Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(3):362-369

ISSN: 2248 –9215
CODEN (USA): EJEBAU

N-nitrosation of N-methylaniline and nitrosamine toxicology in the wistar rats

Ehigbor F. Abebe, *Comfort J. Akpabio and Emmanuel N. Maduagwu

Department of Biochemistry, Basic Medical Sciences, University of Ibadan, Ibadan, Oyo State Nigeria

---

ABSTRACT

The aim of this study is to investigate the urinary excretion of nitrosamine ions in rats, to investigate the effect of precursors of N-nitrosamine on serum enzymes activities and to evaluate the toxicity on liver on exposure to N-nitrosamine precursors. Forty two male wistar rats were divided into six groups and each group was admininistered with varying doses of sodium nitrite and N-methylaniline. The following methods were used; spectrophotometry, thin layer chromatography, cell fractionation, centrifugation, dialysis, histopathology, enzymatic and colorimetry. The RF values of the test samples were similar to those of the test standard, signifying the presence of N-nitrosamine in the urine of the rat. Liver function test, showed significant elevations ($P < 0.05$) in the AST, ALT, ALP and GGT activities in all the test groups compared with the control. Histological evaluation showed severe haemorrhage within the sinusoid, the portal triad was infiltrated by inflammatory cells in both $NaNO_2$ induced group and NMA combined with $NaNO_2$ group, thus showing acute damage compared with the control group which showed no lesion. The present study showed the urinary excretion of nitrosamine formed from N-methylaniline and sodium nitrite, the effect of N-methylaniline and sodium nitrite on serum enzymes and their toxicity on the liver of wistar rats.

Keywords: Enzymes, N-nitrosamine, N-methylaniline, Sodium nitrite, Serum, Urine.

INTRODUCTION

N-Methylaniline is an aniline derivative and a precursor of nitrosamine among others. It is a toxic organic compound with the chemical formula C_6H_5NH (CH_3). The substance exists as a colourless or slightly yellowish viscous liquid, which is insoluble in water and brown when exposed to air. It is used as a latent and coupling solvent and is also used as an intermediate for dyes, agrochemicals and for manufacturing other organic products [Jenkins *et al*, 1972].

N-Methylaniline decomposes on heating and burning, producing toxic fumes including aniline and nitrogen oxides. N-methylaniline reacts violently with strong acids and oxidants, and also affects some plastics. N-methylaniline is harmful if swallowed or inhaled or absorbed through the skin can cause methaemoglobinaemia, central nervous system disorders, eye and skin irritation, liver and kidney damage, gastrointestinal irritation with nausea, vomiting and diarrhoea [Grallal *et al*, 1979].

Nitrite is known to be a precursor of toxic and carcinogenic N-nitrosamines [Bassir and Maduagwu, 1978] and induces cancer in experimental animals [Sen and Baddoo, 1997; Mirvish, 1995]. After ingestion, residual nitrite can form traces of certain N-nitroso compounds in the stomach (where pH < 7) on reacting with secondary amines

which might also be present in the ingested food [O'Neil et al., 1984]. Nitrite can also interact with haemoglobin by oxidation of ferrous ion (Fe^{2+}) to ferric state (Fe^{3+}) preventing or reducing the ability of blood to transport oxygen a condition known as methaemoglobinaemia [Philips, 1971; Tannenbaum, 1984; Jones, 1993].

Nitrosamines are believed to be formed due to the interaction of various nitrosating agents (e.g. nitrite, nitrogen oxide) and amines in the foods [Khodadady *et al* 2012]. Nitrosamine is found in many food stuffs especially beer [Serag and Edrees, 2011], fish by products and in meat and cheese products preserved with nitrite picking salts. Their existence has been confirmed in food products [Wang *et al*., 2005; Luque-Pérez *et al*., 2001], cosmetic products [Schothorst and Somers, 2005; Flower *et al*., 2006], tobacco smoke [Lee, 2007] soil [Pan *et al*., 2006], ground water [Fu and Xu, 1995]. Most N- nitrosamines like N-methylaniline and dimethylaniline have been shown to be carcinogenic in laboratory animals [Preussmann and Stwart, 1984]. The N-nitroso compounds are known to be acutely toxic, mutagenic, teratogenic and carcinogenic. No animal species is known to be resistant to the carcinogenicity effect of nitrosamines and other N-nitroso compounds [Kouda *et al*, 1998; Kamendulis and Corcoran, 1994; Hildeshem *et al*, 2001]. Naturally N-nitrosamines could be found as a result of biological, chemical or photochemical processes [Ayanaba and Alexander, 1974].

The urine is the major routes through which drugs, foreign compounds and their metabolites are eliminated from the body of both animals and human [Magee and Barnes, 1956]. At reasonable concentrations nitrite is rapidly and extensively excreted in urine and thus does not accumulate in tissues [Vandevenne et al, 2000].

In a healthy organism only small amounts of intracellular enzymes are present in the blood plasma, but when an organ is diseased, a greater amount of its enzymes usually escape resulting in an increase, often marked in their activity in the plasma. Changes in serum alkaline phosphatase (ALP), alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT) and also the serum proteins may suggest liver toxicity [Mwanza *et al*, 1997] [Kamendulis and Corcoram, 1994]. These changes could be as a result of cellular damage due to disease conditions or due to hepatocellular damage caused by various foreign compounds [Lum et al, 1972]. The extent of the rise in serum activity of these enzymes depends on the concentration in the tissue, severity of the disease and the rate at which xenobiotics is removed from the plasma [Kaplan, 1985]. The Objective of this research is to investigate the urinary excretion of nitrosamine ions in rats, to investigate the effect of precursors of N-nitrosamine on serum enzymes activities and to evaluate the toxicity on liver tissues on exposure to N-methylaniline and sodium nitrite.

MATERIALS AND METHODS

Chemical and reagents: Sodium nitrite ($NaNO_2$**,** Mol. wt 69), N-Methylaniline (C_6H_5NH (CH_3) Mol. wt 107.15) were obtained from Sigma (USA). Others reagents such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma- glutamyl transferase (GGT) were from Randox Laboratories Ltd, United Kingdom. All the other chemicals and test kits used were of analytical grade**.**

Experimental Animals: The experimental animals used in this work were male albino rats (*Rattus norvegicus*) of the wistar strain. They weighed between 180g and 200g, and were obtained from the animal house of Veterinary Physiology Department, University of Ibadan, where they had been fed commercial rat pellets *ad libitum* and allowed access to clean drinking water. Only those certified free of infection by the Veterinary pathologist were used. They were kept at room temperature (approximately 28^{o}C) and all test animals were acclimatized to their environments before experiments were begun.

Animal treatment: Animals were divided into six groups and were given sodium nitrite [$NaNO_2$] and N-Methylaniline [NMA] orally through intubation.

Group A- received a combination of 50mg of NMA and 200mg of $NaNO_2$/kg.
Group B- received a combination 25mg of NMA and 200mg $NaNO_2$/kg.
Group C- received a combination 25mg of NMA and 100mg $NaNO_2$/kg.
Group D- received normal feed with water (control).
Group E- received 50mg $NaNO_2$/kg.
Group F- received 25mg $NaNO_2$/kg.

Collection of blood samples for serum preparation

The rats were sacrificed within 24 hours after the oral dose of sodium nitrite and N-methylaniline, all the rats were sacrificed by cervical dislocation. Blood was collected with the capillary tubes from the eyes. The blood was collected in dry plastic or glass centrifuge tubes. The blood was allowed to clot and immediately centrifuged at 2500rpm for 15 minutes in a table top centrifuge and serum was collected and preserved in a refrigerator at (at 4^0C) for a short time before the analysis. The activity of Alanine amino transferase (ALT) and Aspartate Amino transferase (AST) were estimated using the method of [Reitman and Frankel, 1957]. The activities of Alkaline Phosphatase (ALP) and Gamma – glutamyl Transferase (γ-GT) were determined in the serum samples using the method of [Klein *et al* 1960] and [Szasz 1969].

Histopathological analysis: Liver samples were immediately collected and fixed in 10% buffered formal saline solution for a period of at least 24 h before histopathological study. Samples were then embedded in paraffin wax and five-micron sections were prepared with a rotary microtome. These thin sections were stained with hematoxylin and eosin (H&E), mounted on glass slides with Canada balsam (Sigma, USA) and observed for pathological changes under a binocular microscope.

Collection of urine samples

The rats were administered with sodium nitrite and N-methylaniline. Then they were placed in metabolic cages with separate facilities for collection of urine. The rats were fed with pellet diet and drinking water was given *ad libitum.* Urine was collected after six hours and the 24 hours product pooled together. The samples were analysed daily or stored in a refrigerator at 10^oC.

Determination of nitrite in urine

The 24-hour urine was clarified by swirling with activated charcoal and filtered through Whatman No. 1 filter paper. 1ml of the filtrate was analyzed for nitrite using the method of [Montgomery and Dymock, 1961].

Thin layer chromatography (TLC)

Thin layer chromatographic plates were prepared first by cleaning the glass plates properly and finally with acetone. 50g of silica gel was mixed with 100ml distilled water in 250ml conical flask and shaken vigorously for about 1 minute. The mixture was then used to coat plates (20 x 20 x 0.25cm) to a thickness of 0.5nm, arranged on Quickest TLC spreaders. Plates were left in air for about 30 minutes and then dried at 110°C in an oven for 3 hours. Plates were stored in TLC plate container and activated just before use.

The test samples were spotted on the thin layer plates alongside with N-methylaniline (NMA), morpholine and dimethylamine + HCL using a micropipette. The spots were allowed to dry and the plates developed in the selected mobile phase (solvent mixture) N-Hexane - diethylether – dichloromethane (4:3:2) to a depth of 0.5 - 1.0cm [Daiber and Preussmann, 1964].The plates were immersed such as to obtain an ascending chromatographic separation. At the end of the run, the solvent was allowed to evaporate from the plates.

Location and identification of the nitrosamines

The developed plate was allowed to dry in air for about 5 minutes and a thin spray with Ninhyrin reagent was applied. The moist TLC plate was irradiated with short wave, UV light (240nm) for a minimum of 15 minutes. The test nitrosamines precursors and urine extract was identified with respect to the positions of the standard on the same plate. Areas containing nitrosamine appeared purple with Ninhyrin reagent.

Data analysis

Statistical analysis was performed using the Microsoft excel 11.0 statistical package, all results were expressed as means ± S.D. Comparisons within each group were performed using Student's *t* test for paired and unpaired data.

RESULTS AND DISCUSSION

Table 1: Thin layer chromatogram of pure nitrosamine standard

Substance streaked on TLC plates	RF Value	Colour under UV Light	Spray reagent Colour
Methylamine	0.879	Blue	-
Morpholine	0.879	Blue	Brown
Diethylamine	0.879	Blue	-
Diethylformamide	0.879	Blue	-
N-methylaniline	0.914	Brown	Yellow
Dimethylamine+HCL	0.750	Blue	Purple
Diphenlnitrosamine	0.664	Blue	Purple
Diphenylamine	0.807	Blue	Purple

Table 2: Thin layer chromatogram of urine samples of rats following oral administration of different doses of NMA and $NaN0_2$

Substance streaked on TLC plates	RF Value	Colour under UV Light	Spray reagent Colour
Test urine A	0.728	Blue	Purple
Test urine B	0.814	Blue	Brown
Test urine C	0.728	Blue	Brown
Control D	-	-	-
Test urine E	0.679	Blue	Purple
Test urine F	0.812	Blue	Purple

Table 3: Serum enzyme activities in rats given different doses of NMA and $NaNO_2$

DIGNOSTIC SERUM ENZYMES	GROUP A	GROUP B	GROUP C	CONTROL D	GROUP E	GROUP F
AST (U/L)	70.75 ± 1.76	61.97 ± 0.22	53.23 ± 3.51	24.00 ± 0.70	45.87 ±1.22	34.25 ± 0.69
ALT (U/L)	89.00 ± 1.41	86.00 ± 2.82	70.50 ± 1.05	13.27 ± 4.35	67.00 ± 1.41	59.50 ± 3.98
ALP (U/L)	314.43 ± 1.17	257.35 ± 1.22	243.57 ± 0.42	56.56 ± 1.46	129.19 ± 1.50	93.49 ± 0.99
GGT (U/L)	39.18 ± 4.26	36.28 ± 2 .52	30.39 ± 0.12	9.26 ± 3.55	27.21 ± 4.59	17.06 ± 1.10

Values are mean ±SD of 5 determinants
There was a significant increase (P-value < 0.05

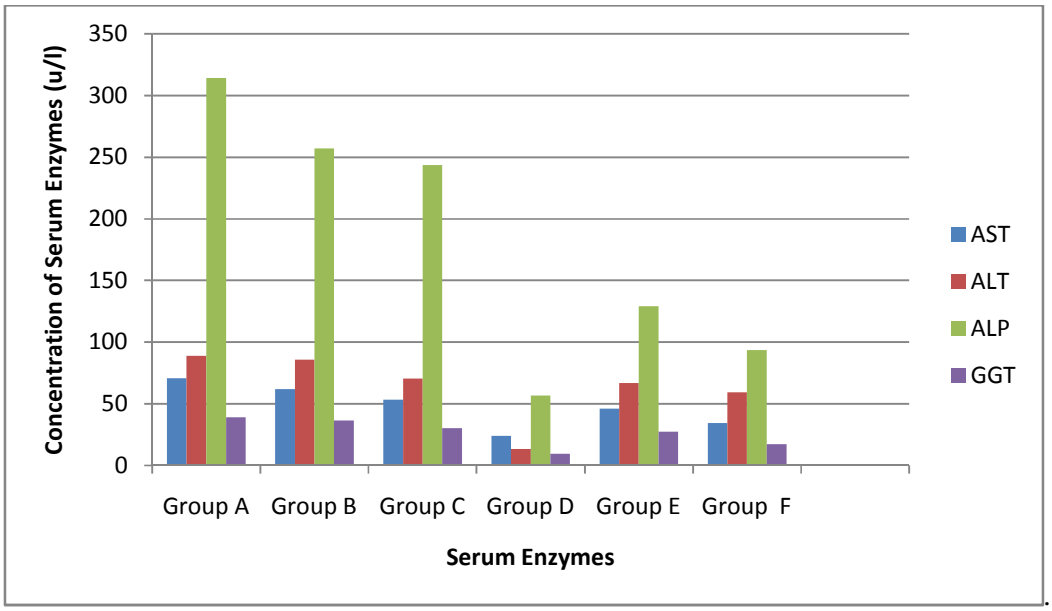
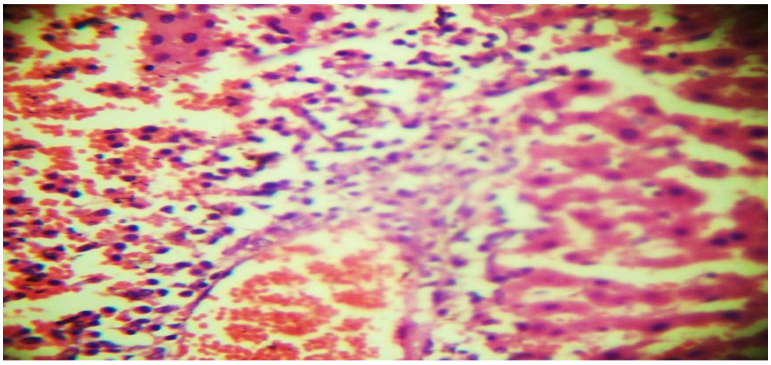


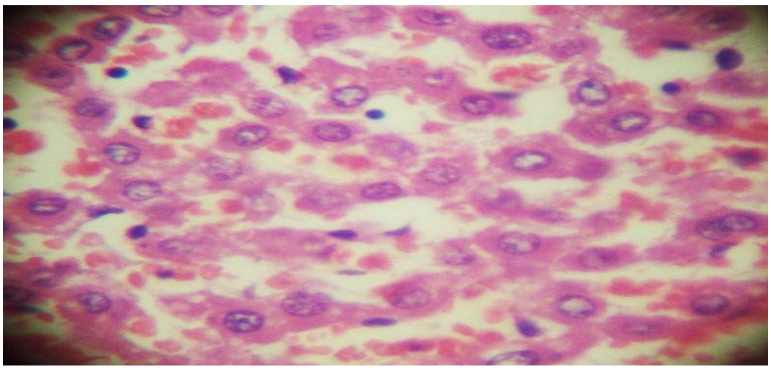
Figure 1: Activity of serum enzymes in rats given different doses of $NaNO_2$ and NMA

Histopathology sections of liver of rats administered with different doses of NMA and $NaNO_2$.



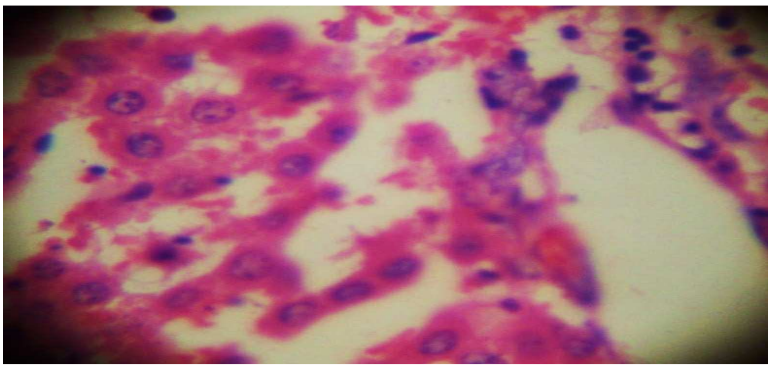
X400

Figure 2: A photomicrograph of liver section of rat given a dose of 50mg/kg of NMA and 200mg/kg of $NaNO_2$ showing severe hemorrhage within sinusoid. The portal triad is infiltrated by inflammatory cells.



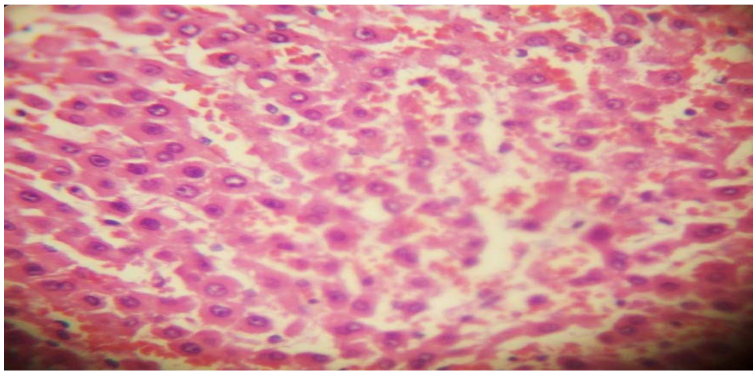
X400

Figure 3: A photomicrograph of liver section of rat given a dose of 25mg/kg of NMA and 200mg/kg of $NaNO_2$ showing infiltration of sinusoid by inflammatory cells.



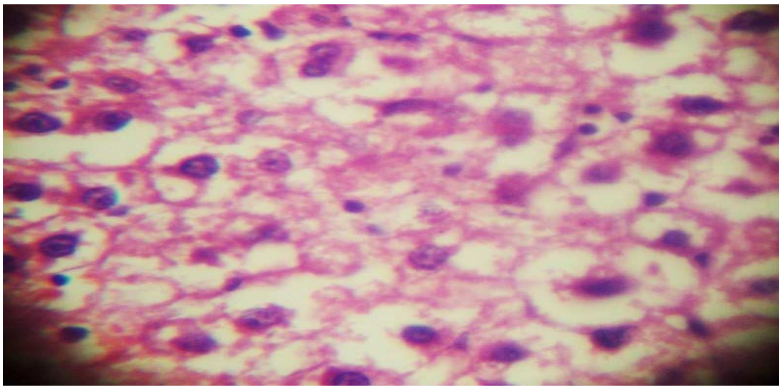
X400

Figure 4: A Photomicrograph of liver section of rat given a dose of 25mg/kg of NMA and 100mg/kg of $NaNO_2$ and showing hemorrhage and portal triad by some leukocytes.



X400

Figure 5: A photomicrograph of liver section of rat given a dose of 50mg/kg of NMA showing normal hepatocytes, haemorrhage is seen within the sinusoid which is attended by some inflammatory cells.



X400

Figure 5: A photomicrograph of liver section of rat given only water (control) showing normal hepatocytes. No hemorrhage is seen.

DISCUSSION

Table.2 shows the results of the streaked substances on TLC plate; there was a relationship in the RF values obtained from the test samples of the wistar rats pretreated with N-methylaniline and $NaN0_2$ with the test standard. The RF values of the various spots detected on TLC for wistar rats were similar to those of test standard. The amine metabolites arising from metabolism of N-methylaniline suggested that the compound was metabolized. 24 hours urine samples of rats given concurrent doses of N-methylaniline plus sodium nitrite and single doses of N-methylaniline and sodium nitrite produce TLC spot, presumes to be a nitrosamine as was clearly shown purple produced by the spray reagent and equally supported by elevation of serum enzymes in responds to the *in vivo* toxicity of the produced nitrosamine.

Ordinarily, liver cell damage is characterized by a rise in serum enzymes like AST, ALT, ALP, etc. (Brautbar and Williams, 2002). Serum enzyme activities, ALP, AST, ALT and GGT levels are widely used in animal studies to diagnose and observe the development of hepatic carcinogenesis. In this study, the values of mentioned parameters in Figure.1 and Table.3 showed significant increase ($P < 0.05$) in the group administered concurrent doses of N-methylaniline plus sodium nitrite and sodium nitrite and N-methylaniline only, when compared with that of the control rats. The toxicological evaluation of the liver showed a steady elevation of the enzymes in rats induced with $NaNO_2$ and NMA. It is known that these enzymes are mainly found on the liver in high concentration and whenever the enzymes are found in high amounts in the serum, it signifies that the liver has problem [Fazilati *et al* 2013]. The high values of the activities of serum transaminases, alkaline phosphatases and γGT, relative to control values are indicative of severe intrahepatic cell damage due to the compound administered. Increased in serum enzymes activities have been reported to occur in hepatotoxicity arising from the presence of foreign chemicals such as nitrosamine in the system. It is reported that increased nitrite level in rat given oral administration of sodium nitrite shows a significant increase in AST, GGT, ALT and AST level [Emman *et al.*, 2006, Akpabio *et al.*, 2013].

Histological evaluation showed hepatic necrosis in both $NaNO_2$ induced group and NMA combined with $NaNO_2$ group, thus showing acute damage compared with the control group which showed no lesion. Morphological changes observed in the livers during necrosis were similar in nitrite induced groups and nitrite plus NMA group. However, rats given concurrent doses of 50mg/kg of N-Methylaniline and 200mg/kg of Sodium nitrite produced severe haemorrhage within the sinusoid; the portal triad was infiltrated by inflammatory cells (Fig. 2). Rats given 25mg/kg of N-Methylaniline and 200mg/Kg of sodium nitrite indicated infiltration of sinusoid by inflammatory cells (Fig. 3). Rats given 25mg of N-methylaniline and 100mg of sodium nitrite/kg showed hemorrhage and portal triad by some leukocytes and centrilobular necrosis affecting about 40% to 60% of the liver lobules (Fig. 4). Rats given a dose of 50mg of N-methylaniline/kg showed normal hepatocytes and few inflammatory cells (Fig. 5).

It has been reported that there was a sequential histological changes when combined dose of N-methylaniline, dimethylaniline and sodium nitrite were administered to rats [Abdo et al, 1990].

The metabolism of N-dimethylaniline has been studied in many species and in human tissues. It involves enzymatic N-demethylation, N-oxidation and ring hydroxylation. Chronic methaemoglobinaemia and erythrocyte haemolysis, with concomitant splenomegaly and other pathological lesions, were observed in mice and rats treated with N-dimethylaniline. The application of 3 grams or more per kg of body weight upon the skin of a rabbit for 1 hour or more always produced death. Increased blood methaemoglobin levels, slight erythropenia, decreased haemoglobin concentration, and reticulocytosis were observed in workers exposed to N, N-dimethylaniline [Markel *et al.*, 1981]. However, chronic exposure of N-methylaniline was observed to cause Heart, kidney, and liver damage, possibly as secondary effects of haemolysis [Abdo et al, 1990].

The study showed the urinary excretion of nitrosamine formed from N-methylaniline and sodium nitrite and it effects on serum enzymes and liver of wistar rats.

ABBREVIATION

ALT : Alanine aminotransferase
ALP : Alkaline Phosphatase
AST : Aspartate aminotransferase
G : Gramme
NMA : N-methylaniline
$NaNO_2$: Sodium nitrite
RPM : Revolution per minute
SD : Standard deviation
TLC : Thin Layer Chromatography
UV : Ultraviolet
µL : Microlitre
γ-GT : Gamma-glutamyl transferase
RF : Retardation Factor

Acknowledgement
The authors are grateful to the Biochemical Toxicology Laboratory and Central Laboratory, University of Ibadan for supplying the N-nitrosamine precursors and the equipment used for this research work and also to the Veterinary Physiology farm for the animals supplied.

REFERENCES

[1] Akpabio CJ, Efuruibe AA, Ogunsola MO, Adeleke GE, Maduagwu EN, *European Journal of Expt. Bio.*, **2013,** 3(2):94-103.
[2] Abdo KM, Jokinen MP, Hiles R, *Toxicol. Environ. Health*, **1990,** 29, 77-88.
[3] Ayanaba A. Alexander M, *J.* E*nvironmental quality*. **1974, 3,** 83-89.
[4] Bassir O**,** Maduagwu EN, *J. Agric Fd. Chem*, **1978**. **26**, 200.
[5] Brautbar N, Williams J. 2002. *Int. Journ. Hyg. Environ. Health.*, **2002,** 205: 479-491. PMID: 12455270.
[6] Daiber D, Preussmann R, *Z. Anal. Chem.* **1964 206**, 334 - 352.
[7] Eman GE, Helal & Fahmy GG, *Elsaid. Research Journal of Medicine and Medical Sciences* **2006,** 1.3: 85-89.
[8] Fazilati M, European *Journal of Experimental Biology*, **2013**, 3(1):97-103

[9] Flower CS, Carter A, Earls R, Fowler S, Hewlins S, Lalljie M, Lefebvre J, Mavro D, Small NV, *Int. J. Cosmet. Sci*. **2006,** 28: 21-33.
[10] Fu CG, Xu HD, **1995.** Analyst, 120: 1147.
[11] Gralla EJ, Bus JS, Reno F, Cushman BNJ, *Toxicol. Appl. Pharmacol*. **1979,** 48:A97.
[11] Hildesheim A, Dosemeci M, Chan CC, Chen CJ, Cheng YJ, Hsu MM, Chen IH, Mittl BF, Sun B, Levine PH, Chen JY, Brinton LA, Yang CS, *Cancer Epid. Biomarkers Prev*. **2001, 10,** 1145-1153.
[12] Jenkins FP, Robinson JA, Gellatly JBM, Salmond GWA, *Food Cosmet. Toxicol*.**1972,** 10:671-679.
[13] Jones T, 1993. *In Practice* **1993**, 15: 146-149.
[14] Kamendulis LM, Corcoran CB, *J. Pharmacol . Exp. Ther*.**1994, 271**, 1695 - 1698.
[15] Kaplan MM, Chang NC, Chan N M, Research Monograph,**1985,**17(in Early identification of alcohol abuse).(NIAAA) p.186.
[16] Khodadady M, Shahryari T, Dorri H, Sharifzadah GR, Ziyazade A, *European Journal of Experimental Biology*, **2012,** 2 (6):2120-2124.
[17] Klein B, Read PA, Babson LA, *Clin. Chem*, **1960,** 6: 269-275.
[18] Kohda KH, Anjo T, Mochizuki M, Kawazoe Y, Okado M, *Gann*.**1982 73**, 517 - 521.
[19] Lee HL, Wang S, Lin DP, Hsieh H, *Talanta*, **2007,** 73: 76.
[20] Lum G, Gambino SR, *Clin. Chem*.**1972,** 18(4); 358-362.
[21] Luque-Pérez E, Rios A, Valcárcel M, *Fresenius J. Anal. Chem.*, **2001,** 371: 891- 895.
[22] Magee P N, Barnes J M, *Br. J. Cancer*, **1956, 10**, 114 - 122.
[23] Markel H, WiIcox T, Health Hazard Evaluation Report **1981,** No. HHE- 79-104-838.
[24] Mirvish SS, Ramm MD, Sams JP, Babcock DM, *Cancer Res*, **1988,** 48: 1095-1099.
[25] Montgomery H A C, Dymock J F, *Analyst (Lond.)* **1961, 86**, 414.
[26] Mwanza A, Madej A, Kindahl H, Lundeheim N, Einarsson S, *Journal of Vet. Med; Physiology, Pathology*, Clinical Medicine, **2000,** 47, 193-200.
[27] O'Neill IK, Borstel RC, Miller CT, Long J, Bartsch H, IARC Scientific Publication. **1984,** 57; Oxford University Press: Lyon.
[28] Pan X B, Zhang SB, Cox TA, Anderson, Cobb GP, *J. Chromatogr.A*, **2006,** 1107(1-2): 2-8.
[29] Phillips WEJ, *Food and Cosmetics Toxicol*. **1971,** 9:219-228.
[30] Preussmann R, Stewart BW, ACS Monograph. *American Chemical Society*, **1984,** 182: 643 -822.
[31] Reitman S, Frankel S, *Am. J. Clin. Pathol*. **1957, 28**, 56 - 63.
[32] Schothorst RC, Somers HHJ, *Anal. Bioannal. Chem*., **2005,** 381:681-685.
[33] Sen NP, Baddoo PA, Seaman SW, *J. Food Sci*.**1986, 51**, 821 - 825.
[34] Sen NP, Baddoo PA, J.Food Saf, **1989,** 9: 183-191.
[35] Serag H, Edrees G, *European Journal of Experimental Biology*, **2011,** 1(4):87-92
[36] Szasz G, Clin.Chem., **1969,**15: 124-136.
[37] Tannenbaum SR, Origin in human. *Science*, **1979**, **205**, 1333 - 1335.
[38] Vandevenne M, Vandenbussche H, Vestraete A, *Acta Clin Belg*.**2000,** 55:323-333.
[39] Vermeer IT, Henderson LY, Mooner E J, Engels LG, Dallinga JW, Van maanen JM, Kleinjans JC, *Toxical lett*, **2004, 154.**175-182.
[40] Wang J, Chan WG, Haut SA, Krausss MR, Izac RR, Hempfling WP, *J. Agric. Food Chem.*, **2005,** 53(12): 4686-4691.