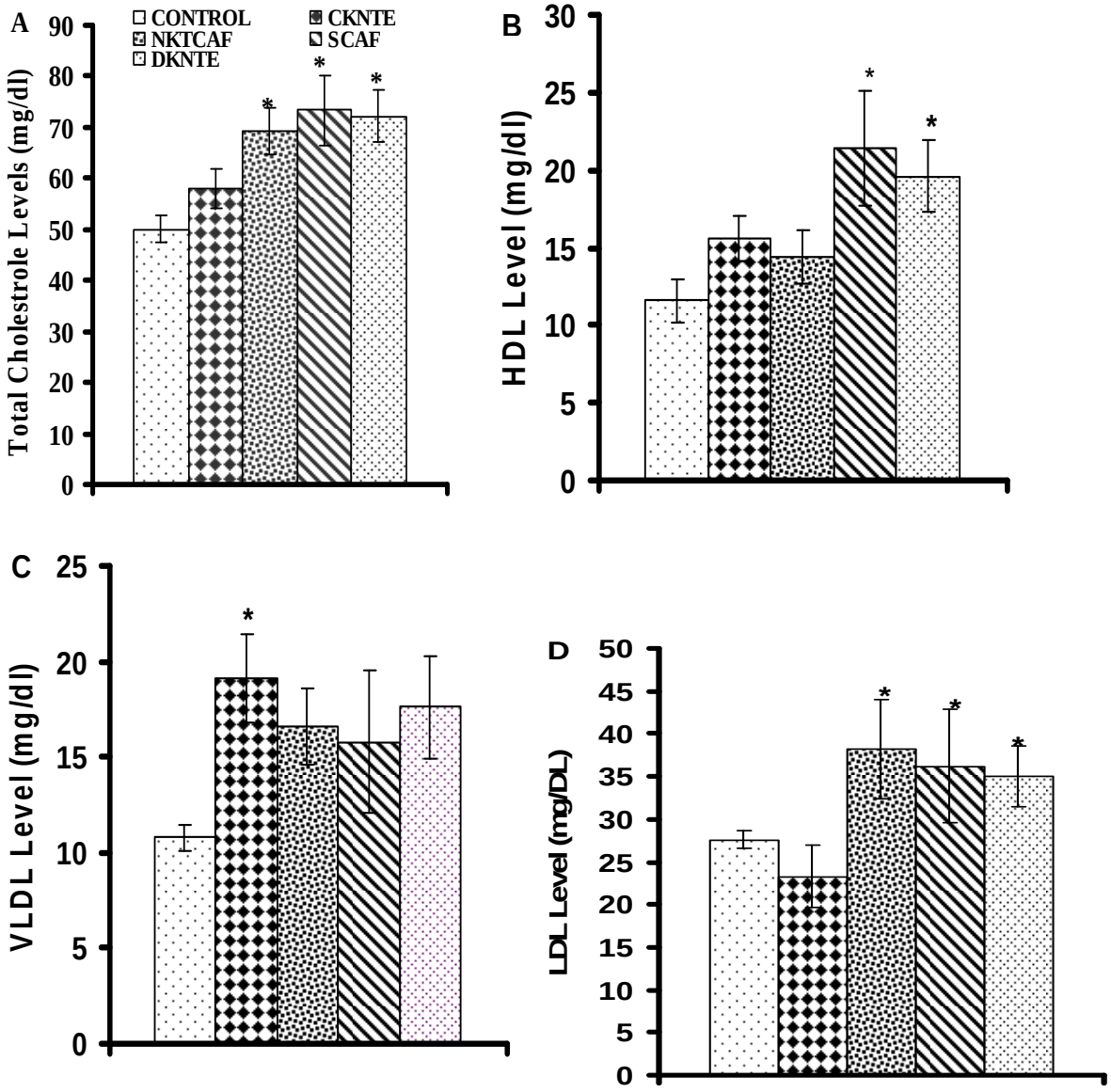

Figure 5. Effects of chronic consumption of normal saline (Control), crude extract of kolanut (CKNTE), caffeine isolated from kolanut (NKNCAF), synthetic caffeine (SYCAF) and decaffeinated kolanut extract (DKNTE) on blood plasma levels of (A) Total cholesterol, (B) high density lipoprotein (HDL), (C) 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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