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Protective Effect of Silymarin on Cisplatin-induced Nephrotoxicity in Rats

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Abstract: Cisplatin (CDDP) is a potent anticancer agents used for the treatment of solid tumors. However, its clinical use is often limited by its adverse effects including nephrotoxicity. The present study was designed to estimate if silymarin, a bioflavonoid with antioxidant potential can inhibit or at least ameliorate the alteration in some renal structures induced by cisplatin in rats or not. Five equal-sized groups (18 rats each) of male Sprague Dawley rats [Control, vehicle; cisplatin (5 mg/kg); silymarin (50 mg/kg) 2 h after cisplatin injection; and silymarin (50 mg/kg) 2 h before cisplatin injection] were used. Results revealed that cisplatin produced animal behavioral and morphological changes, as well as cellular and subcellular changes in kidneys. The most important changes were: decreased body weight, increased kidney wet weight, atrophied glomeruli, dilated urinary space, loss of PCT brush borders, hypertrophied podocyte pedicels, thickened glomerular basement membrane as well as tubular cell vacuolization. Post-treatment of silymarin 2 h after cisplatin however, significantly increase the body weight returning it to normal value, yet it failed in complete protection against the pathological alteration caused by cisplatin. Pre-treatment with silymarin 2 h before cisplatin significantly decreased the histological and ultrastructural changes induced by cisplatin and appear highly protective. These results suggested that the effects of cisplatin on glomerular and renal tubular cells morphology could be totally or to a great extent inhibited by silymarin.

Key words: Cisplatin, histopathology, kidney, silymarin, ultrastructure

INTRODUCTION

The human kidneys are primarily involved in filtering and concentrating various substances and chemical agents that may reach a high concentration and become toxic (Loh and Cohen, 2009). Nephrotoxicity is an inherent adverse side effect of the anticancer drugs for solid and hematologic malignancy (Kintzel, 2001). Antimetabolites, alkylating agents and anthracyclines are commonly used anticancer drugs resulting in nephrotoxicity (Erkut *et al*., 2008). Renal tubular damage is a well-known renal complication induced by anticancer drugs (Kakihara *et al*., 2003). The rate of glomerular damage may have been underestimated because tubular dysfunction can mask glomerular dysfunction (Ikarashi *et al*., 2004). It was reported that the mechanisms of chemotherapy-induced renal dysfunction generally include damage to vasculature or structures of the kidneys (Kintzel, 2001). He also added that, patients with cancer are frequently at risk of renal impairment secondary to disease-related and iatrogenic causes.

The anticancer drug cisplatin (CDDP) is a very effective platinum compound in the treatment of a variety of cancer (Kintzel, 2001). He also added that its clinical use is associated with severe side-effects; including renal impairments of which nephrotoxicity the most common side effect (Kintzel, 2001). Nephrotoxicity of CDDP has been recognized as the most important dose-limiting factor (Mora Lde *et al*., 2003). Although wide investigations have been conducted on the general organ toxicity of this anticancer drug (Pal *et al*., 2008), the exact mechanisms of nephrotoxicity induced by CDDP are still not fully elucidated. Stewart *et al*. (1982) reported that cisplatin is preferentially taken up and accumulated in the kidney cells. Nevertheless the major site of renal injury is the proximal convoluted tubule as reported by Kuhlmann *et al*. (1998). Therefore, the enhanced production of Reactive Oxygen Species (ROS) (Saad *et al*., 2004), oxidative stress, (Saad *et al*., 2004) and the decrease in antioxidant enzymes (Mora Lde *et al*., 2003) in kidneys have been implicated in the pathogenesis of cisplatin induced renal injury (Yilmaz *et al*., 2004). However, the involvement of oxidative stress in cisplatin induced toxicity is further supported by the fact that many antioxidants prevent cisplatin induced nephrotoxicity (Ajith *et al*., 2002; Lee *et al*., 2007).

Cytoprotective agents can be applied in therapy to ameliorate functional renal disorders (Behling *et al*., 2006). Behling *et al*. (2006) also added that

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cytoprotection is also considered as a suitable tool to elucidate the pathogenesis of chemically induced injury. Silymarin is a flavonoid extracted from *Silybum marianum*, that has already successfully been applied as a protective agent in various clinical and both *in-vivo* and *in-vitro* experimental models of hepatotoxicity (Laekeman *et al*., 2003; Eminzade *et al*., 2008) and nephrotoxicity to a certain extent (Karimi *et al*., 2005). Silymarin possesses antioxidant property that seems to be due to their ability to scavenge free radicals and to chelate metal ions (Borsari *et al*., 2001). Silymarin has been shown to be safe in animal models and no significant adverse reactions are reported in human studies (Hogan *et al*., 2007). The study presented here attempted to evaluate nephroprotective effects of the flavonoid silymarin if present on acute CDDP toxicity.

MATERIALS AND METHODS

Male Sprague Dawley rats (*Rattus norvegicus*), 150 g each were used in this work. Animals were housed in cages (4 animals/cage), under standardized laboratory conditions with controlled light-dark cycle, temperature of 23±2°C and relative moisture 60-70%. Animals had free access to tap water and to standard food diet *ad libitum*. All animals were adapted to handling and cages repeatedly during a 5-day period prior to experiment.

Cisplatin [(CDDP) or *cis*-Dichlorodiammine Platinum (II)] was obtained as yellowish crystalline powder, soluble in physiological saline solution and purchased from Sigma-Aldrich, CAS Number 15663-27-1 (P4394 Sigma). Silymarin was purchased from Sigma-Aldrich Chemical Co. (S0292).

Experimental design and procedures: The animals were divided into five equal sized groups (12 rats/each). In the first group (GIa) animals received no chemical treatment. The second group of animals (GIb) served also, as controls and were dosed with vehicle solutions only (propylene glycol and saline; 75/25 v/v). In the third group (GIIa) Animals were i.p injected with single dose of cisplatin dissolved in normal saline (5 mg/kg) at the beginning of the experiment (Mansour *et al*., 2006). In the fourth group (GIIb) animals were i.p injected with single dose of cisplatin (5 mg/kg), followed after 2 h by i.p. injection of silymarin (50 mg/kg/day) dissolved in vehicle solution as in the second group (Karimi *et al*., 2005). In the fifth group (GIIc) animals were i.p injected with silymarin (50 mg/kg/day) dissolved in vehicle solution as in the second group, 2 h before CDDP injection.

Animal behavior and body weight of all rats were recorded 4 times weekly. For histological evaluation, randomly selected rats were killed by decapitation after 2 weeks and the kidney tissues were immediately removed, weighed and washed with saline, cut into small pieces then dropped in 10% buffered formalin and dehydrated in ascending grades of ethanol concentration, cleared in xylene and processed in paraffin wax for embedding. Histological sections (5-6 µm thick) were stained with hematoxylin and eosin method and examined with light microscope.

For ultrastructural study, anaesthetized rats from all groups were rapidly dissected and then perfusion fixation with formalin-glutaraldehyde fixative ($_4F_1G$) in phosphate buffer was performed. Kidney tissues (1 mm^3) were removed and dropped into $_4F_1G$ buffered with 0.1 M phosphate (pH = 7.4) at 4°C. Samples were post-fixed in 2% OsO4 for 2 h at 4°C in the same buffer. The specimens were washed and dehydrated at 4°C through a graded series of ethanol. Tissues were then treated with propylene oxide solution and embedded in a mixture of 1:1 of Epon-Araldite. Specimens were embedded for 1 h. Polymerization was done in the oven at 65°C for 24 h. Ultrathin sections (50 µm) were cut on LKB ultratome, then mounted on copper grids, double stained with uranyl acetate and lead citrate and investigated on JEOL 100CX TEM.

The statistical significance was evaluated by one-way ANOVA using SPSS Version 16 and the individual comparison were obtained by LSD method. Values were considered statistically significant when $p<0.05$.

RESULTS

Effects of chemical used on behavior, body weight and kidney wet weight: Animals of all groups showed no obvious symptoms or signs of toxicity throughout the experiment. Moreover; they did not exhibit any case of mortality or death. Rats dosed with single injection of cisplatin (GIIa) (5 mg/kg) also, showed no adverse effects and remained alert as control groups until the 10th day of the experiment, where they became slightly nervous, less active, with minimal loss of furring. However, rats treated either with silymarin 2 h after cisplatin injection (GIIb), or received the same dose of silymarin 2 h before cisplatin injection (GIIc); exhibited no behavioral changes or clinical symptoms apart from increasing activity especially after the 7th day of the experiment.

It was noticed that in cisplatin treated rats, water and pellet diet consumption was decreased, if compared to normal. Also, a significant decrease in the body weight gain after 2 weeks (186.08±5.08 g) as compared to control (299.02±6.68 g) was noticed. However, the body weight of rats exposed to cisplatin and silymarin (GIIb and GIIc) were found to be equal 219.3±3.47 g and 218.86±4.88 g respectively, i.e. similar to that of controls. Moreover, a significant increase in cisplatin treated groups and significant depression in both groups receiving silymarin before and after cisplatin in kidney wet weight as well as relative weight to body weight was detected, i.e. returned value to normal (Table 1).

Table 1: Effects of cisplatin and silymarin on kidney wet weight and relative kidney to body weight ration among control and experimental rats[1]

	Kidney wet weight		Relative kidney to body weight ratio	
Group	0 Week[2]	2 Weeks	0 Week[2]	2 Weeks
Control GIa	2.30±0.31[a]	2.32±0.13[a]	0.010±0.005[a]	0.0094±0.007[a]
Vehicle GIb	2.33±0.23[a]	2.16±0.10[a]	0.010±0.004[a]	0.008±0.006[a]
Cisplatin GIIa	2.37±0.34[a]	2.68±0.12[b]	0.013±0.007[a]	0.0144±0.008[b]
Silymarin 2 h after cisplatin GIIb	2.36±0.30[a]	2.43±0.04[a]	0.011±0.004[a]	0.010±0.0017[a]
Silymarin 2 h before cisplatin GIIc	2.34±0.29[a]	2.39±0.06[a]	0.011±0.004[a]	0.010±0.002[a]
Results of one way ANOVA (Dose)	F = 0.04	F = 3.5	F = 0.04	F = 3.5
	p>0.05	p<0.05*	p>0.05	p<0.05*

[1]Data are means±SE (n = 6). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Table 2: Morphometric measurements of thickness of renal filtration barrier, pedicel length and filtration slit width (μm) among control and experimental group[1]

Groups	Thickness of renal filtration barrier (μm)	Pedicel length (μm)	Filtration slits width (μm)
Control GIa	0.26±0.01[a]	0.58±0.02[a,c]	0.02±0.003[a,d]
Vehicle GIb	0.22±0.01[a]	0.46±0.04[a,d]	0.01±0.0003[a,d]
Cisplatin GIIa	0.48±0.03[b]	0.67±0.05[a,c]	0.06±0.01[b,c]
Silymarin 2 h after cisplatin GIIb	0.32±0.02[a]	0.52±0.04[a,d]	0.03±0.001[a,d]
Silymarin 2 h before cisplatin GIIc	0.28±0.05[a]	0.51±0.04[a,d]	0.04±0.007[a,c]
Results of one way ANOVA	F = 8.56	F = 3.28	F = 6.91
	p<0.05*	p<0.05*	p<0.05*

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Histopathological and ultrastructural findings: Examination of hematoxylin and eosin stained kidney sections of control rats after 2 weeks from the beginning of the experiment, revealed normal basic structure. Kidney sections showed a large number of renal corpuscles and numerous urinefrous tubules within the cortex (Fig. 1a). Renal corpuscles appeared morphologically normal with double walled Bowman's capsule surrounding the glomerulus. However, between the two layers of Bowman's capsule is preserved a narrow urinary space. Ultrastructurally, it was noticed that the visceral layer of Bowman's capsule of renal corpuscle consists of podocytes (Fig. 1b) with large eccentric kidney shaped nuclei, fairly dense cytoplasm and numerous small bell shaped pedicels that appeared in direct contact with the renal filtration barrier and were separated by filtration slits (Table 2). However, glomeruli possessed numerous capillary loops that were lined with flattened fenestrated endothelial cells resting on the glomerular basement membrane. The capillaries loops were supported by mesangial cells having small densely stained nuclei with electron dense mesangial matrix (Fig. 1b). Moreover, light microscopic preparations showed that Proximal Convoluted Tubules (PCT) of normal kidneys have narrow lumen (Fig. 1a); occupied by striated brush borders and a regular basal lamina lined by a single layer of pyramidal shaped cells (Table 3). Ultrastructurally these cells attained numerous closely packed and regularly oriented microvilli (Fig. 1c) and possessed large spherical centrally or basally located nuclei with evenly distributed chromatin. Mitochondria were numerous apically; exhibiting rounded shapes with dense matrices and transverse cristae (Fig. 1c; Table 4). Poorly developed RER and few free ribosomes scattered within the cytoplasm were observed. On the other side, light microscopical observation revealed that Distal Convoluted Tubule (DCT) attained a wide lumen (Fig. 1a), numerous smaller sized cuboidal cells. Besides, measurements showed that DCT attained larger dimensions than those recorded in PCT (Table 3). Ultrastructurally these cells attained few short apical microvilli within the lumen. Nuclei were spherical euchromatic and basally located. Mitochondria were small in size (Table 4) located within basal infoldings that appeared pronounced in most cells (Fig. 1d).

Worth mentioning that vehicle administration (GIb) did not affect significantly any of the cellular investigated in kidneys sections in control group (GIa) (Fig. 2 a-d). At the ultrastructural level also, vehicle group revealed normal subcellular structures including elongated mitochondria that were regularly arranged within basal infoldings in most PCT cells (Fig. 2b) (Table 4).

In the current study, kidney sections of rats injected with cisplatin (GIIa); showed more intense characteristics of chronic nephropathy when compared to controls after 2 weeks. These changes included: hypertrophied renal corpuscles (5.73±0.17 μm), with reduced glomerular cellularity (Fig. 3a) and maximally and significantly dilated urinary space (0.68±0.10 μm). Significant

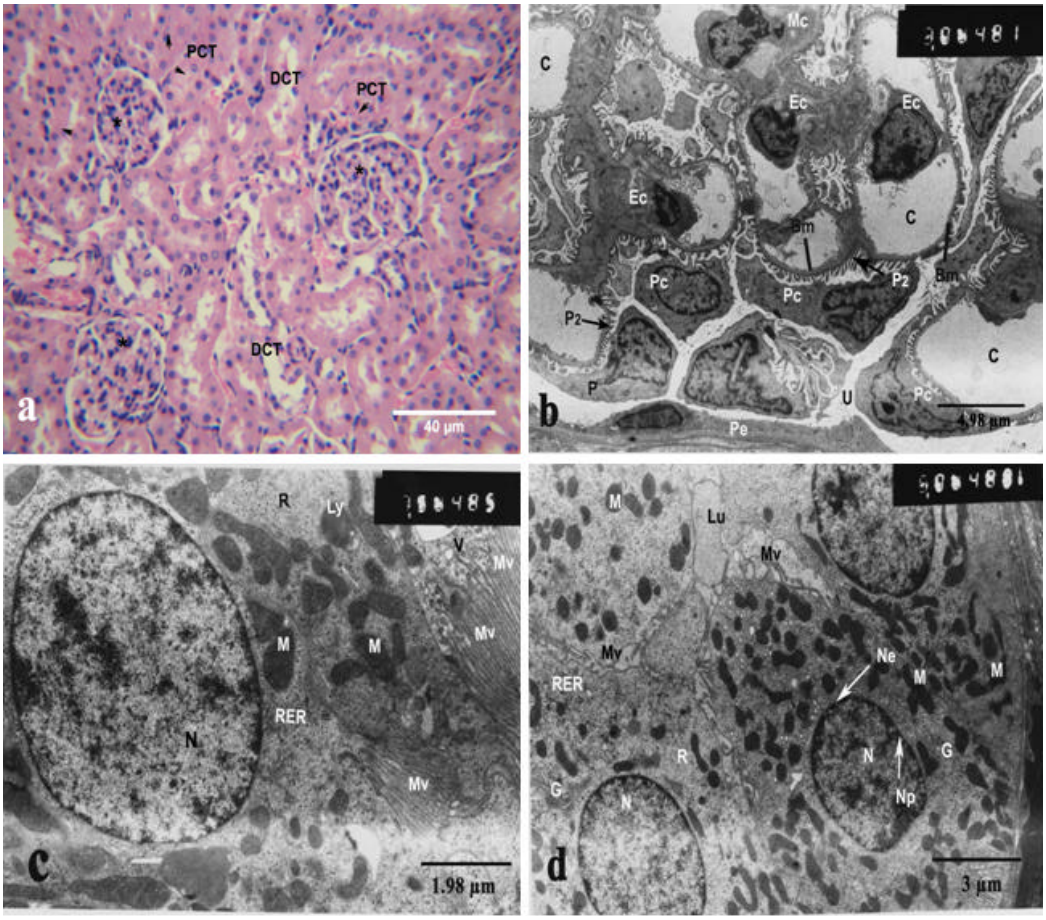

Fig. 1 (a-d): a) Light micrographs. Sections of normal rat kidneys after 2 weeks. Showing, Renal corpuscle (asterisk), brush borders of PCT (arrow head), distal convoluted tubule DCT (Hematoxylin and eosin stained sections). b-d) Electron micrographs. Control group after 2 weeks. b) Showing parts of renal corpuscle, Parietal epithelium (Pe), Urinary space (U), Podocyte (Pc) with secondary foot processes (P2), Endothelial cells (Ec), renal filtration barrier Basement membrane (Bm), Capillary loops (C), Mesangial cells (Mc). c) Part of PCT with numerous Microvilli (Mv), oval basal Nucleus (N); organized Mitochondria (M), RER, Ribosomes (R), Vesicles (V); Lysosomes (Ly). d) Part of DCT: spherical apical Nucleus (N), Nuclear envelope (Ne), Nuclear pores (Np), Mitochondria (M), RER, Golgi bodies (G), few short Microvilli (Mv), Lumen (Lu)

ultrastructural changes were greatly detected among renal structures after 2 weeks (Fig. 3b-d) including atrophied endothelial cells. Most of the podocytes revealed swollen and fused pedicels in parallel with significant increase in filtration slit width as compared to those of control (Table 2). However, slight thickening in renal filtration barrier that appeared highly irregular was detected (Table 2). Moreover, mesangial cells decreased in their number and some of them showed dense pyknotic nuclei and matrices as well as reduced dimensions (Fig. 3b). Moreover, it was observed that cisplatin treatment revealed focal and severe PCT tubular degenerative features including, significant reduction in the mean number of cells/tubules (11.00±0.57) accompanied with a significant increase in their mean width compared to controls (Table 3). Their tubular lumen appeared wide and contained cellular debris and most of them showed swollen outlines. Light preparations showed also, mononuclear cellular infiltration among renal tubules. Ultrastructurally, PCT revealed severed signs of tubular necrosis after cisplatin administration including, harshly fragmented and elongated microvilli, apical cytoplasmic blabbing and vacuolization (Fig. 3c) and thickened basement membrane. In addition, nuclei of most PCT cells attained corrugated nuclear envelope with numerous nuclear pores (Fig. 3c). These preparations showed rounded mitochondria with increased diameter (compared to control) possessing obscure cristae and located at the apical cellular part (Table 4). Also

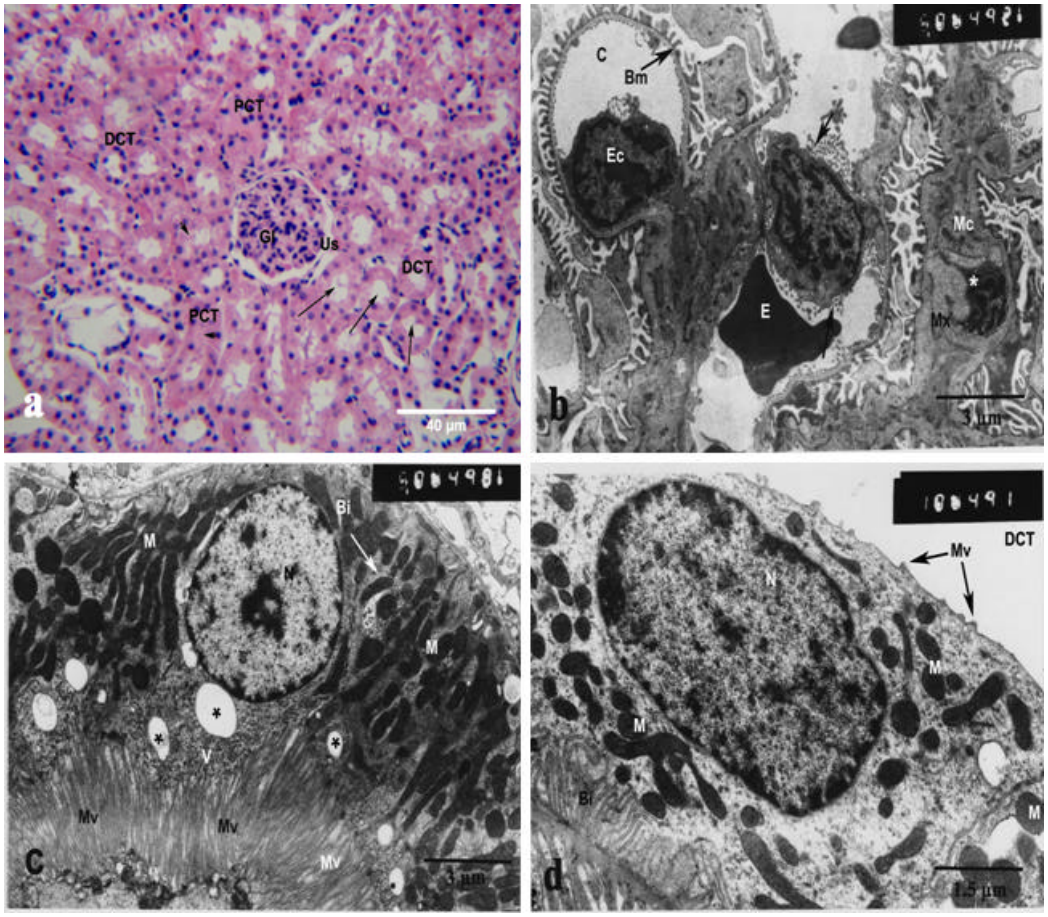

Fig. 2 (a-c): a) Light micrographs of sections of rat kidneys in vehicle administrated groups after 2 weeks; showing Glomerulus (Gl), with narrow Urinary space (Us), Proximal Convoluted Tubule (PCT), with brush borders (arrow head), Distal Convoluted Tubule (DCT) with wide lumen (arrow). (Hematoxylin and eosin stained section). b-d) Electron micrographs, sections of kidneys of male Sprague Dawley rats in vehicle administrated group after 2 weeks. b) Parts of renal corpuscle; Capillary (C): Endothelial cells (Ec), Erythrocyte (E), Mesangial cell (Mc), dense mesangial Matrix (Mx) and numerous processes (arrow); pyknotic nucleus (asterisk); regular Basement membrane (Bm). c) Part of PCT: rounded basal Nucleus (N); numerous condensed Mitochondria (M), numerous long Microvilli (Mv), Vesicles (V); lipid droplet (asterisk), numerous Basal infoldings (Bi). d) Part of DCT: oval apical Nuclei (N), with even chromatin distribution, dense Mitochondria (M), few short Microvilli (Mv), Basal infoldings (Bi)

numerous lysosomes were commonly detected. Light microscopy however, showed that most DCT showed few changes and revealed significant decrease in cellular number and moderate change in dimensions (Table 3). Ultrastructurally, few tubular changes were pronounced in DCT cells where their nuclei appeared euchromatic attaining different sizes with uneven arrangement and highly corrugated nuclear envelope (Fig. 3d). Mitochondria were pleomorphic in shape, with electron dense matrices and were highly disorganized and scattered irregularly throughout the cytoplasm (Table 4). Basal infoldings appeared greatly disrupted and irregular (Fig. 3d), whereas numerous desmosomes were greatly pronounced apically.

Further light microscopical examinations revealed that after 2 weeks of experiment, silymarin when administrated 2 h after cisplatin (GIIb), showed little pathological changes, if compared to those observed in rats administrated cisplatin alone (Fig. 4a), where renal corpuscle significantly showed normal dimensions (4.71±0.17 µm) and appeared more regular with moderate glomerular cellularity, minimal blood congestion and slightly narrow urinary space (0.31±0.10 µm). Rare tubular necrosis and inflammatory cells among renal tubules were detected (Fig. 4a). Ultrastructurally post treatment of silymarin revealed resemblance to those recorded in control group (GIa) (Fig. 4b-d). Glomerular capillary loops occupied with

Table 3: PCT and DCT tubules (μm) changes in response to treatment among control and experimental group[1]

	PCT		DCT	
Groups	Length (μm)	Width (μm)	Length (μm)	Width (μm)
Control GIa	1.66±0.14[a]	6.40±0.19[a]	1.74±0.1[a]	7.23±0.15[a]
Vehicle GIb	1.64±0.15[a]	6.33±0.19[a]	1.62±0.1[a]	7.92±0.4[a]
Cisplatin GIIa	2.64±0.09[b]	7.63±0.29[b]	2.66±0.1[b]	7.90±0.4[a]
Silymarin 2 h after cisplatin GIIb	1.71±0.07[a]	7.12±0.2[b]	2.31±0.1[b]	7.61±0.2[a]
Silymarin 2 h before cisplatin GIIc	1.52±0.07[a]	5.76±0.21[c]	2.25±0.1[b]	7.52±0.2[a]
Results of one way ANOVA	p<0.05*	p<0.05*	p>0.345	p>0.05
	F = 9.8	F = 29.7	F = 1.112	F = 11.26

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Table 4: Morphometric measurements of mitochondrial dimensions of proximal tubular cells of kidneys (μm) among control and experimental group[1]

	Elongated mitochondria		Rounded mitochondria
Groups	Length (μm)	Width (μm)	Diameter (μm)
Control GIa	3.16± 0.19[a]	0.34±0.02[a]	0.48±0.02[a]
Vehicle GIb	2.95±0.17[a]	0.38±0.01[a]	0.55±0.01[a]
Cisplatin GIIa	2.64±0.27[a]	0.37±0.8[a]	0.75±0.04[b]
Silymarin 2 h after cisplatin GIIb	2.75±0.12[a]	0.58±0.02[a]	0.81±0.07[b]
Silymarin 2 h before cisplatin GIIc	2.74± 0.24[a]	0.47±0.0[a]	0.84±0.04[b]
Results of one way ANOVA	F = 0.99	F = 1.95	F = 13.71
	p>0.05	p>0.05	p<0.05*

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

normal endothelial cells and few erythrocytes. It is of interest that atrophied endothelial cells were minimally detected. Podocytes showed normal cellular and nuclear outlines and their pedicels, filtration slits, renal filtration barrier thickness returned significantly to normal values (Table 2). In addition, mesangial cells remained irregular with dense nuclei and matrices (Fig. 4b). Besides, pyknotic nuclei were detected among few mesangial cells (Fig. 4b). On the other sides, mild to moderate tubular necrosis were detected among PCT and DCT of this group especially at the level of light microscope. PCT cells/tubules increased significantly (15±0.88) in number accompanied with a significant increase in their mean tubular width (Table 2), (returning values to normal). PCT appeared slightly organized with regular lumen occupied by evident brush borders; a large number of epithelial cells were less hypertrophied (compared to cisplatin group) (Fig. 4a). Mononuclear cellular infiltrates was less common among most renal tubules. Ultrastructurally, most PCT cells showed as normal numerous elongated microvilli, attained minimal apical vacuolization and endocytotic vesicles (Fig. 4c). Nuclei appeared euchromatic, oval in shape with segregated and centric nucleoli. Mitochondria appeared rounded in shape and distinct transverse cristae with either dense or light matrices and showed normal dimension (Table 4). Basal infoldings appeared regular possessing numerous elongated mitochondria in between (Fig. 4c). Numerous free ribosomes and moderate number of lysosomes were scattered throughout the cytoplasm (Fig. 4c). In addition, hematoxylin and eosin stained kidney section of this group revealed that DCT did not show significant tubular damage (Fig. 4a) and their dimensions returned significantly to normal values (Table 2). Moreover, most DCT cells revealed higher cellular and nuclear organization with great reduction of cytoplasmic vacuolization. Ultrastructurally, silymarin posttreatment showed that most DCT preserved regular cellular and nuclear arrangement and structures, few cells showed reduced microvilli, rounded heterochromatic nuclei and organized cytoplasmic organelles (Fig. 4d).

Similarly, pretreatment with silymarin 2h before cisplatin (GIIc) exhibited few changes compared to GIIb (Fig. 5 a-d) after 2 weeks. At the level of light microscopy, majority of renal corpuscles returned to normal dimensions (4.48±0.16 μm) along with narrow urinary space (0.30±0.08 μm), regular glomerular cellularity and minimal erythrocytes leakage (Fig. 5a). Electron microscopic observation showed well preserved cell structures and organelles in most kidney samples of this group. However, some minor alterations were observed among renal corpuscles including few glomerular capillary loops with mild congestion. Podocytes achieved significant recovery ultrastructural features as they appeared normal (Fig. 5b) with less swollen pedicels and more regular renal filtration barrier basement membrane (Table 2). In addition to the well preserved cell structures and organelles already recorded in group Iib, pretreatment with silymarin

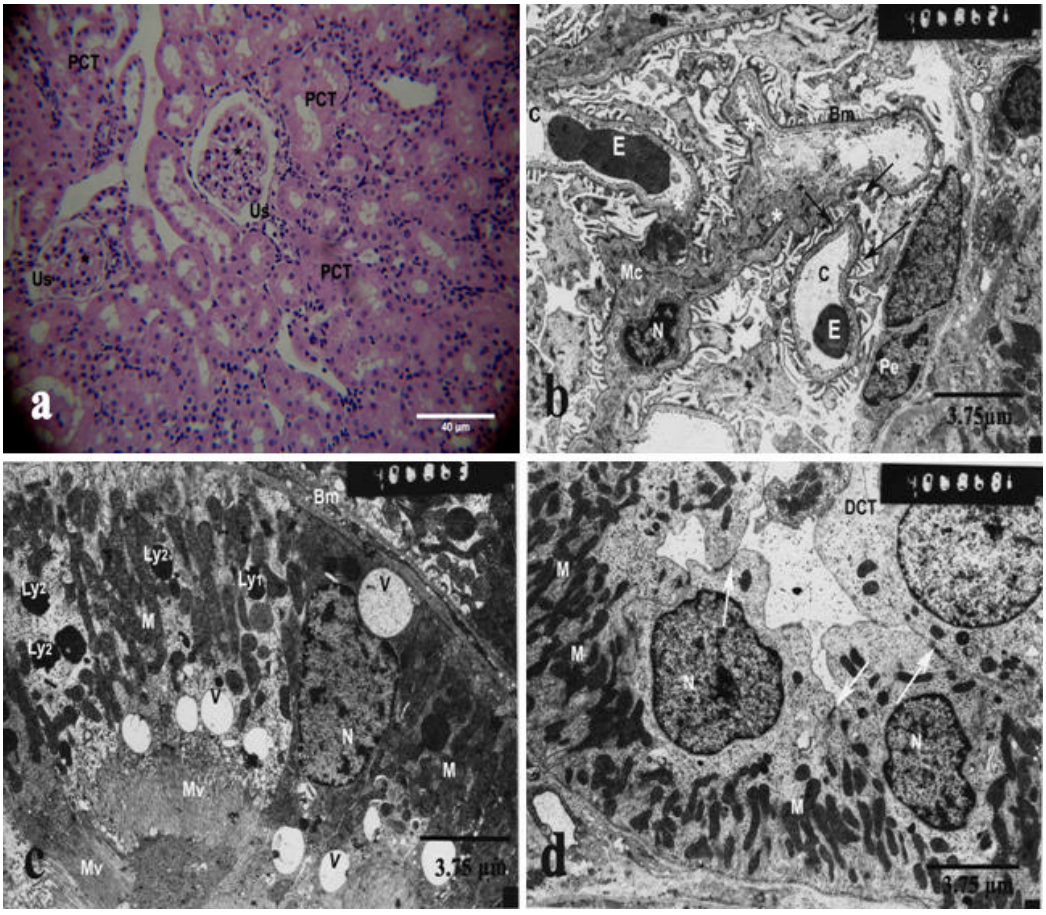

Fig. 3 (a-c): a) Light micrograph of sections of rat kidneys in cisplatin treated group after 2 weeks; showing shrunk glomerulus (asterisk), wide Urinary space (Us) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidneys of cisplatin injected male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: Capillaries (C) with deformed Erythrocytes (E). Mesangial cell (Mc): small irregular Nucleus (N). Ballooned secondary foot processes (arrow), thickened Basement membrane (Bm) with disruption (asterisk); cleaved nucleus of Parietal cell (Pe) c) PCT cells with few Microvilli (Mv), increased apical and basal Vacuoles (V), irregular Nucleus (N); numerous disorganized Mitochondria (M), numerous primary (Ly1) and secondary (Ly2) lysosomes, thickened Basement membrane (Bm). d) Part of DCT: absence of microvilli, increased tight junctions (arrow); severe cellular blabbing with irregular Nuclear outlines (N) of different sizes, pleomorphic Mitochondria (M) with swelling profiles

exhibited the highest minimal frequency of pyknotic nuclei among mesangial cells (Fig. 5b).

Pretreatment with silymarin treatment succeeded in keeping PCT dimensions as well as number of cell/tubules as normal values (15±0.82) when observed at the level of light microscope. Tubular necrosis was completely absent among a large number of PCT examined; lumen appeared narrow with brush border arising from a well organized pyramidal shaped PCT cells (Fig. 5c). On the other side, silymarin pretreatment revealed normal histological features including significantly elevated PCT dimensions when compared to their corresponding in cisplatin group (Table 3). Ultrastructurally, PCT cells appeared highly organized with minimal loss of microvilli, complete absence of cytoplasmic vacuolization and regular basal infoldings (Fig. 5c). PCT cells illustrated normal rounded and basally located nuclei. Most mitochondria appeared normal with regular dimensions (Table 4), attaining dense matrices and transverse cristae and distributed apically above and basally within basal infoldings (Fig. 5c).

Moreover, light microscopic observation showed that DCT maintained normal number of cells and their dimensions significantly approaches those of controls (Fig. 5a) and most of them exhibited regular

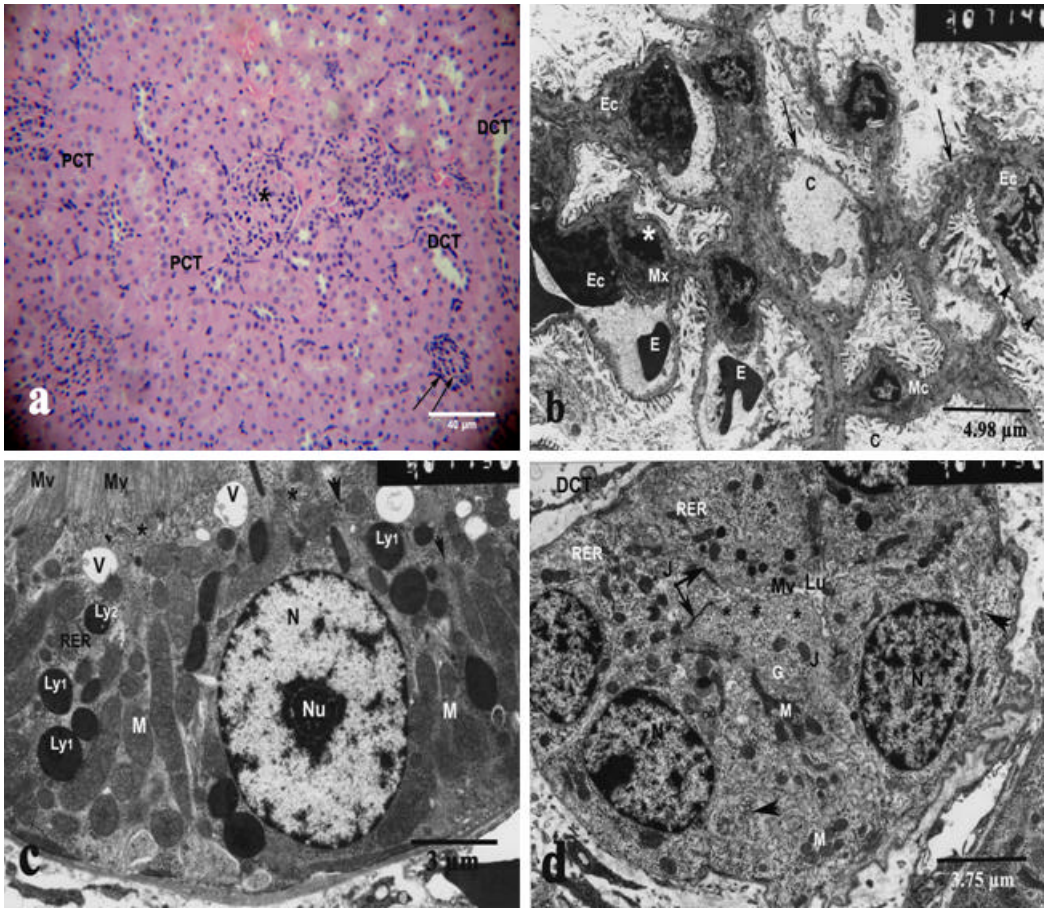

Fig. 4 (a-c): a) Light micrograph of sections of rat kidneys treated with silymarin 2 h after cisplatin of male Sprague Dawley rats after 2 weeks; showing normal renal corpuscles (asterisk), tubules (PCT and DCT), few mononuclear infiltration (double arrow) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidney treated with silymarin 2 h after cisplatin of male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: Capillary (C) lumen congested with few Erythrocytes (E), Endothelial cell (Ec); Mesangial cell (Mc) with dense mesangial Matrix (Mx): pyknotic nucleus (asterisk); reduced secondary processes (arrow); regular basement membrane (arrow head). c) PCT cell: numerous Microvilli (Mv), pinocytotic vesicles (*), apical Vacuoles (V), ribosomes (arrow head), oval basally located Nucleus (N) with centric and segregated Nucleolus (Nu); numerous organized Mitochondria (M) with transverse cristae; RER, moderate number primary (Ly1) and secondary lysosomes (Ly2). d) Part of DCT: Microvilli (Mv), apical vesicles (asterisk), tight Junctions (J), oval Nuclei (N), more organized dense Mitochondria (M), RER, Golgi bodies (G).

architecture with wide lumen. DCT attained normal cellular and tubular appearance (Table 3). Ultrastructurally, pretreatment of silymarin inhibited the hazardous effect of cisplatin and resulted in minimal changes in DCT where most cells appeared normal cellular and cytoplasmic appearance similar to those recorded in control group (Gla) Most cytopalsmic organelles and mitochondria appeared organized (Fig. 5d).

DISCUSSION

In the current study, the proposed plan aimed to assess and examine the possibility of silymarin to prevent the alterations induced by cisplatin in kidney tissues of male Sprague Dawley rats, which were used as biological test animals.

Cisplatin is an effective chemotherapeutic agent for a wide variety of tumors (Park *et al*., 2009). Nevertheless, it has several side effects including hepatotoxicity (Mansour *et al*., 2006; Pratibha *et al*., 2006) and nephrotoxicity (Park *et al*., 2009). Mora Lde *et al*. (2003) showed that, a decrease in antioxidant enzymes resulted from cisplatin induced tissue toxicity. They added also that the development of therapies to prevent the appearance of cisplatin-induced tissue toxicities has focused on administration of antioxidants along with

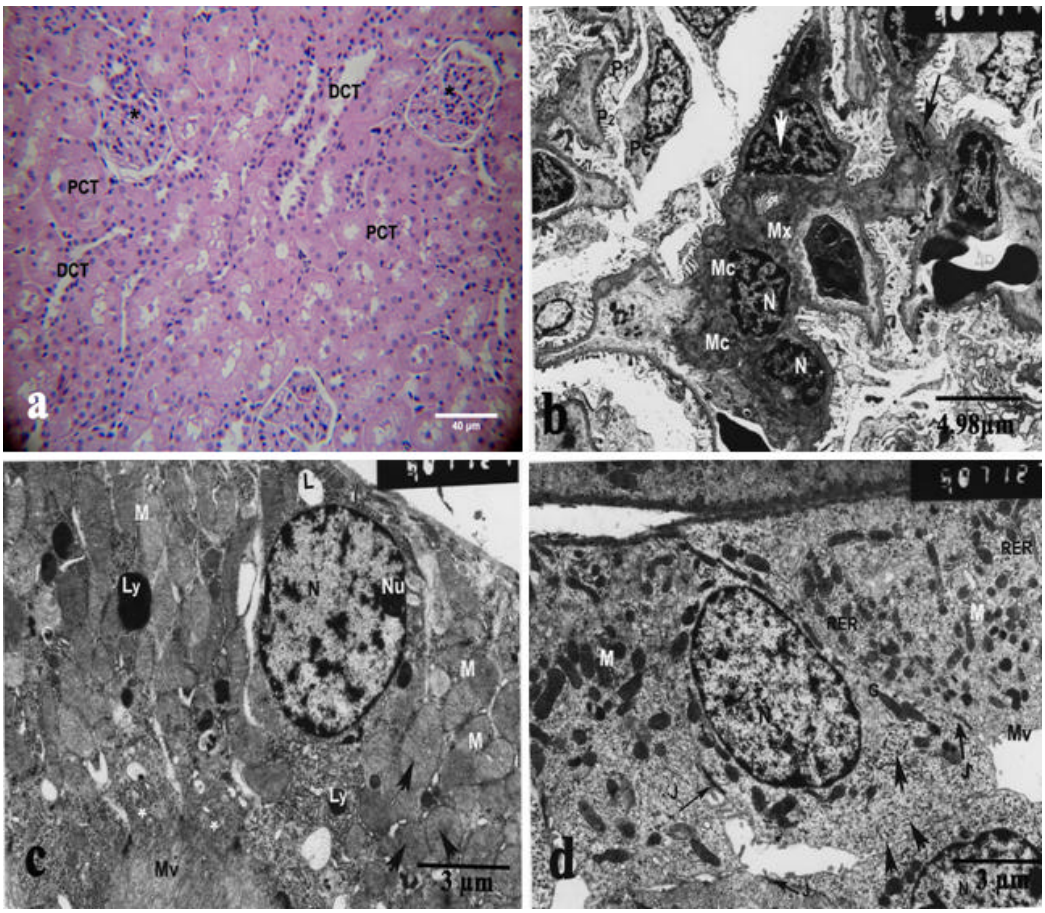

Fig. 5 (a-c): a) Light micrograph of sections of rat kidneys treated with silymarin 2 h before cisplatin of male Sprague Dawley rats after 2 weeks; showing regular renal corpuscles (asterisk) and tubules (PCT, DCT) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidneys treated with silymarin 2 h before cisplatin of male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: regular mesangial cell (Mc) with dense matrix (Mx): cleaved nucleus (arrow head), pyknotic nucleus (arrow); podocytec (Pc) with organized outline and processes (P1 and P2). c) PCT cell: numerous Microvilli (Mv) and vesicles (asterisk), rounded basally located Nucleus (N) with marginated Nucleolus (Nu); numerous organized Mitochondria (M) with transverse cristae (arrow head), Lipid droplet (L), Lysosomes (Ly). d) Organized part of DCT: Microvilli (Mv), tight Junctions (J), euchromatic Nucleus (N), numerous well oriented dense Mitochondria (M), RER, Golgi bodies (G), ribosomes (arrow head)

cisplatin treatment. Thus, many studies dealing with the protective effects using extracts of natural products and dietary antioxidant against cisplatin induced tissue toxicities have been reported (Behling *et al*., 2006; Mansour *et al*., 2006).

Silymarin, the root extract from *Silybum marianum*, is known to have hepatoprotective effect against numerous liver diseases (Eminzade *et al*., 2008). Karimi *et al*. (2005) reported that silymarin has antinephrotoxic activity against cisplatin induced nephrotoxicity in albino rats.

In the present investigation, a single dose of cisplatin (5 mg/kg), in male rats resulted in significant body weight reduction and decreased food intake that was most pronounced after 2 weeks. In accordance with our results, Chirino *et al*. (2004) suggested that i.p. administration of a single dose of cisplatin to male Wistar rats (7.5 mg/kg) after 3 days significantly decreased their body weight. Confirming our results, Shimeda *et al*. (2005) stated that cisplatin has been shown to decrease total body weight in male rats. During the course of the present study, post-treatment and pre-treatment of silymarin (50 mg/kg) remarkably prevented the reduction in body weight induced by cispaltin, thus leading to normal body weight. Our results were in agreement with the results of Gaedeke *et al*. (1996), who noticed that daily i.v. injections of silibinin (active compound of silymarin) (200 mg/kg) to female Wistar rats succeeded in the complete inhibition of the hazardous effect of cisplatin on the experimental animals' body weight over a period of 11 days.

Contradicting our results, Shimeda *et al.* (2005) stated that daily oral administration of capsaicin antioxidant (10 mg/kg) for 6 consecutive days, increased male Sprague Dawley body weight, yet it failed in the complete recovery, i.e. return to normal body weight.

In the present study, our results showed that cisplatin induced kidney damage characterized by a significant increase in the wet kidney weight manifested by a significant elevation in kidney weight to body weight ratio after 2 weeks from the beginning of the experiment. Our results were in accordance with those reported by Shimeda *et al.* (2005), who indicated a significant increase in the kidney weight to body weight ratio in cisplatin treated male S.D. rats (5 mg/ kg). However, our results were opposing those reported by Lee *et al.* (2007) who showed that cisplatin treatment resulted in a significant decrease in kidney weight as a percentage of the total body weight. In the present work, both post- and pre- treatments of silymarin significantly prevented changes in kidney wet weight as well as kidney weight to body weight ratio values to normal after 2 weeks. Confirming our results, Gaedeke *et al.* (1996) and Shimeda *et al.* (2005) who stated that silibinin and capsaicin respectively, used as antioxidant against cisplatin-induced nephrotoxicity resulted in significant protection against cisplatin by decreasing kidney to body weight ratio. During the present work, it could be elucidated that alterations of organ-body weight ratio in cisplatin intoxicated rats could be attributed to tissue damage and altered in their functions. This is in agreement with results reported previously by Lee *et al.* (2007) and Park *et al.* (2009).

The alterations in renal structures detected in rat models correlate well with the nephrotoxic effects of cisplatin in patients treated with antitumor agent (Daugaard *et al.*, 1988a, 1988b). In the present investigation, a single dose of cisplatin (5 mg/kg, i.p.), in rats resulted in the deterioration of renal corpuscle structure and increased tubular necrosis after 2 weeks. Moreover, cisplatin administration revealed that most renal corpuscles appeared hypertrophied with diminished glomeruli congested with erythrocytes and dense mesangial cells and dilated urinary space. In agreement with our results, Chirino *et al.* (2004) showed that cisplatin treatment induced mesangial cells contraction. However, our results contradicted those reported by Chirino *et al.* (2004), who suggested that the alteration in glomerular function cannot be attributed to structural damage since glomeruli exhibited normal appearance in cisplatin treated rats (7.5 mg/kg, i.p., for 3 days).

The kidneys accumulate and retain platinum complexes to a greater extent than other organs, perhaps via mediated transport and it is the main excretory outlet for either intravenous or intraperitoneal cisplatin (Arany and Safirstein, 2003). The underlying mechanism of cisplatin-induced nephrotoxicity is still not well known but many recent *in vitro* and *in vivo* studies indicate an important role for the reactive oxygen metabolites in the pathogenesis of this effect (Matsushima *et al.*, 1998). Behling *et al.* (2006) suggested that cisplatin acts mostly on the PCT of the kidney. Arany and Safirstein (2003) reported that proximal tubular epithelial cells take up this antitumor agent and this actively leads to higher concentrations than those found in the plasma; thus, cisplatin toxicity in PCT is morphologically characterized by tubular necrosis. In the present study, cisplatin caused structural alterations characteristics of acute tubular necrosis in both PCT and DCT after 2 weeks. This is in accordance with those results reported by Karimi *et al.* (2005) who stated that male Wistar rats receiving single dose of cisplatin (3 mg/kg) for 5 days showed severe tubular necrosis among kidney sections. Moreover, in the present work, PCT showed cytoplasmic debris, denudation of PCT basement membrane, swollen PCT cell with open face and pyknotic nuclei, vacuolated cytoplasm; intercellular edema and mononuclear infiltration. DCT appeared with few tubular changes. Thus, our description were in general agreement with those reported by Chirino *et al.* (2004), who observed PCT tubular necrosis, cytoplasmic vacuolization and intercellular edema in cisplatin treated male Wistar rats (at dose level 7.5 mg/kg, i.p., for 3 days). Similarly, Morigi *et al.* (2004) and Behling *et al.* (2006), reported similar description in acute cisplatin nephrotoxicity. After 2 weeks from the beginning of the present experiment, histological features of chronic nephropathy as indicated by degenerated and highly congested glomeruli were detected among kidney sections of this group. Confirming our results, El-Abd and Okda (2007), suggested that male rats receiving ribavirin (at dose level 12 mg/kg twice day) for 3 weeks, demonstrated highly congested capillaries with sever hemorrhage along with atrophied renal glomeruli. Besides, in the present study cisplatin injection resulted (after 2 weeks) in complete absence of PCT brush border, hypertrophied PCT cells with numerous cytoplasmic degenerative vacuoles, detachment of basement membrane and increased cellular infiltration. DCT exhibited similar atrophied profiles but were less severe. Similar to the present results, were those reported by Behling *et al.* (2006), who focused on the histopathological features of chronic nephropathy induced by cisplatin for 20 days in male Wistar rats including tubular atrophy and dilatation.

In the current study, glomerular and tubular atrophy was less intense in rats administrated silymarin 2 h after cisplatin (GIIb). After 2 weeks, silymarin post-treatment showed attenuation of glomerular atrophy that revealed minimal erythrocytes leakage and slightly dilated urinary space. Confirming our description, El-Abd and Okda (2007), suggested that male rats receiving i.p., injection

of silymarin (250 mg/kg for 1, 2 and 3 weeks) reduced and improved histopathological lesions induced by ribavirin (broad spectrum antiviral drug) specifically atrophied renal glomeruli. In the present study, renal tubules of this group exhibited moderate microvilli, normal shaped cells with minimal cytoplasmic degenerative vacuoles, mild to moderate tubular necrosis as well as inflammatory cell infiltration. Our results were in great accordance with those reported by Karimi *et al*. (2005), who stated that post-treatment with silymarin (50 mg/kg, i.p.) 2 h after cisplatin for 5 days, resulted in mild to moderate renal cellular injury. In agreement with Behling *et al*. (2006) who reported that gavage of administration of flavonoid quercetin (50 mg/kg) to male wistar rats receiving cisplatin (5 mg/kg) after 5 and 20 days showed reduction of acute tubular necrosis including, focal areas of broken basement membrane, swelling and flattening of PCT cells with brush border loss, diffuse interstitial edema and interstitial inflammatory cell infiltrate. In the present work, 2 weeks after silymarin treatment did not exhibit complete protection against histopathological changes induced by cisplatin. Thus, glomeruli as well as renal tubules exhibited normal cellular architecture yet very mild glomerular atrophy and tubular necrosis were rarely detected. Induction of nephrotoxicity by cisplatin is assumed to be a rapid process involving reaction with proteins in the renal tubules (Montine and Borch, 1990). Rao and Rao (1992) stated that renal damage occurs within 1 h after cisplatin administration. It is important that the protective agent is present in renal tissue before damage occurs. This might explain why complete protection did not result in our study when silymarin were given after administration of cisplatin.

In the present investigation, pre-treatment with silymarin 2 h before cisplatin administration resulted in inhibition or complete protection against cisplatin induced damage after 2 weeks; glomeruli appeared with normal dimensions, mild renal cellular injury were noted in few foci. Confirming our finding were those reported by Behling *et al*. (2006), who studied the complete protective effect of silymarin when administrated 2 h before cisplatin injection in male albino rats. However, the present work focused on the total protective and preventive effect of this treatment that significantly increased after 2 weeks, since glomerular as well as renal tubules showed normal histological features and dimensions. Our results concur with those previous reported by Gaedeke *et al*. (1996) who demonstrated that female Wistar rats receiving pre-treatment with silibinin (200 mg/kg, i.v.) 1 h prior to cisplatin administration for 11 days significantly decreased both proximal tubular and glomerular damage induced by cisplatin. Tubular defects resulting from cisplatin treatment have been ascribed largely to the generation of free radicals (Hannemann and Baumann, 1998). Cisplatin-induced damage could be increased by depleting cells of protective radical scavengers like gluthathione or superoxide dismutase (Sadzuka *et al*., 1992).

Electron microscopical examinations revealed many ultrastructural alterations in kidney preparations of cisplatin-treated animals. Since PCT were more affected than DCT, we believe that a relationship between the action of cisplatin used and the function of these tubules could be suggested. This agrees with the findings of Morigi *et al*. (2004), who studied the effect of cisplatin on the kidney of albino mice. In this study, it was also noted that cisplatin induced alterations in renal corpuscles including destructed and atrophied endothelial cells; hypertrophied podocytes with elongated, swollen and fused podocyte pedicels and widened filtration slits; thickening and highly irregular renal filtration barrier. These data are in close correlation with those reported by Kohn *et al*. (2002), who studied the nephrotoxic effect of cisplatin on kidney glomerular components in guinea pigs. Moreover, in the present study, mesangial cells (minimal in number and with slight depression in their dimensions according to morphometric measurements) appeared irregular with bizarre shaped nuclei and dense matrices. Confirming our results are those reported by L'Azou *et al*. (2002) who demonstrated that cadmium used as a nephrotoxin agent in mesangial cell culture, induced masangial glomerular cell contraction that was evident by decrease in the mesangial cells surfaces. It is conceivable that even a minor reduction in the mesangial cell area considerably affects the filtering surface of the glomeruli and could explain the decreased glomerular filtration (Rodriguez *et al*., 2000). Additionally, the present work showed serious PCT tubular lesions with loss of microvilli, pyknotic nuclei, cytopalsmic vacuolization, increased lysosomes and altered mitochondrial structure and arrangement. On the other hand, less tubular changes including minimal microvilli, pyknotic nuclei and organelles disorganization, were pronounced in DCT cells but to a lesser extent. Morigi *et al*. (2004), revealed great similarities to a certain extent with our results, where cisplatin resulted in focal and severe tubular changes in PCT and DCT cells, in male albino mice. In the present study, 2 weeks after cisplatin administration exhibited progressive and increasing glomerular cells, PCT and DCT tubular damage manifested by reduced podocytes with highly hypertrophied foot process and sharply dilated filtration slits. PCT cells with complete fragmented microvilli and increased frequency of pyknotic nuclei, cytoplasmic vacuolization, myelin figures and altered mitochondria. In agreement with our

findings, Shalaby *et al.* (2006) who studied the chronic effect of cisplatin in kidneys of male albino mice over 21 days and showed similar degenerative changes in PCT cells.

In the present study, the protective effect of silymarin was confirmed when renal tissues were observed by electron microscope. After 2 weeks, both post-treatment and pre-treatment with silymarin showed minimal changes in renal cellular structures in most kidney samples. However, some minor to moderate alteration including: slightly congested capillary loops; minimal atrophied endothelial cells; irregular mesangial and podocytes cells; PCT with reduced microvilli, moderate cytoplasmic vacuolization, irregular mitochondria; DCT cells with pyknotic nuclei and disorganized organelles. The protective effect of pretreatment of silymarin was greatly evident than those in post-treatment group, as it resulted in the complete protection and well preservation of the renal corpuscle structures as well as PCT and DCT cells structures and arrangement that did not exhibit significant differences from those in control groups.

In contrast post-treatment with silymarin reflected moderate protection with minimal alteration in renal glomerular structures and PCT cells such as partial loss of microvilli and cytoplasmic vacuolization. These findings were similar to a certain extent to those reported by Morales *et al.* (2006), who suggested that kidneys of male wistar rats receiving flavinoids i.p. quercetin (at dose level 50 mg/kg/daily for nine weeks) showed well preserved cell structures in most kidney samples including minor alteration in PCT cells such as partial loss of microvilli and isolated vacuoles could be observed.

Conclusion: The present work has shown that experimental administration of cisplatin in rats was greatly associated with many biological, histological and ultrastructual changes. Although the exact mechanism by which silymarin prevent to a great extent, cisplatin toxicity remained to be elucidated, yet our present findings suggest that silymarin protects against acute cisplatin nephrotoxicity and may be considered as a potentially useful candidate in the combination with chemotherapy by acting in the kidney as a potent scavenger of free radicals thus preventing the toxic effect of cisplatin both the histological and ultrastructural levels.

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Stoichiometric Relationship Between Short Chain Fatty Acid and *in vitro* Gas Production of Semi-arid Browses of North-Eastern Nigeria

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Abstract: The *in vitro* gas production of semi-arid browse species were evaluated. The relationship between *in vitro* gas measured on incubation of tannin-containing browses in buffered rumen fluid and calculated from Short Chain Fatty Acid (SCFA) production was investigated. Crude Protein (CP) content in the browses ranged from 13.96-21.421% Dry Matter (DM). The NDF and ADF were 33.31-58.81 and 21.16-31.39 g/100 g DM respectively. The ash content of the browses ranged from 10.76-17.76 (% DM). The content of phenolic and Saponin were 0.32-0.48 and 2.02-2.78 mg/g DM. Total Condensed Tannin (TCT) ranged from 0.32-2.96 mg/g DM. The TCT was significantly correlated ($p<0.05$) with gas production ($r = 0.95$; $p<0.05$). The Metabolizable Energy (ME) and Organic Matter Digestibility (OMD) were 3.31-6.23 (MJ/Kg DM) and 30.64-55.44 (% DM). A good relationship ($R^2 = 0.99$; $p<0.05$) was observed between measured *in vitro* gas production and that calculated from SCFA. The relationship between *in vitro* gas measured on incubation of browse leaves and that calculated from SCFA allows prediction of SCFA from gas production. The study showed that the leaves of the browse forages had nutritive value and therefore, may serve as potential supplements for ruminants in Nigeria.

Key words: Stoichiometric, *in vitro*, browse, tannins, short chain fatty acids, phenolic

INTRODUCTION

Browsable plants, beside grass, constitute one of the cheapest sources of feed for ruminants. The diversity and distribution of browse plants in Nigeria have received early attention in studies carried out for the north (Saleem *et al*., 1979), southwest (Carew *et al*., 1980) and middle belt (Ibeawuchi *et al*., 2002) Nigeria. Tropical browse have been shown to contain varying quantities of condensed tannin and other anti-nutritional substances in their biomass that affect their optimal utilization by animals (Odenyo *et al*., 1999). The tree and shrub legume forages are rich in most essential nutrients such as proteins and minerals and tend to be more digestible than the grasses and crop residues. This necessitates the evaluation of the nutritional characteristics of the forages in order to maximize their use in ruminant diets.

The *in vitro* gas production technique as modified by Menke and Steingass (1988) is widely used to evaluate the nutritive value of feeds resources consumed by ruminants especially tree and shrub legume forages, particularly to estimate energy value of straws (Makkar *et al*., 1999), agro industrial by-products (Krishna and Gunther, 1987), compound feeds (Aiple *et al*., 1996) and various types of tropical feeds (Krishnamoorthy *et al*., 1995). The gas produced on incubation of cereal straws (Blummel and Ørskov, 1993), cereal grains (Opatparakit *et al*., 1994) and different classes of feed (Blummel *et al*., 1999) in buffered rumen fluid was closely related to the production of Short Chain Fatty Acids (SCFA) calculated using the stoichiometry outlined by Wolin (1960), which was based on carbohydrate fermentation. Little work has been done to investigate the effect of proteins and fats on stoichiometry of gas production. Cone and Van Gelder (1999) reported a poor correlation between measured and calculated gas volume on incubation of starch and glucose with increasing levels of casein. The objective of the present study was to assess the contents of phenolic compounds, *in vitro* gas production and stoichiometrical relationship between measured gas production and that calculated from SCFA production on incubating tropical browse species.

MATERIALS AND METHODS

Forage samples: Four indigenous browse samples (leaves) commonly consumed by ruminants animals were used in this study. The species were: *Ficus polita*, *Ficus thonningii*, *Batryospermum paradoxum*, *Kigalia africana*, *Celtis integuifolis*, *Khaya senegalensis*, *Leptadenia lancifolia* and *Ziziphus abyssinica*. All forages were harvested from Gwoza local government area of Borno State Nigeria. The area is located at 11.05° North and 30.05° East and at an elevation of about 364 above sea level in the North Eastern part of Nigeria. The ambient temperature ranges between 30°C and 42°C being the hottest period (March to June) while its cold between November to February with temperatures ranging between 19-25°C. The browse forages were harvested from at least 10 trees per each specie selected at random in four locations with the study area at the end of the season. The harvested

sample were then pooled for each individual tree species and then oven dried at 105°C for 24 h to constant weight and ground to pass through a 1.0 mm, sieve. The samples were then sub-sample to obtain three samples for each tree species and used for the laboratory analysis.

Chemical analysis: Browse species were analyzed for Dry Matter (DM), Crude Protein (CP), Ether Extract (EE), Crue Fibre (CF) and ash according to AOAC (2005). The leaves samples were also analyzed for Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), according to Van Soest *et al.* (1991). Phenolic was determine using Folin Ciocalteu as describe by (Makkar, 2000). Saponins and total condensed tannin will be determined as reported by (Babayemi *et al.*, 2004). Total condensed tannin was determined as reported by (Polshettiwar *et al.*, 2007).

***In vitro* gas production study:** *In vitro* incubation was carried out using the method of Menke and Steingass (1988) in 30 ml buffered rumen fluid. Samples (200 mg DM) were incubated in triplicate. Rumen fluid was taken before the morning feed from three non lactating, non-pregnant West African Dwarf (WAD) female sheep through sunction method. The animals were fed with (*Panicum maximum*) *ad libitum* and 40% concentrate. Rumen fluid was collected into a pre-warmed insulated bottle, homogenized in a laboratory blender, strained using Cheese cloth with a pore size of 100 µm and then filtered through glass wool. All handling was carried out with continuous flushing by CO_2. The well-mixed and CO_2 flushed rumen fluid was added to the buffered mineral solution, which was maintained in a water bath at 39°C and mixed. Buffered rumen fluid (30 ml) was dispensed into each syringe containing browse samples. The syringes were immediately placed in a water bath at 39°C. Three syringes containing 30 ml inoculum served as blanks.

Statistical analysis: Metabolizable Energy (ME) was calculated as ME = 2.20 + 0.136GV + 0.057 CP + 0.0029 CF (Menke and Steingass, 1988). Organic Matter Digestibility (OMD%) was assess as OMD = 14.88 + 0.889 GV + 0.45 CP + 0.651 XA (Menke and Steingass, 1988). Short Chain Fatty Acids (SCFA) as 0.0239 GV-0.0601 (Getachew *et al.*, 1999) was also obtained, where GV, CP, CF and XA are total gas volume, crude protein, crude fibre and ash respectively. Data obtained were subjected to analysis of variance. Where significant differences occurred, the means were separated using Duncan multiple range F-test of the SAS (1988) options.

RESULTS

Chemical composition of browse forages: The chemical composition and fibre fraction of the browse species are presented in Table 1. There was wide variation in the chemical composition of the roughages, with CP ranging from 13.96-21.42% DM, Ash from 10.70-17.96 %DM, NDF from 33.31-58.81 g/100 g DM, ADF from 21.16-31.39 g/100 g DM, TCT, PHE and SAP have these values of 0.25-2.96 mg/g DM, 0.32-0.48 mg/g DM and 2.02-2.78 mg/g DM respectively.

***In vitro* gas production:** Figure 1 shows the *in vitro* gas fermentation of the selected browses respectively. Net gas production was highest in *Z. mucronata* and tend to increase with increase in incubation time up to 48 h. It was however observe that gas production pattern of *A. tortilis*, *L. leucocephala* and *M. oleifera* were similar. Metabolizable Energy (ME), Organic Matter Digestibility (OMD), Short Chain Fatty Acids (SCFA) are shown in Table 2. The ME ranged between 3.31 MJ/Kg DM in *L. leucocephala* and 6.23 MJ/Kg DM in *Z. mucronata*. There was significant differences ($p<0.05$) in the ME among the browse forages. *Z. mucronata* was significantly ($p<0.05$) higher in ME than the other browse forages. The ME in *A. tortilis* and *L. leucocephala* was similar ($p>0.05$) below the value of *Z. mucronata*. The OMD ranged from 30.64% in *L .leucocephala* to 55.44% in *Z. mucronata*. The OMD increased significantly ($p<0.05$) with increase in gas production. Highly significant ($p<0.001$) correlations were observed between gas production and TCT ($r = 0.93$, $n = 4$). There were negative relationship between saponin and gas production ($r = 0.10$, $n = 4$); short chain fatty acids and gas production ($r = 0.01$, $n = 4$). The result also revealed a weak correlation between phenolic content of the browses and gas production ($R^2 = 0.44$, $n = 4$); Total condensed tannin and gas production ($R^2 = 0.12$, $n = 4$).

Table 1: Proximate composition of some selected browse species forages of semi-arid (% DM); NDF, ADF (g/100 g DM); TCT (mg/g)

Browse forages	DM	CP	Ash	NDF	ADF	TCT	PHE	SAP
Acacia tortilis	72.33^{c}	13.96^{c}	10.76^{c}	48.62^{b}	21.16^{c}	0.32^{b}	0.48	2.02
Leucaena leucocephala	89.63^{b}	19.42^{b}	17.96^{a}	58.81^{a}	25.52^{b}	2.96^{a}	0.37	2.02
Moringa oleifera	88.22^{b}	21.42^{a}	17.60^{a}	33.31^{c}	31.39^{a}	0.25^{b}	0.45	2.16
Ziziphus mucronata	92.26^{a}	19.23^{b}	15.43^{b}	58.67^{a}	22.89^{c}	0.72^{b}	0.32	2.78
Means	85.61	18.51	15.44	49.85	25.24	1.06	0.41	2.25
SEM	2.19	0.73	0.95	3.42	0.97	0.55	0.09	0.98

^{a,b,c}Means in the same column with different superscript differ significantly ($p<0.05$). Dry matter; CP = Crude Protein; NDF = Neutral Detergent Fibre; ADF = Acid Detergent Fibre; TCT = Total Condensed Tannin; PHE = Phenolic; SAP = Saponin

Table 2: Net gas volume, metabolizable energy, organic matter digestibility, short chain fatty acid of semi-arid browse forages

Browse forages	NGV	ME	OMD	SCFA
Acacia tortilis	2.83[c]	3.47[c]	30.68[b]	0.01[c]
Leucaena leucocephala	1.16[c]	3.31[c]	30.64[b]	-0.03[d]
Moringa oleifera	8.16[b]	4.33[b]	40.94[a]	0.13[b]
Ziziphus mucronata	25.50[a]	6.23[a]	55.44[a]	0.55[a]
Means	9.41	4.33	39.42	0.17
SEM	2.43	0.28	3.35	0.06

Net Gas Volume (NGV = ml/200 mg DM), Metabolizable Energy (ME = MJ/Kg DM), Organic Matter Digestibility (OMD = %), Short Chain Fatty Acids (mmol)

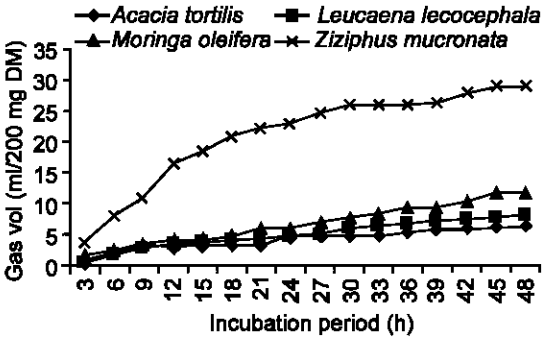

Fig. 1: Gas production of semi-arid browses

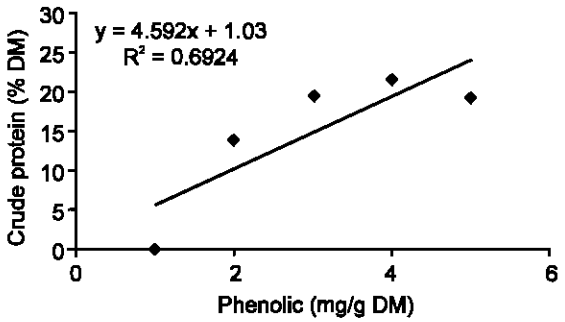

Fig. 2: Relationship between crude protein phenolic of semi-arid browses

DISCUSSION

The CP contents of the browses studied had a similar range as those from West Africa (Rittner and Reed, 1992). The results of the current study, those of Rittner and Reed (1992) and Njidda *et al.* (2009) indicate that most tropical browse species are high in CP and can be used to supplement poor quality roughages to increase productivity of ruminant livestock in tropical regions. The inverse relationship between CP and phenolic compounds indicates that considerable attention should be given in germplasm evaluation programmes to avoid selection against materials of high CP content. The result is similar (r = 0.87, n = 37) to the findings of Getachew *et al.* (2002) who further suggested that studies are required to understand the physiological mechanisms of plants that lead to the inverse

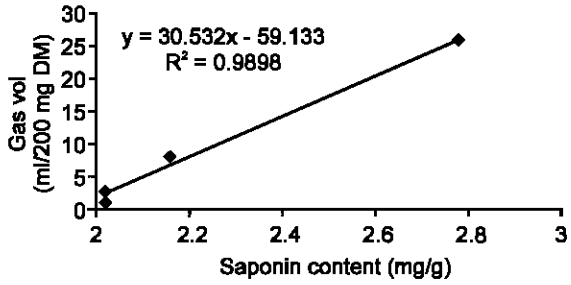

Fig. 3: Relationship between gas production and saponin of semi-arid browses

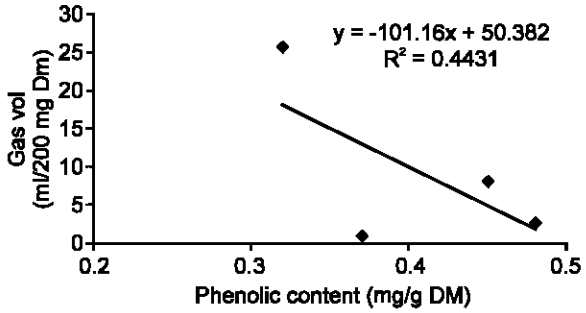

Fig. 4: Relationship between gas production and phenolic of semi-arid-browses

relationship between contents of CP and phenolic compounds and hence to make decisions in plant selection and screening programmes. Though a positive correlation was observed between CP and *in vitro* dry matter digestibility of the same browses (Njidda and Nasiru, 2010). The correlation between the change in gas production in the presence of tannin binding agent and phenolic contents of browses was consistent with those of (Khazaal *et al.*, 1994; Tolera *et al.*, 1997; Wood and Plumb, 1995). The relatively weak correlation between CT and gas production (R^2 = 0.41, n = 4), observed in the present study and that reported by others (Wood and Plumb, 1995; Abdulrazak *et al.*, 2000) could be due to the variation in structural and biological activity of tannins. The condensed tannins values by butanol-HCl method do not appear to reflect the biological activity. From the relationships between phenolic components and gas production observed in this study (Fig. 4), it can be concluded that tropical browses with less than approximately 45 and 20 g/kg of total phenol and tannin respectively are not likely to produce significant adverse effects on ruminant livestock. There was a positive correlation between metabolizable energy calculated from *in vitro* gas production together with content with metabolizable energy value of conventional feeds measured *in vivo* (Menke and Steingass, 1988). The equation of Menke and Steingass (1988) used to predict the metabolizable energy value of feeds from *in vitro* gas production has

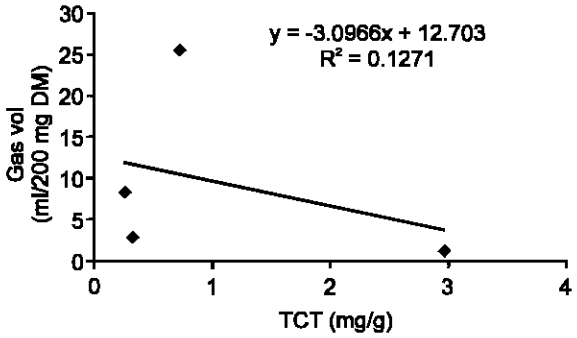

Fig. 5: Relationship between gas production TCT of semi-arid browses

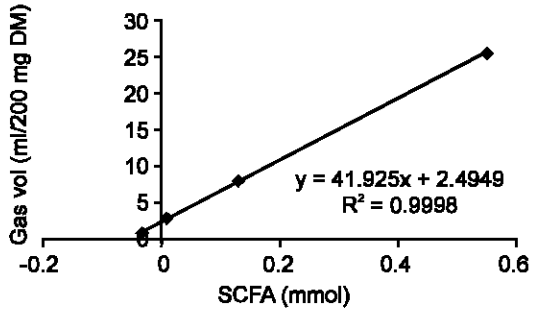

Fig. 6: Relationship between gas production SCFA of semi-arid browses

not been validated for tannin-containing tropical browses. However, since gas production on incubation of feeds in buffered rumen fluid is associated with feed fermentation, the low gas production from browses could be related to low feeding value of these feeds. The correlation between *in vitro* gas productions measured after 24 h incubation of tropical browses and that calculated from SCFA was similar to that reported for conventional feeds (Blummel *et al*., 1999). About 94% of the variation in the *in vitro* gas production on incubation of browse leaves was explained by SCFA produced, which mainly comes from carbohydrate fermentation. These results suggest that the SCFA production from sources other than carbohydrates is negligible. The negative relationship (r = 0.001, n = 4) observed in the study is similar to the report of Cone and Van Gelder (1999) who used different proportions of casein and carbohydrate sources (glucose and starch) and reported a poor correlation between gas measured and calculated from SCFA. These poor correlations could be due to the highly fermentable carbohydrate sources that drastically changed the molar proportions of SCFA, indicating the pattern of fermentation of pure substrate does not reflect the normal fermentation pattern that occurs in the rumen. The results of the relationship between gas volume calculated from the SCFA and measured using the *in vitro* gas method of (Menke and Steingass, 1988) confirm the close relationship between SCFA production and gas volume liberated on fermentation of browse species with wide range of CP (77-300 g/Kg) and phenolic contents (Phenolic from 17-250 g/Kg DM and TP from 7-214 g/Kg DM respectively). From the results observed in the present study, SCFA production could be predicted from *in vitro* gas production. This relationship indicates that the presence of tannins does not influence the prediction of SCFA from *in vitro* gas production and that Wolin's stoichiometry also holds good for tannin-containing browses. Attempts have been made to predict the SCFA production using mathematical models (Pitt *et al*., 1999). However, such models involve several variables and variations in these variables could affect the prediction of SCFA. The close association between the *in vitro* gas and SCFA production would allow the determination of the amount of apparently fermented substrate (substrate used for SCFA, CO_2, CH_4 and H_2O production) for tannin-containing browses from the stoichiometrical relationship between *in vitro* gas and SCFA using the approach outlined in Getachew *et al*. (1998).

Conclusion: Semi-arid browses contain considerable amounts of phenolic compounds that reduced *in vitro* gas production. The close association between SCFA and gas production may allow the use of the relationship between SCFA and gas production to estimate the SCFA production from gas values, which is an indicator of energy availability to the animal. Since SCFA measurement is important for relating feed composition to production parameters and to net energy values of diets, prediction of SCFA from *in vitro* gas measurement will be increasingly important in developing countries where laboratories are seldom equipped with modern equipment to measure SCFA.

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Effect of Some Dietary Oils and Fats on Serum Lipid Profile, Calcium Absorption and Bone Mineralization in Mice

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Abstract: Amount and type of fats in the diet have an important effect on bone health and lipid profile. This study was conducted to investigate the effect of different types of dietary oils and fats on lipid profile, calcium absorption and bone mineralization in male mice. Mice weighing 25±5 g were divided into nine groups and fed on diets without oils or fats (control group) and containing soybean oil, corn oil, olive oil, palm oil, sunflower oil, butter, animal fat or margarine. Mice fed on diet containing soybean oil or olive oil had the lowest levels of TG, TC, LDL-c and HDL-c as compared to the other groups. Diets with palm oil, olive oil, sunflower oil, butter, animal fat or margarine caused significant decreases in the serum level of calcium as compared to the effect of diet without oils or fats. Mice fed diet containing olive oil, butter or animal fat had significant increase in bone density, while those fed diet containing soybean oil, corn oil, sunflower oil or margarine had significant decreases in femur bone density, compared to the control group. The apparent calcium absorption was significantly increased by feeding diets containing soybean oil, corn oil, palm oil, olive oil, sunflower oil, butter or animal fat. Dietary intake of vegetable oils improved lipid profile while butter, animal fat and margarine had the opposite effect. Butter and animal fats increased calcium and phosphorus deposition in femur bone more than vegetable oils.

Key words: Oils, fats, lipid profile, calcium, bone density

INTRODUCTION

Bone is a connective tissue, which continuously undergoes remodeling. Bone remodeling involves bone formation by osteoblasts and bone resorption by osteoclasts. Bone undergoes continuous remodeling with regular resorption and deposition of calcium into newly deposited bone (AAPCN, 1998). The bone matrix is composed of organic and inorganic components. The organic components include collagen and glycoprotein while the inorganic components include minerals such as calcium and phosphorus. Both the organic and inorganic components provide strictness and strength to the bone (Annemieke *et al*., 1997). Adequate calcium intake is recommended for the development of high peak bone mass and for the prevention of osteoporoses (Kanis, 1999). On the other hand, consideration should be given not only to the intake of adequate calcium, but also, to the absorptive efficiency of the ingested calcium, because intestinal calcium absorption is influenced by many factors (Mjyazawa and Yoshida, 1991).

Dietary fats and oils are known as macronutrients and provide concentrated source of energy for human metabolic processes. In addition, they are the main source of fat-soluble vitamins (Sanchez-Muniz and Bastida, 2006). Dietary oils and fats are composed of different types of Fatty Acids (FA). Fatty Acids are Saturated (SFA), Monounsaturated (MUSFA) and Polyunsaturated (PUSFA). Evidence has been demonstrated that dietary fats can have important effects on bone health. Studies in animals indicate that high-fat diets can adversely affect bone (Hoffman *et al*., 1999). Saturated fatty acids in particular, may have effects that could weaken bone health (Parhami, 2003). A number of study founded that long-chain polyunsaturated fatty acids influence bone mass in various animal models (Watkins *et al*., 2000). A variety of mechanisms may account for the effects of dietary fats on bone, including alterations in calcium absorption, prostaglandin synthesis, osteoblasts formation and lipid oxidation (Haag *et al*., 2003).

It is well known that, the amount and the type of fats in the diet can have important effects on bone health and lipid profile. Therefore, the main objective of the present study were to investigate the effect of adding different types of oils and fats to the diet on serum lipid profile, calcium absorption and bone mineralization in male mice.

MATERIALS AND METHODS

Animals: Male mice of Swiss strain weighing 25±5 g were purchased from Laboratory Animal Colony, Ministry of Health and Population, Helwan, Egypt.

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Oils and fats: Soybean oil, corn oil, olive oil, palm oil, sunflower oil, butter, animal fat and margarine were purchased from the local market, Cairo, Egypt.

Kits: Kits for biochemical analysis of serum lipid profile, calcium, phosphors and magnesium were obtained from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Giza, Egypt.

Preparation of basal diet: The basal diet (AIN-93G) (Reeves *et al*., 1993) was formulated without oils or fats. The following oils or fats were added to the diet: soybean oil, corn oil, olive oil, palm oil, sunflower oil, butter, animal fat and margarine. Diets were formulated to meet recommended nutrients levels for mice as showed in Table 1.

Table 1: Composition of the modified AIN-93G diet

Ingredient	Content (g/kg)
Casein	200.000
Maize starch	529.486
Sucrose	100.000
*Oil or fat	70.000
Fibers	50.000
Mineral mix.	35.000
Vitamin mix.	10.000
L-Cystine	3.000
Choline chloride	2.500
Butyl hydroquinone	0.014

*Oils or fats were: Soybean oil, Corn oil, Olive oil, Palm oil, Sunflower oil, Butter, Animal fat, Margarine and without any source of oils or fats

Experimental design: The experiment was carried out on forty-five male mice, weighing approximately 25±5 g. Mice were housed in an air-condition room at 22-25°C, under 12-light/dark cycle, fed on the basal diet and tap water *ad libitium*. Animals fed on the basal diet for one week for acclimatization before starting the experiment. After acclimatization period, mice were divided into nine groups, of five animals each, and fed on the following experimental diets: (I) diet without oils or fats source and without change in total calories by replacing calories of fats by starch and sucrose (control group), (II) diet contains soybean oil, (III) diet contains corn oil, (IV) diet contains palm oil, (V) diet contains olive oil, (VI) diet contains sunflower oil, (VII) diet contains butter, (VII) diet contains animal fat, (IX) diet contains margarine.

Determination of calcium balance: During the last week of the experimental period, all mice were housed in individual metabolic cages containing a grid-floor and a facility for separate collection of feces and urine. To acclimatize the mice in the new environment, they were housed in these cage two days before the beginning of four days metabolic study for the determination of net dietary calcium absorption. During the last 4 days, food consumption was determined on a daily basis over the 4-d metabolic balance-study period. Urine and fecal samples (24 h) of each animal were collected. The urine volume for each animal was recorded. Portions of the urine samples were acidified with 12 M HCL and stored at -20°C until required for analysis. The fecal samples of each animal were dried for 12 h at 100°C. The diets and dried fecal samples were ash-dried at 700°C for 12 h. The diet and fecal ashes were solubilized with 6N HC1 solution (Yang *et al*., 2008). Calcium concentrations were measured using atomic absorption spectrophotometer (model 3300) (Perken, 1982).
Apparent Ca absorption, apparent Ca-absorption rate, and apparent Ca balance were calculated using the following equations respectively:

$$\text{Apparent Ca absorption} = \text{Ca intake in diet} - \text{Fecal Ca}$$

$$\text{Apparent Ca absorption rate (\%)} = \frac{\text{Ca absorption}}{\text{Ca intake in diet}} \times 100$$

$$\text{Apparent Ca balance} = \text{Ca intake in diet} - (\text{fecal Ca} + \text{urinary Ca})$$

Determination of femur length, volume and density: The soft tissue in the right femur was removed and the length of each right femur was measured with a vernier caliper. Femur volume and density were calculated using Archimedes' principle. In brief, the femur was cut out at the mid-diaphyses and the marrow was washed out. Each bone was placed in an unstoppered vial filled with deionized water, and the vial was placed for 90 min in a vacuum desiccator. The desiccator was agitated periodically to ensure that the trapped air completely diffused out of the bone. The bone was removed from the vial, dried by blotted paper, weighed, and placed again in the vial containing deionized water. The bone was reweighed in a suspended vessel and should be not completely immersed in water before equilibrated at room temperature, and the density (g per cm^3 of bone volume) was calculated (Doyle and Cashman, 2003).

Determination of femur mineral contents: The right femur was dried overnight at 100°C. The femur was then incinerated for 12 h at 1000°C in Muffle apparatus to obtain ash. Then, ash was solubilized with 6 N HCL (Yang *et al*., 2008), quantitatively transferred into volumetric flask and completed to 100 ml HCL. The solutions were used for analysis of Ca and Mg concentrations using atomic absorption spectrophotometer (model 3300). However, phosphorus content was determined Spectrophotometericily (Perken, 1982).

Biochemical analysis: At the end of the experimental period (6 weeks), animals were fasted for 12 h then killed. Blood samples were collected from the portal vein into dry clean centrifuge tubes and left to clot at room

temperature. Serum was separated using centrifuge at 3000 rpm for 15 min. Serum was used for the estimation of lipid profile as Triglyceride (TG), Total Cholesterol (TC), Low-density Lipoprotein (LDL-c) and High-density Lipoprotein (HDL-c) as well as minerals such as Calcium (Ca), Phosphorus (P) and Magnesium (Mg).

Statistical analysis: Results were expressed as mean±SE. All data from the experiment were examined statistically by one-way analysis of variance with computerized SPSS package program (SPSS 9.00 software for Windows) by ANOVA test. A p-value <0.05 was considered statistically significant (Snedecor and Cochran, 1980).

RESULTS

Serum lipid profile: The results in Table 2 showed that mice fed on diets supplemented with corn oil, butter, animal fat or margarine had significant increases in serum TG levels, whereas groups fed on diets supplemented with soybean oil, palm oil, olive oil or sunflower oil had significantly lower in T.G levels than in the control group. Soybean oil and olive oil diets caused significantly lower in T.C; however, butter, animal fat and margarine diets caused significantly higher level as compared to non-oils or fats diets. There were not significant differences for groups fed on diets containing corn oil, palm oil and sunflower oil as compared to control group. Fed on diets containing soybean oil, corn oil, palm oil and olive oil caused a significant decreased in LDL-c, while fed on diets containing butter, animal fat and margarine caused a significant increased as compared to fed on diet without oils or fats. The serum HDL-c level appeared to be more significantly lower of mice fed on diets supplemented with soybean oil, palm oil, olive oil, butter, animal fat and margarine, however there was significantly higher of mice fed on diet containing sunflower oil as compared to mice fed on diet without oils or fats.

Serum calcium, phosphorus and magnesium: The effect of some dietary oils or fats on serum calcium, phosphorus and magnesium is depicted in Table 3. Results demonstrated that diets supplemented with palm oil, olive oil, sunflower oil, butter, animal fat or margarine caused significant decreases in the serum level of calcium in mice, whereas diet supplemented with corn oil had no effect as compared to the effect of diet without oils or fats. Diet supplemented with soybean oil caused a significant increase of serum calcium level as compared to the other groups. With regard to serum phosphorus level, data showed that mice fed on diets containing soybean oil, corn oil, palm oil, olive oil, sunflower oil, animal fat or margarine had significant decrease as compared to mice fed on diet without oils or fats and that containing butter. Serum levels of magnesium were significantly decreased in groups fed on soybean oil, olive oil, animal fat or margarine, while significantly increased in groups fed on diets containing sunflower oil or butter as compared to groups fed on diets without oils or fats, corn oil or palm oil.

Table 2: Effect of some dietary oils and fats on serum TG, TC, LDL-c and HDL-c in mice

	Parameters as Mean±SE			
Groups	TG (mg/dL)	TC (mmol/L)	LDL-c (mg/dL)	HDL-c (mg/dL)
Group (1) diet without oils or fats	254.20±1.46^{e}	14.05±0.79^{cd}	234.25±10.85^{b}	147.60±10.63^{b}
Group (2) diet with soybean oil	153.80±1.28^{g}	8.38±0.57^{f}	113.80±3.20^{e}	82.50±2.89^{d}
Group (3) diet with corn oil	282.80±1.39^{d}	11.88±0.54^{de}	170.40±13.07^{d}	132.20±7.41^{bc}
Group (4) diet with palm oil	236.00±0.32^{f}	11.22±0.37^{def}	173.40±10.40^{d}	123.00±1.82^{c}
Group (5) diet with olive oil	154.20±1.28^{g}	10.80±1.12^{ef}	128.80±6.36^{e}	120.40±10.30^{c}
Group (6) diet with sunflower oil	236.00±1.76^{f}	14.90±0.02^{c}	218.60±5.46^{bc}	217.00±11.14^{a}
Group (7) diet with butter	420.00±5.39^{c}	22.62±2.08^{b}	292.40±14.14^{a}	121.20±5.46^{c}
Group (8) diet with animal fat	433.20±0.74^{b}	22.26±1.15^{b}	292.50±7.35^{a}	114.20±6.55^{c}
Group (9) diet with margarine	479.60±3.14^{a}	27.64±0.58^{a}	293.40±17.01^{a}	110.80±6.39^{c}

Means±SE in each column with different superscript letters differ significantly at p<0.05

Table 3: Effect of some dietary oils and fats on serum calcium, phosphorus and magnesium in mice

	Parameters as Mean±SE		
Groups	Calcium (mg/dL)	Phosphorus (mg/dL)	Magnesium (mg/dL)
Group (1) diet without oils or fats	8.05±0.32^{b}	24.28±0.42^{a}	1.64±0.01^{c}
Group (2) diet with soybean oil	11.58±0.30^{a}	18.87±0.36^{b}	0.77±0.004^{d}
Group (3) diet with corn oil	7.65±0.31^{b}	17.50±1.17^{b}	1.54±0.13^{c}
Group (4) diet with palm oil	6.40±0.59^{cd}	17.56±0.80^{b}	1.72±0.15^{c}
Group (5) diet with olive oil	4.60±0.42^{ef}	12.53±0.28^{c}	0.93±0.03^{d}
Group (6) diet with sunflower oil	6.56±0.41^{c}	19.66±0.43^{b}	2.78±0.02^{b}
Group (7) diet with butter	5.36±0.15^{de}	24.70±1.75^{a}	4.45±0.34^{a}
Group (8) diet with animal fat	4.21±0.19^{f}	9.77±0.40^{d}	0.29±0.003^{e}
Group (9) diet with margarine	4.49±0.41^{ef}	17.29±0.56^{b}	0.95±0.05^{d}

Means±SE in each column with different superscript letters differ significantly at p<0.05

Femur bone length, volume and density: Effects of dietary oils and fats on femur bone length, volume and density in mice are recorded in Table 4. Data showed that mice fed on diet containing soybean oil had a shorter femur bone length (18.00±0.47 mm) than the control group (20.40±0.51 mm) that fed diet without oils or fats. Mice fed on the other tested oils and fats showed no significant changes in femur bone length. Concerning femur bone volume, mice fed different oils or fats had a significant decrease in femur bone volume as compared to those fed on diet without oils and fats, while those fed on diet containing margarine had non-significant increase in femur bone volume. With regarded to femur bone density, there were significant increase in bone density in mice fed diet containing olive oil, butter or animal fat. While those fed diet containing soybean oil, corn oil, sunflower oil or margarine had significant decreases in femur bone density, compared to the control group fed diets containing no oils and fats.

Concentrations of calcium, phosphorus and magnesium in femur bone of mice: As shown in Table 5, feeding mice on diets containing soybean oil, palm oil, olive oil, sunflower oil, butter or animal fat caused significant increases in calcium concentration in femur bone compared to the control group fed diet without oils and fats. Regarding concentrations of phosphorus, the diets only containing soybean oil, olive oil, sunflower oil, butter or animal fat induced significant increases, compared to the control group. Feeding mice diets containing soybean oil or margarine produced significant decrease in magnesium concentration in femur bone, while those feed diet containing butter showed significant increases in magnesium, compared to the control group. Mice fed on diets containing the other test oils or fats showed non-significant changes in the concentration of magnesium in femur bone.

Daily calcium intake, fecal calcium and urinary calcium: Results in Table 6 revealed that there was no significant difference in calcium intake for groups fed on diets containing tested oils or fats as compared to the group fed on diet without oils or fats. Mice fed on diet supplemented with corn oil has significantly higher calcium intake more than mice fed on olive oil, animal fat or margarine diets. Concerning fecal and urinary calcium execration, there were significant decreases in their levels in mice fed on diets containing oils or fats as compared to that without both of them.

Apparent calcium absorption, apparent calcium absorption ratio and apparent calcium balance: Effect of feeding mice for six weeks on diets supplemented with some dietary oils or fats on apparent calcium absorption, apparent calcium absorption ratio and apparent calcium balance is recorded in Table 7. The apparent absorption, apparent absorption ratio and apparent balance of calcium were significantly increased by feeding diets containing soybean oil, corn oil, palm oil, olive oil, sunflower oil, butter or animal fat.

Table 4: Effect of some dietary oils and fats on femur bone length, volume and density in mice

	Parameters as Mean±SE		
Groups	Length (mm)	Volume (cm^3)	Bone density (g/cm^3)
Group (1) diet without oils or fats	20.40±0.51^{ab}	0.74±0.01^{a}	0.84±0.004^{b}
Group (2) diet with soybean oil	18.00±0.47^{c}	0.40±0.004^{c}	1.54±0.17^{b}
Group (3) diet with corn oil	21.40±0.40^{a}	0.44±0.01^{c}	1.53±0.13^{b}
Group (4) diet with palm oil	19.00±0.76^{bc}	0.60±0.003^{b}	1.04±0.02^{b}
Group (5) diet with olive oil	20.80±0.74^{ab}	0.22±0.004^{d}	3.23±0.72^{a}
Group (6) diet with sunflower oil	19.80±0.66^{bc}	0.40±0.003^{c}	1.60±0.18^{b}
Group (7) diet with butter	19.00±0.45^{bc}	0.16±0.003^{d}	4.02±0.60^{a}
Group (8) diet with animal fat	21.00±0.32^{a}	0.24±0.01^{d}	3.35±0.60^{a}
Group (9) diet with margarine	20.60±0.75^{ab}	0.80±0.01^{a}	0.77±0.02^{b}

Means±SE in each column with different superscript letters differ significantly at $p<0.05$

Table 5: Effect of some dietary oils and fats on concentrations of calcium, phosphorus and magnesium in femur bone of mice

	Concentrations of (Mean±SE)		
Groups	Calcium (mg/g)	Phosphorus (mg/g)	Magnesium (mg/g)
Group (1) diet without oils or fats	232.13±4.16^{e}	99.54±1.71^{d}	29.03±2.99^{bc}
Group (2) diet with soybean oil	254.99±3.48^{c}	114.69±1.15^{bc}	15.54±1.72^{d}
Group (3) diet with corn oil	239.28±1.60^{de}	102.60±0.66^{d}	25.36±0.93^{c}
Group (4) diet with palm oil	248.63±4.52^{cd}	102.44±1.33^{d}	25.06±0.96^{c}
Group (5) diet with olive oil	267.37±3.84^{b}	117.20±1.62^{b}	26.35±2.46^{bc}
Group (6) diet with sunflower oil	253.54±3.81^{c}	111.49±1.58^{c}	25.10±1.52^{c}
Group (7) diet with butter	280.72±2.98^{a}	122.78±1.24^{a}	33.28±1.27^{a}
Group (8) diet with animal fat	266.56±4.23^{b}	116.51±1.95^{b}	31.29±1.18^{ab}
Group (9) diet with margarine	235.13±1.39^{e}	98.63±0.79^{d}	16.32±1.72^{d}

Means±SE in each column with different superscript letters differ significantly at $p<0.05$

Table 6: Effect of some dietary oils and fats on daily calcium intake and calcium excretion in feces and urine in mice

	Parameters as Mean±SE		
Groups	Calcium intake (mg/day)	Fecal calcium (mg/day)	Urinary calcium (mg/day)
Group (1) diet without oils or fats	31.50±0.79^{abc}	12.64±0.72^{a}	0.58±0.001^{a}
Group (2) diet with soybean oil	31.50±1.87^{abc}	6.90±0.53^{c}	0.44±0.01^{b}
Group (3) diet with corn oil	35.20±1.15^{a}	7.52±0.01^{c}	0.39±0.02^{cd}
Group (4) diet with palm oil	33.00±0.94^{abc}	7.26±0.39^{c}	0.37±0.001^{d}
Group (5) diet with olive oil	30.90±1.64^{bc}	4.02±0.50^{d}	0.25±0.002^{f}
Group (6) diet with sunflower oil	35.00±1.13^{ab}	6.59±0.40^{c}	0.29±0.002^{e}
Group (7) diet with butter	35.00±1.37^{ab}	3.30±0.29^{d}	0.19±0.001^{g}
Group (8) diet with animal fat	30.50±1.59^{c}	3.56±0.37^{d}	0.37±0.001^{d}
Group (9) diet with margarine	29.00±0.61^{c}	10.05±0.48^{b}	0.42±0.01^{bc}

Means±SE in each column with different superscript letters differ significantly at p<0.05

Table 7: Effect of some dietary oils and fats on apparent calcium absorption, apparent calcium absorption ratio and apparent calcium balance in mice

	Parameters as Mean±SE		
Groups	Apparent calcium absorption (mg/day)	Apparent calcium absorption ratio (%)	Apparent calcium balance (mg/day)
Group (1) diet without oils or fats	18.86±1.88^{c}	59.98±2.36^{d}	18.27±0.29^{d}
Group (2) diet with soybean oil	24.62±1.40^{b}	77.84±0.98^{b}	24.17±1.87^{c}
Group (3) diet with corn oil	28.48±1.07^{ab}	78.96±1.40^{b}	28.10±1.40^{ab}
Group (4) diet with palm oil	25.74±1.50^{b}	77.90±1.36^{b}	25.45±1.06^{bc}
Group (5) diet with olive oil	26.88±0.77^{b}	87.00±0.63^{a}	26.64±1.51^{bc}
Group (6) diet with sunflower oil	28.41±1.20^{ab}	81.24±0.66^{b}	28.03±0.77^{ab}
Group (7) diet with butter	31.70±1.29^{a}	90.60±0.73^{a}	31.51±1.20^{a}
Group (8) diet with animal fat	26.94±0.53^{b}	88.43±1.36^{a}	26.58±1.28^{bc}
Group (9) diet with margarine	18.95±1.88^{c}	65.35±2.36^{c}	18.52±0.54^{d}

Means±SE in each column with different superscript letters differ significantly at p<0.05

There were no significant changes in calcium apparent absorption and balance in the group fed on margarine diet as compared to the control group.

DISCUSSION

This study aimed to investigate the effect of different types of dietary oils and fats on lipid profile, calcium absorption and bone mineralization in male mice. Our results with regard to addition of soybean oil to the diet, agreed with previous studies founded that soybean oil reduced serum cholesterol and lipoproteins in different aged rats fed on hypercholesterolemic diets (Choi *et al*., 1993) and it improves serum lipid profile (Ramadan *et al*., 2008). These results may be possibly explains on the basis that soybean oil is rich in UNSFAs, especially PUSFAs. Polyunsaturated fats stimulate the catabolic rate of LDL-cholesterol, thus resulting in the reduction of serum LDL-cholesterol (Choi *et al*., 1993). Concerning addition of corn oil to the diet, our finding are similar to some extent with previous reports showing that rats fed a corn oil-rich diet had higher values of serum TG (Asadi *et al*., 2008) and significantly decreased in serum HDL-c (Shad *et al*., 2002). Feeding rats on diet containing palm oil significantly improved lipid profile as it reduced serum TG, TC and lipoproteins (Choi *et al*., 1993; Oluba *et al*., 2008) and decreased serum HDL-c concentrations compared with coconut oil (Scholtz *et al*., 2004), these results agreed with our findings. The pronounced effect of palm oil may be attributes to its antioxidant properties. Palm oil is rich in antioxidant vitamins tocotrienols and unsaturated analogue of tocopherols. Tocotrienols had a hypocholesterolemic effect probably through the inhibition of cholesterol synthesis (Choi *et al*., 1993; Karaji-Bani *et al*., 2006). Improvements in lipid profile in mice fed on the diet containing olive oil may be explains on the basis that olive oil is a rich source of MUSFA that improves lipid profile. The primary MUSFAs in the diet are oleic (C18:1, n-9) and palmitoleic (C16:1, n-9) acids. Olive oil is excellent source of oleic acid. Previous studies demonstrated that olive oils containing a large fraction of MUSFAs and a substantial amount of PUSFAs promote a better triacylglycerol clearance from the blood (Beynen *et al*., 1987). In additionally, a diet with olive oil is a good source of monounsaturated fatty reduced serum TG, LDL-c concentrations with respect to diets rich in SFAs (Hayes *et al*., 1994). Healthy heart effects from olive oil are attributed to its higher contents of monounsaturated fats and its higher ingredients of antioxidants (including: chlorophyll, carotenoids and the polyphenolic compounds: tyrosol, hydrotyrosol and oleuropein), all of these compounds have free radical scavenging ability and protect vitamin E found in olive oil (Morello *et al*., 2007; Puela *et al*., 2004). Diet rich in olive oil, has much more favorable effects on blood lipid profile and plasma lipoproteins compared with coconut

oil (Mroueh *et al*., 2009). With regard to the effect of sunflower oil, our findings agreed with Kris-Etheton and Yu (1997) who reported that HDL-c production was greater in young rats fed on safflower than in those fed on palm oil. These results may be related to the type of fatty acids in sunflower oil, which is rich in PUSFAs. Polyunsaturated fatty acids are effective in lowering serum cholesterol.

Concerning, the effect of butter, animal fat, or margarine on serum lipid profile, our findings agreed with previous reported showing that, butter and margarine produced a significant rise in serum TC in normocholesterolemic women (Wardlaw and Snook, 1990) and increased serum TC, LDL-c and decreased HDL-c (Lichtenstein *et al*., 1993). These results may be explained on basis that the high SFAs and low PUSFAs contents in butter, which is an important contributing factor to raising serum cholesterol level. More recently, the *Trans* fatty acids resulting from the partial hydrogenation of vegetable oils (margarine) produced undesirable serum lipoprotein profiles (Mensink and Katan, 1990). Therefore, the hypercholesterolemic effects of margarine are to decreased proportion of PUSFAs, increased proportion of SFAs and in part to the independent effect of *trans* fatty acids. Feeding mice on diets containing butter and animal fat diet had lower serum level of TG, TC and LDL-c as compared to those feeding margarine diets. These results explained on based antiatherogenic effect of Conjugated Linoleic Acid (CLA) content of butter and animal fat. Milk and animal fat from ruminant animals are natural sources of CLA (Chin *et al*., 1992). CLA have beneficial effects on the atherosclerotic process by reducing plasma total and LDL cholesterol, the LDL/HDL cholesterol ratio (atherogenic index) and triglyceride levels in rabbits fed on atherogenic diet (Lee *et al*., 1994). Other study reported that the CLA-fed hamsters exhibited lower levels of plasma total cholesterol and triglycerides (Yurawecz *et al*., 1999).

Our results with regard to the effect of different dietary oils or fats on bone mineralization revealed that soybean oil increased serum calcium concentration, while decreased phosphorus level. It also increased calcium and phosphorus concentration in femur bone and increased bone density. Increased calcium concentration in the serum caused by adding soybean oil to the diet could be possibly explained by the reduced fecal and urinary calcium excretion that reported in the presented study. These findings were partially similar to previous study founded that soybean oil in the diet have a role in the prevention of osteoporosis as it reduce bone loss and increase bone density (Jie *et al*., 2000). These results may be attributed to soybean oil contents PUSFAs, which are beneficial in inhibits the activity of osteoclasts and enhance the activity of osteoblasts in animals (Watkins *et al*., 1997).

The effect of corn oil on serum calcium, phosphorus and magnesium levels and fecal, urinary calcium excretion, bone density and apparent calcium absorption and balance, explained on the basis that corn oil is rich in PUSFAs, which elevate femur calcium content and enhance calcium balance (Mollard and Weiler, 2006).

With regard to effect of palm oil on bone health, our results were to some extant similar to the previous study explained the effect of palm oil on bone turnover in thyrotoxic rats on the basis of its content of vitamin E which reduced bone resorption to a greater extent than bone formation (Ima-Nirwana *et al*., 1993). In addition to, vitamin E improves bone calcium content in both the left femur and the fifth lumbar vertebra. Its deficiency cause loss of bone calcium in growing female rats and this could be due to increased free radical activity or decreased calcium availability for bone deposition. Supplementing animals with palm oil containing a mixture of tocopherol and tocotrienols was effective in preventing the loss in bone calcium (Norazlina *et al*., 2002).

The positive effects of olive oil may be due to its higher contents of MUSFAs, which had a positive associated with bone mineral density (Trichopoulou *et al*., 2002). Olive oil prevents the bone loss and improves bone mineral density in rats (Puela *et al*., 2004).

The beneficial effects of sunflower oil on bone health in mice were confirmed by increased retention of calcium and phosphorus in femur bone. It caused a significant decrease in serum levels of calcium and phosphorus as well as fecal and urinary calcium excretion. In addition to, it increased bone density, femur calcium and phosphorus contents and apparent calcium absorption and balance. Therefore, calcium retained in the bone. Recent study reported that sunflower oil is rich in polyunsaturated fatty acids, which elevate femur calcium content, enhance calcium balance, increased calcium absorption efficiency and enhanced calcium bioavailability in rats (Perez-Granados *et al*., 2006), these results agreed with our results.

In contrast, our results indicated that butter, animal fat and margarine diets significantly decreased serum concentration of calcium and fecal and urinary calcium excretion. Therefore, calcium retained in the bone, as showed by the increased in bone density, apparent calcium balance and calcium and phosphorus content induced by addition of butter or animal fat to diet. However, margarine in diet did not affect significantly the bone density, femur calcium and phosphorus content. These results indicate that the type of fat in the diet play an important role on bone health. Watkins *et al*. (1996) indicated that growing animals fed saturated fat enriched diets had significantly greater bone formation rate compared to those given soybean oil. The beneficial effects of margarine (hydrogenated oil) on bone mineral content, mechanical and histological properties might be attributable to their decreased PGE2 production-

induced bone resorption (Liu *et al*., 2003). The positive effects of butter and animal fat on calcium absorption and bone calcium content may be related to its content of CLA (Yurawecz *et al*., 1999). CLA occurs naturally in many foods, though the primary sources are foods from ruminant animals such as beef, lamb and dairy products (Decker, 1999). In growing animals given butterfat, bone formation was increased and the production of ex vivo bone prostaglandin (a potent stimulator of bone resorption) decreased (Watkins *et al*., 2000). The increase in the rate of bone formation may be attributed to reduce the level of arachidonic acid and PGE2 production as well as higher levels of IGF-1 in bone (Watkins and Seifert, 2000). Reduced rate of PGE2 production from CLA might be due to a competitive inhibition of n-6 PUFA elongation that results in a lower amount of available substrate for cyclooxygenase, the enzyme necessary for PGE2 production (Watkins *et al*., 1997). CLA may directly or indirectly alter cyclooxygenase-2 (the inducible form of cyclooxygenase) activity or expression and therefore affect PGE2 production (Brownbill *et al*., 2005).

Conclusion: This study concluded that dietary intake of vegetable oils are more beneficial than animal fats (butter and beef fat) and hydrogenated oils (margarine), especially olive oil and sunflower oils, as they improve lipid profile and bone mineral contents in mice. It is known that fatty acid composition of food is more associated with variations in the plasma total cholesterol level. Therefore, dietary intake of vegetable oils, which are rich in unsaturated fatty acids, reduces the risk of atherosclerosis and coronary heart disease, while saturated fatty acids have the opposite effect. However, data revealed also that butter and animal fats are more beneficial than vegetable oils for osteoporosis as they increase calcium and phosphorus deposition in femur bone and reduce fecal and urinary calcium excretion in mice.

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Microbiological Safety of Raw Milk in Khartoum State, Sudan: 2- Khartoum-North City

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Abstract: Sixteen random samples of raw cow's milk were collected from Khartoum North in Sudan. Samples were analyzed for microbiological properties included total plate count (TPC), total coliforms (TC), fecal coliforms (FC), *Staphylococcus aureus*, Salmonella, lactic acid bacteria (LAB), spore forming bacteria (SFB) and yeast. The results showed higher counts for all the microorganisms studied. Average of TPC, TC, FC, S. *aureus*, SFB, LAB and Yeast were 9.88×10^6, 5.43×10^4, 1.56×10^4, 1.2×10^6, 1.23×10^2, 7×10^4 and 9.63×10^5 cfu/mL, respectively. The microbial profiles found had non-conformance to the standards. Based on the exceedingly high microbial counts found in this study, it could be concluded that this milk type poses a serious health risk in the study areas.

Key words: Cow milk, pathogenic and indicator bacteria, human food

INTRODUCTION

Cow's milk has long been considered a highly nutritious and valuable human food and is consumed by millions daily in a variety of different products. Its nutrient composition makes it an ideal medium for bacterial growth and therefore it can be considered one of the most perishable agricultural products because it can so very easily be contaminated (Bryan, 1983, Bramley and McKinnon, 1990; Heeschen, 1993). Raw Milk (RM) often contains microorganisms which may cause food borne diseases (Adesiyun *et al*., 1995; Steele *et al*., 1997; Headrick *et al*., 1998).

Because of the specific production it is impossible to avoid contamination of milk with micro-organisms therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003). He stated that the number and types of microorganisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health. Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al*., 2008). He also stated that it is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk. Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley and McKinnon, 1990).

The main objectives of this study were to investigate the microbial quality of cow milk and detect the pathogenic bacteria and enumerate the bacteria that may cause changes in raw cow milk in Khartoum North, Sudan and the distribution of those bacteria.

MATERIALS AND METHODS

Microbiological analysis: Samples of cow milk were obtained from Khartoum north district. Milking was done manually twice a day at 7.00am and 5.00pm. A total of 16 samples of raw cow milk were collected at four locations. At each location, samples of approximately 500 ml were taken aseptically from the bulk milk container into sterile glass bottles. The milk was collected within 15 min of milking at ambient temperatures and was analyzed immediately after arrival at the laboratory (Microbiology Laboratory, Food Research Centre, Khartoum North). All methods of analysis were carried out according to Harrigan and MacCance (1976), unless otherwise indicated.

Sample treatment: Representative 10 ml were aseptically mixed with 90 ml distilled water and homogenized by shaking. Subsequent decimal dilutions were prepared with the same diluents and in all cases duplicate-counting plates were prepared of appropriate dilutions.

Total count of mesophilic aerobic bacteria (TC): was enumerated according to Harrigan and MacCance (1976) in pour plates of plate count agar (Oxoid), after incubation at 37°C for 2 days.

Lactic acid bacteria (LAB): was enumerated according to Harrigan and MacCance (1976). Appropriate dilutions were plated on De Man, Rogosa and Sharpe medium (MRS, Merck, Germany), after incubation at 37°C for 3 days.

***Staphylococcus aureus*:** *Staphylococcus aureus* was performed on Baird-Parker Agar (Oxoid). The plates were incubated at 37°C for 48 h.

Table 1: Microbiological parameters of raw cow milk samples collected from different sources in Khartoum North

Region	No. samples	TB	TC	FC	Staph	SFB	LAB	Yeasts
		cfu/ml						
Kh. North (Morning)	8	9.88×10^6	5.43×10^4	1.56×10^4	1.20×10^6	1.23×10^2	7×10^4	9.63×10^5
Kh. North (Night)	8	9.42×10^6	5.11×10^4	1.23×10^4	0	1.12×10^2	6.4×10^4	4.3×10^5

TB: Total Bacterial Count; TC: Total Coliforms; FC: Fecal Coliforms; SFB: Spore Forming Bacteria; Staph: *Staphylococcus aureus*, LAB: Lactic Acid Bacteria

Enumeration of total coli forms: Presumptive test was done using MacConkey broth (Oxoid) and tubes were incubated at 37°C, examined for gas production and growth after 24 h. A confirmation test was done using BGB broth for total coliform and EMB agar (Oxoid) for *E.coli* and incubated at 37°C for 18-24 h. Two typical colonies from each EMB plate were picked and transferred to plate count agar slants for morphological and biochemical tests (Harrigan and MacCance, 1976).

Enumeration of total spore forming bacteria: The colony count method to determine the total spore forming bacteria was followed as described by Harrigan and MacCance (1976). A test tube of suitable dilution is heated in water bath at 80°C for 10 min to destroy vegetative cells. The tube is cooled and 1 ml from this dilution was aseptically transferred into sterile Petri dishes. To each plate melted Starch Milk Agar (SMA) was added. The plate's inoculums were mixed with the medium and allowed to solidify. The plates were incubated at 37°C for 2 days.

Yeast: Yeasts were enumerated by surface plating on malt extract agar (Oxoid) with 0.01% chloramphenicol as bacterial inhibitor and incubated aerobically at 25°C for 2-3 days (Harrigan and MacCance, 1976).

RESULTS AND DISCUSSION

Hygiene quality was determined by the enumeration of total bacterial, total coliforms, faecal coliforms and *Staphylococcus* sp. The result (Table 1) indicated high contamination of milk samples: TBC (6.5×10^4 cfu/mL to 2.17×10^7 cfu/mL and an average 1×10^7 cfu/mL, TC: 3.6×10^3 cfu/ml to 15.25×10^4 cfu/mL with an average of 5.93×10^4 cfu/mL, FC: 3.3×10^3 cfu/ml to 2.8×10^4 cfu/mL with an average 1.56×10^4 cfu/mL.

The rates of *S. aureus* found in the examined milk samples are very variable "1.26×10^5 to $4.3 \times l0^6$ germs/mL with an average of 1.2×10^6 *S. aureus*/mL. This higher contamination was probably originated from cow's udder. This result is higher than those found by Hamama (1989); Fook *et al.* (2004) and the cows milk with normal food. The contamination of the milk by *S. aureus* is often original but can also occur after handling draft in non-hygienic conditions. *Staphylococcus aureus* is a poor competitor and is readily outgrown by lactic acid-producing microorganisms, so its growth is limited in raw milk (Holsinger *et al.*, 1997; Asperger, 1994). Raw milk may contain microorganisms pathogenic to man and their source may lie either within or out side the udder. Pathogenic bacteria may present in raw milk as a direct consequence of udder disease. Among the organisms commonly producing mastitis are *Staphylococcus aureus* and *Escherichia coli* and all are pathogenic (Sinell, 1973). Contamination of raw milk by pathogenic bacteria from source external to the udder may be caused by *salmonellae* strains, which produce many out breaks of *enteritis* (Robinson *et al.*, 1979).

The average values of coliform counts/ml of milk samples collected from Khartoum north was 5.43×10^4 cfu/ml this result is in agreement with the finding of Mutukumira *et al.* (1996), who found the coliform bacteria 3.2×10^2 to 2.3×10^5. Saitanu *et al.* (1996) examined and found that the total coliform count of <1000 cfu/ml.

Total bacterial counts or total aerobic colony counts are used to estimate viable bacterial populations in milk and reflect the hygienic practices used in the production and handling of the milk (Houghtby *et al.*, 1994). The results of this investigation are in agreement with the finding of Lee *et al.* (1983) conducted an experiment in Seoul (Korea) and found that the bacterial count in raw milk ranged from 4×10^6 to 2.7×10^7 cfu/ml.

Yeasts are not commonly the cause of defect in dairy products unless they ferment lactose. In this case, they can grow rapidly and produce a characteristic yeasty or fruity flavor and obvious gas (Davis and Wilbey, 1990). They also produce metabolites, e.g. short-chain fatty acids and other compounds, with known toxic effects against undesired micro-organisms in the intestinal tract (Jacobsen and Narvhus, 1996).

Conclusion: The microbiological quality was only marginally acceptable with respect to the total bacteria count. Nevertheless, the presence of pathogenic and indicator bacteria, such as *E. coli*, coliforms and *S. aureus* indicate that to the growth of these organisms may lead to a hazard against public health. Therefore practice and regulations, such as on-site pasteurization and implementation of HACCP following established standards, should be introduced to facilitate the production of cow milk of high quality and safety.

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Plasma Glucose, Protein and Cholesterol Levels of Chicks or Birds Maintained on Pawpaw (*Carica papaya*) Seed Containing Diet

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Abstract: Proximate analysis of dehusked *Carica papaya* seeds indicated the presence of the following constituents: Ash, 6.0%; fat, 14%; fiber, 18%; protein 23.1% and carbohydrates, 28.6%. Plasma assays of glucose, protein and cholesterol in the blood of birds maintained on the diet were determined and compared with the control. The birds were grouped into A (control), B (Intermediate group) and group C (test group). The parameters assayed exhibited the following results: Group A (190.05±1.61) range 42.70 mg/dl; 6.39±0.15) range 0.80 g/dl and (107.68±12.50), range 42.70 mg/dl; for glucose, protein and cholesterol respectively. For the intermediate group (group B), the following were derived for the parameters: (194.90±1.60) range 3.20 mg/dl; 6.41±0.05 (range 0.4 mg/dl) and 114.13±18.30 (range 25.0 mg/dl). For group C (test group), the following values were derived: 201.75±5.50 (range 19.50 mg/dl) 6.40±0.20 (range 0.80 mg/dl) and 104.98±3.30 (range 14.10 mg/dl) respectively. The average weight gain for the respective groups were: 268±0.18 (range 0.23 kg) for group A; 278±0.04 (range 0.13 kg) for group B and 2.96±0.08 (range 0.34 kg) for group C. The amount of food consumed were as follows: 5.13±1.40 (range 4.5 kg), 6.17±2.60 (range 6.4 kg) and 7.70±3.8 (range 8.8 kg) respectively; while the feed efficiency values were as follows: 0.37±0.03 (range 0.22), 0.33±0.01 (range 0.31) and 0.31±0.06 (range 0.49 respectively. Statistical analysis at $p \geq 0.5$ indicated no significant difference in the means of the parameters investigated. Dehusked seeds of *Carica papaya* could be nutritionally, economically and therapeutically beneficial in poultry farming and management.

Key words: *Carica papaya*, plasma glucose, protein, cholesterol

INTRODUCTION

The quest for alternative sources of energy has been the paramount occupation of scientists all over the universe to sustain biological life in this global architecture. The source of energy to animal life has facilitated researches in different fields of knowledge as a bid to reducing cost and achieving longer life span. It is an obvious fact that man depends on plants for food and medicinal uses. *Carica papaya*, otherwise known as pawpaw is a tropical American plant (Purseglove, 1974). It has spread to all tropical and sub-tropical countries (Samson, 1980). Glucose and other monosaccharides are primary sources of energy to animal cells. The nerves, the lung tissues and the brain use glucose as their source of fuel. The level of blood glucose depends on the balance between the intake of carbohydrates and the endogenous glucose synthesis and release by the liver on one hand and storage, utilization and excretion on the other (Baron *et al*., 1993). The blood of chicken contains about 200-250 mg of glucose per 100 ml of blood, which originates from digested carbohydrates. Protein makes up about three to a quarter of the body solids which include the structural proteins, enzymes, nucleoproteins, protein in the form of hemoglobin, proteins of muscles and other forms that perform specific functions. Plasma contain over one hundred types of proteins with great diversity of physiochemical characteristics and physiological roles (Baron *et al*., 1993). Most of the plasma proteins consists of albumin, globulin and fibrinogen. At physiological pH of 7.4; plasma proteins maintain the water balance of blood, transportation and storage of a large variety of ligands such as fatty acids, calcium, bilirubin and hormones such as thyroxine.

Plasma cholesterol is normally assayed because of the strong correlation between its high levels in the blood and the incidence of cardiovascular diseases in humans (Nelson and Cox, 2005). It is the precursor of bile acids and steroid hormones in the body such as the corticosteroids, sex hormones and vitamin D. It is typically a product of animal metabolism (Ganong, 1991; Baron *et al*., 1993). Cholesterol can be obtained from the diet or can be synthesized *de novo*. Approximately, about 1 g/day of cholesterol of the body arises by synthesis where as 0.3 g/day is provided by diet. Atherosclerosis is characterized by the deposition of cholesterol esters and other lipids in the connective tissues of the arterial walls. The rabbit, pig and cat are resistant. Cholesterol is an amphipathic lipid and as such, an essential structural component of membranes. *Carica papaya* is one of the genera and is cultivated throughout the tropics. The pawpaw fruit is a large fleshy, hollow berry, weighing 0.5-2.0 kg per fruit. Based on the chemical analysis, the fruit contains 88% water, 10% sugar

(carbohydrate), 0.5% proteins, 0.1% fat, 0.7% fiber, 0.6% oil and 0.1% acids (Purseglove, 1974). It is rich in vitamins A and C (Ekeke *et al*., 2001). *Carica papaya* contains many biologically active compounds which include Chymopapain and papain, which aid in digestion and milk clotting (Foyet, 1972). It has been used industrially in brewing and wine making. *Carica papaya* has been estimated to contain the following compounds: alkaloids, butanoic acid, methylbutanoate, carpaine, flavonoids, pseudo carpaine, papain, chymopapain-a and -b, tannins, α-linolenic acid, nicotine, benzylisothiocyanate, etc. Papain latex is probably of more interest to livestock producers as it is applied as anti-helminthic (Suhalla *et al*., 1994). Water extracts of papaya seeds decreased *Ascardia galli* infections in chicks by 41.7% (Kumar *et al*., 1991). *Carica papaya* extracts have been used to cure several forms of ailments in several parts of the globe, depending on the part of the tree, sex, age, extraction method and chiefly, the type of ailment.

The research work aims at providing alternative source of nutrient energy for poultry animals and equally, the reduction in the cost effectiveness of animal nutrition and husbandry.

MATERIALS AND METHODS

Materials used in this work included an array of laboratory reagents and equipment for analysis, $CuSO_4$, H_2SO_4, 99% Ethanol, Potassium sodium tartarate, Dihydroxybutanedioate, water, Glucose oxidase, glucometer, Peroxidase, Mutarotase, 4-Aminoantipyrine, p-Hydroxybenzene sulphonate, Cholesterol oxidase, all of analytical grade, purchased from Sigma Biochemicals, London.

Dietary materials: Powdered maize, powdered cake, fish meal, Palm Kernel Cake (PKC), bone meal, wheat offal, Premix, Methionine, Lysine, Pawpaw seeds (dehusked) and salt.

Drugs: Intraoccular, Gamboro, Lasota, Multivitallite, etc.

Proximate analysis: The recommended methods of the Association of Official Analytical Chemists (AOAC, 1990) were used for the determination of moisture, ash, crude lipid, crude fibre and nitrogen content.

Determination of crude lipid: Two grams (2 g) in triplicate of dried sample were weighed into the porous timble and its mouth plugged with cotton wool. The timble placed in the extraction chamber which was suspended above the weighed receiving flask containing petroleum ether (bp 40-60°C) and below a condenser. The flask was heated for eight hours to extract the crude lipid. The flask containing the crude was disconnected from the Soxhlet, the oven dried at 100°C for 30 min, cooled in a dessicator and weighed. The difference in weight is expressed as percentage of crude lipid content.

Determination of crude fiber: The crude fiber was determined as the organic residue left after treating the sample under standard conditions with petroleum ether and then boiled in 1.25% H_2SO_4 (w/v) and 1.25% NaOH (w/v) solutions. The residue after crude lipid extraction was used for this assay. Crude fiber content was expressed as percentage loss in weight on ignition.

Determination of nitrogen content of Pawpaw: Micro-Kjeldahl method was used to determine the nitrogen content of the sample. Two grams of dried powdered sample was placed in a 100 cm^3 Kjeldahl digestion flask. A kjeldahl digestion tablet and 10 ml of concentrated tetraoxosulphate (vi) acid were added and the sample digested gently until frotting stopped. The mixture was boiled until the digest become clear. The content was filtered into a 100 ml volumetric flask and made up to 100 ml with distilled water. 10 ml of the aliquot solution and 20 ml of 45% NaOH solution were put into a distillation flask and steam distilled. The ammonia liberated was collected over 50 ml, 20% boric acid mixed indicator solution, cooled and titrated with standard 0.01 M HCl solution. Blank determination was carried out in similar manner.

Determination of crude protein and available carbohydrate: Crude protein was estimated by multiplying the sample percentage nitrogen content by a factor of 6.25. Available carbohydrate was calculated by the difference method by subtracting the total or sum of crude protein, crude lipid, crude fiber and ash from the 100% DW sample.

Design of experiment: A total of twenty birds of age two months (broilers) were used. They were divided into five in each group, kept in a room of dimension 1.5 m^2. The birds were assumed to have been on commercial diet (Starter mash) for few weeks and the formulated diet for few more weeks prior to purchase. After purchase, the birds were maintained on a commercial feed (Broiler finisher) for one week. Then, the birds were fed as follows: Group A (control) were fed diet containing 64.33 g of maize. Group B (Intermediate group) were fed diet containing pawpaw seeds, substituting ¼ of the maize component of the control diet. Group C were fed diet containing pawpaw seeds substituting ½ of the maize in the control. Water was given to the birds *ad libidum*. The individual weights of the birds were taken prior to the commencement of the dietary substitution of maize with the dehusked pawpaw seeds.

Ration formulation: This was carried out to determine the nutrient requirement of the stock whose ration is to be formulated, to access the nutrient content and availability of foodstuff to be used. Ration formulation was carried out by the Pearson Square Method (Obioha, 1992).

Metabolizable energy of pawpaw: The energy value of pawpaw seed is calculated by the Atwater factors of 4, 9 and 4 as reported by Onyeike and Osuji (2003). The value of protein content is multiplied by 4; that of lipid by 9 and that of total carbohydrate by 4. The sum of these values is expressed in Kcal/100 g sample. The metabolizable energy of pawpaw seed is 332.8 Kcal/g. The wet weight of one dehusked pawpaw seed is 0.0075 g and a husked seed weighed 0.01 g.

Metabolizable energy of maize: The metabolizable energy of maize was calculated as shown below. According to Obioha (1992) maize has 10% protein, 4.6% fat and 83% carbohydrate. Hence, carbohydrate = 83 x 4 = 332; fat = 4.6 x 9 = 41.4; protein = 10 x 4 = 40; Total = 413.4 Kcal/g.

Composition of diet fed on chicks or birds

Materials	Control (A) %	Group (B) %	Group (C) %
Groundnut cake	20.63	20.63	20.63
Palm Kernel Cake (PKC)	4.42	4.42	4.42
Fish meal	4.40	4.42	4.42
Maize	64.33	48.25	32.17
Pawpaw	-	16.08	32.17
Bone meal	2.00	2.00	2.00
Wheat offal	2.00	2.00	2.00
Premix	0.50	0.50	0.50
Methionine	0.50	0.50	0.50

Blood sample collection: Blood samples were collected from each of the birds of the three respective groups. This was done by using a syringe to draw 1.0 ml of blood from the veins of the wing side (right wing).
The blood samples were collected using anticoagulant bottle or container. The samples were later centrifuged to obtain the plasma used in the test.

Quantitative determination of total plasma glucose: This was determined using glucose oxidase test method.

Quantitative determination of total plasma protein: This was done by the Biuret method of Lowry *et al.* (1951).

Quantitative determination of total plasma cholesterol: This was carried out by the methods of Holvey (1972).

RESULTS

The results of the various assays are shown in Table 1-6.

Table 1: Proximate analysis of pawpaw seeds

Parameter	Percent (%)	Parameter	Percent (%)
Moisture	10.5	Ash	6.0
Fat	14.0	Protein	23.1
Fiber	18.0	Carbohydrates	28.6

Table 2: Plasma glucose, protein and cholesterol concentrations of group A (Control)

Group	Glucose (mg/dl)	Protein (mg/dl)	Cholesterol (mg/dl)
A_1	198.10	6.35	119.00
A_2	165.00	6.40	105.80
A_3	189.40	6.20	93.30
A_4	207.70	6.60	121.60
Average	190.05±24.40	6.39±0.15	10768±12.50
Range	42.7	0.80	28.30

Table 3: Plasma glucose, protein and cholesterol concentrations of the intermediate group (B)

Group	Glucose (mg/dl)	Protein (mg/dl)	Cholesterol (mg/dl)
B_1	194.90	6.20	98.30
B_2	196.50	6.60	116.60
B_3	193.30	6.25	123.30
B_4	194.90	6.60	118.30
Average	194.90±1.60	6.41±0.05	114.13±18.30
Range	3.30	0.40	25.00

Table 4: Plasma glucose, protein and cholesterol concentrations of group (C), the test group

Group	Glucose (mg/dl)	Protein (mg/dl)	Cholesterol (mg/dl)
C_1	199.00	6.60	111.60
C_2	213.00	6.00	97.50
C_3	201.50	6.25	100.80
C_4	193.50	6.60	110.30
Average	201.75±5.50	6.40±0.20	104.98±3.30
Range	19.50	0.80	14.10

DISCUSSION

From the statistical analyses of all the results obtained for plasma glucose, protein and cholesterol levels in the control group, intermediate group and the test group fed pawpaw seed containing diet; there is virtually no significant difference in the mean (X) of the parameters investigated. The relative constant plasma protein levels of the chicks or birds in the control groups, intermediate and test groups could be as a result of adequate utilization of the dietary materials in the groups. According to Anosike (1994), the requirements for dietary protein arises from the requirements for certain amino acids which are considered essential for the body. This requirement was met by the constant level of proteins in the diet fed to the animals in the three groups. The Metabolizable Energy (ME) of the diet fed the experimental animals indicated a value of 580.7 kcal/g.

Table 5: Summary of parameters and values

Group	Glucose (mg/dl)	Protein (mg/dl)	Cholesterol (mg/dl)
A	190.05±24.40; Range = 42.70	6.39±0.15; Range = 0.80	107.68±12.50; Range = 28.30
B	194.90±1.60; Range = 3.30	6.41±0.05; Range = 0.40	114.13±18.30; Range = 25.00
C	201.75±5.50; Range = 19.50	6.40±0.20; Range = 0.80	104.98±3.30; Range = 14.10

Table 6: Weight gain per bird per group

Group	Weight (kg)	Group	Weight (kg)	Group	Weight (kg)
A_1	2.73	B_1	2.75	C_1	2.85
A_2	2.72	B_2	2.84	C_2	3.11
A_3	2.54	B_3	2.71	C_3	3.10
A_4	2.77	B_4	2.77	C_4	2.77
Average	2.69±0.18	2.78±0.04		2.96±0.08	
Range	0.28 kg	0.13 kg		0.34 kg	

This calculation was eventuated by the observation of Neishein *et al.* (1972) that the efficiency of feed utilization increases as the energy of the diet increases. Though maize has 83% carbohydrate, while pawpaw has 39.20%, the experimental animals have half the carbohydrate content of maize, that is 41.15 g in addition to that of pawpaw. It has been observed that feed efficiency does not depend on carbohydrate content of the feed alone (Obioha, 1992). The relative constant level of plasma cholesterol as seen in the chicks studied in both the control and experimental groups could be attributed to both hereditary and dietary factors, since the chicks came from the same stock, probably; there are not much variations in their endogenous biosynthesis of cholesterol. This is in accordance with the observation of Mayes (1993), that hereditary factors play the greatest role in determining an individual blood cholesterol concentration. The level of blood or plasma cholesterol depends on a gamut of factors especially the dietary intake of saturated fatty acids with polyunsaturated and monounsaturated fatty acids, determines individual's blood cholesterol. Pawpaw seeds as obtained, are also rich in lipids. These exogenous sources of cholesterol. Inhibit exogenous biosynthesis (Guyton, 1991). Considering the weight gain in the groups, it seems that the efficiency in feed utilization of the birds is accountable for this. Naturally, animals tend to loose weight when inadequate source of metabolizable energy is efficiently supplied. However, the general performance of the animals (birds) in the test group is a clear indication of non-toxicity of the pawpaw seeds. Specifically, toxic substances tend to inhibit protein synthesis We recommend that *Carica papaya* seeds can comfortably and confidently be incorporated into poultry feeds. From the results obtained, the birds that were on pawpaw seed-containing diets were vibrant and healthy, displaying voracious appetite for the diet. One of the benefits of this study is that pawpaw seeds that were neglected or otherwise regarded as waste, may prove useful in animal sciences and husbandry for human nutrition and medication. Only 50% substitution for maize, produced this excellent finding. The reduction in cost effectiveness of feeds and the improvement on the health of the birds is an excellent breakthrough for poultry farmers if the findings are successfully applied.

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Isolation of *Enterobacter sakazakii* from Powdered Foods Locally Consumed in Nigeria

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Abstract: The presence *Enterobacter sakazakii* was investigated from powdered food samples using enrichment procedure of Enterobacteriaceae Enrichment Broth (EEB), Violet Red Bile Glucose Agar (VRBGA) and Tryptic Soy Agar (TSA). A total of 140 food samples were tested. *E. sakazakii* was isolated from 20/70 powdered infant foods, 13/50 powdered milk and 5/20 milk-based products, making a total of 27.1% prevalence. The isolates were tested for their susceptibility to 15 antibiotics. The results showed that streptomycin was the most effective (94.7%), followed by ofloxacin (92.1%), levofloxacin (84.2%), ciprofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%). The organism was resistant to rifampicin and amoxicillin.

Key words: Food, *Enterobacter sakazakii*, antibiotic, streptomycin, infant milk

INTRODUCTION

Enterobacter sakazakii is a gram negative, facultative anaerobic, straight rod-shaped bacterium. It belongs to the family *Enterobacteriaceae* and genus *Enterobacter* that contains a number of species including *E. agglomerans, E. cloacae, E. aerogenes* and *E. gergoviae*. It is a coliform having dimensions of 3 µm in length and 1 µm in width. The cells are motile by peritrichous flagella and do not form spores (Farmer *et al*., 1980; Farmer and Kelly, 1992).

Enterobacter sakazakii is an opportunistic human pathogen that has been implicated in severe forms of septicemia (Lai, 2001), necrotizing enterocolitis (Van Acker *et al*., 2001) and meningitis (Bar-Oz *et al*., 2001), especially in neonates with mortality rate varying from 40-80% (Muytjens *et al*., 1988). The International Commission for Foods, due to the seriousness of pathologies with *E. sakazakii* has ranked the organism as "severe hazard for restricted populations, life threatening or substantial chronic sequelae or long lasting" (ICMSF, 2002).

E. sakazakii has been isolated from a wide range of foods including ultra high-temperature treated milk (UHT milk), cheese, meat, vegetables, grains, sorghum seeds, rice seeds, herbs, spices, fermented bread, fermented beverage, tofu and sour tea (Gassem, 2002; Leclercq *et al*., 2002; Iversen and Forsythe, 2003). Despite this, studies have confirmed the connection between neonatal *E. sakazakii* infection and infant milk formulas (Muytjens *et al*., 1988; Biering *et al*., 1989; Simmons *et al*., 1989; Nazarowec-White and Farber, 1997b; Van Acker *et al*., 2001).

The US Food and Drug Administration (FDA, 2002) has issued an alert to health care professionals about the risk associated with *E. sakazakii* infections among neonates fed with milk-based infant formula. The alert stated that a major contribution to the avoidance of *E. sakazakii* infections in premature babies and neonates is the prevention of contamination of infant milk formula during production and bottle preparation.

Several outbreaks have occurred in neonatal intensive care units as a result of infections by the organism: in May/ June 1994 in France where 13 neonates were infected and three died, June/ July 1998 in Belgium where 12 neonates developed necrotizing enterocolitis and two twin brothers died and a more serious one in Tennessee in 2001 (CDC, 2002). These outbreaks were traced to contaminated powdered infant formula by the organism (Van Acker *et al*., 2001). But most recently in 2008, a total of five babies were lost in New Mexico due to *E. sakazakii* infection (CDC, 2009). These have alerted the US Centers for Disease Prevention and Control (CDC) on the consumption of powdered infant formula, and have brought the organism to limelight.

E. sakazakii is an emerging food borne pathogen that had been discovered in the developed countries. It is likely that there is a significant under-reporting of infections in all countries. The absence of reports is probably due to a lack of awareness of the problem rather than an absence of illness. Since infant formula is widely used, the presence of *E. sakazakii* in infant formula and its potential effects in infants could as well be a significant public health problem in most countries. This study provides an information on the determination on the incidence of *E. sakazakii* in powdered infant foods and antibiotic susceptibility pattern of the organism.

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MATERIALS AND METHODS

Collection of samples: A total of 140 different commercial food samples from different manufacturers were purchased from retail stores across Benin City, Nigeria. The samples composed of 70 powdered infant foods (recommended for from birth to one year old infants), 50 powdered milk and 20 milk-based products. The samples were labeled appropriately and approved by National Agency for Food and Drug Administration and Control (NAFDAC).

Detection and Isolation of *Enterobacter sakazakii*: The method of Food and Drug Administration (2002) for the isolation of the pathogen was used. For the pre-enrichment step, 10 g of each powder was aseptically dissolved in 50 ml of sterile water pre-warmed to 40°C, to make a solution in the 60ml screw-capped bottles. From the bottle, 10-fold dilution was made using sterile test tubes. The test tubes were screwed. The tubes were incubated at 36°C for 24 h in an incubator.

For the enrichment step, 90 ml of Enterobacteriaceae Enrichment Broth (EEB) was prepared into the 100 ml screw-capped bottles. The incubated tubes (10 ml each) were aseptically inoculated into the bottles containing 90ml of EEB. A bottle was left uninoculated to act as a control. The bottles were incubated at 36°C for 24 h in an incubator.

The selection step was carried out by aseptically streaked a loop full of the incubated bottles into a duplicate plates of Violet red bile glucose agar. The plates were then incubated at 36°C for 24 h in an incubator. From the cultured plates, purple colonies were picked using sterile inoculating loop and sub-cultured into Tryptic Soy Agar (TSA) plates in duplicates by streaking. The plates were incubated at 25°C for 72 h in an incubator.

The isolated colonies were further detected by biochemical methods.

Antibiotic susceptibility test: Antibiotic susceptibility test was performed using Kirby-Bauer disc diffusion method. The medium used for this test is Tryptic soy agar. Prepared plates containing the medium were dried in hot air oven for about five minutes. A sterile inoculating loop was used to pick isolated colonies and emulsified into 5 ml of sterile nutrient broth. After proper homogenization, the broth was poured into the agar surface to make a lawn of growth. The surface of the agar plate was allowed to dry for 5 min. A sterile forceps was used to place the antibiotic discs unto the inoculated plates. The antibiotic discs were in two sets and each set was used for isolation. The antibiotics contained in the discs were: streptomycin (30 µg), norfloxacin (10 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), levofloxacin (20 µg), gentamycin (10 µg), rifampicin (20 µg), amoxicillin (20 µg), ofloxacin (10 µg), pefloxacin (10 µg), augmentin (30 µg), cephalexin (10 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (30 µg), ampicillin (30 µg).

The plates were incubated at 37°C for 24 h in an incubator. After incubation, the diameter of the zone of inhibition was measured in millimetres using a ruler. All results were recorded appropriately and interpreted using the National Committee for Clinical Laboratory Standards (NCCLS) interpretation chart (NCCLS, 2002).

RESULTS

A total of 140 food samples were tested for the presence of *E. sakazakii*, 38 samples were found positive. The positive strains of *E. sakazakii* formed yellow colonies on Tryptic soy agar after 48 h of incubation at 25°C and satisfied the biochemical screening.

Table 1 indicates the number of *E. sakazakii* isolates obtained from each food brand. The organism was detected in all the food brands except in Bournvita. Among the powdered infant foods, SMA was the most prevalent, while Frisocrem and Nutrend were the least prevalent for the organism. Peak choco had the highest prevalence among the milk-based products, with Bournvita had zero prevalence.

The result in Table 2 showed that 38(27.1%) of the total 140 powdered foods were positive for *E. sakazakii*, with powdered infant foods having the highest frequency of 33.9%, followed by powdered milk (32.7%) and milk-based products (31.4%) having the least.

On testing the 38 isolates from the samples using 15 antibiotics in antibiotic susceptibility discs, almost all the isolates were sensitive to streptomycin (94.7%), followed by ofloxacin (92.1%), levofloxacin (84.2%), ciprofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%) in the decreasing order (Table 3).

Cephalexin (42.1%), norfloxacin (31.6%), amoxicillin-clavulanate (31.6%) was poorly sensitive; while nalidixic acid (10.5%), trimethoprim-sulfamethoxazole (10.5%), chloramphenicol (7.9%), ampicillin (5.3%) were resistant. But rifampicin (0%) and amoxicillin (0%) were strongly resistant.

DISCUSSION

Enterobacter sakazakii is an emerging food-borne pathogen that had been linked with infantile meningitis, septicemia and necrotizing colitis transmitted through the consumption of contaminated powdered infant foods and other milk products (Lai, 2001; Van Acker *et al*., 2001; Bar-Oz *et al*., 2001).

This study was set up to investigate the incidence of this organism in powdered foods and its products. *E. sakazakii* was isolated from the three food types-powdered infant food, powdered milk and milk-based products, in 27.1% of samples. Among the three food types, powdered infant food had the highest frequency.

Table 1: *Enterobacter sakazakii* isolated from powdered foods

Food samples/brand	No. of samples	SP (+) with *E. sakazakii*	SN (-) with *E. sakazakii*
Powdered infant food			
SMA	10	4	6
NAN	10	3	7
Peak 123	10	3	7
Nutrend	10	2	8
Soya	10	3	7
Cerelac	10	3	7
Frisocrem	10	2	8
Powdered milk			
Peak	10	3	7
Cowbell	10	3	7
Nunu	8	2	6
Coast	8	1	7
Blue boat	7	2	5
Jago	7	2	5
Milk-based product			
Milo	4	1	3
Ovaltine	4	1	3
Peak choco	4	2	2
Cowbell choco	4	1	3
Bournvita	4	0	4

SP = Samples Positive (+) with *E. sakazakii*
SN = Samples Negative (-) with *E. sakazakii*

Table 2: Total (percentage) contamination of the sample type

Sample type	Total number	No. (%) of sample contaminated
Powdered infant food	70	20 (33.9)
Powdered milk	50	3 (32.7)
Milk-based product	20	5 (31.4)
Total	140	38 (27.1)

These results correlated with the works of (Muytjens *et al*., 1988; Biering *et al*., 1989; Simmons *et al*., 1989; Noriega *et al*., 1990; Nazarowec-White and Farber, 1997b; Iversen and Forsythe, 2003; Shaker *et al*., 2007), who had found a direct relationship between infant formula and *E. sakazakii*. Muytjens *et al*. (1988) tested 141 samples of powdered infant milk formula manufactured in different countries. They found that *E. sakazakii* and other *Enterobacteriaceae* were isolated from 14.1 and 52.2% of the total samples respectively. Nazarowec-White and Farber (1997b) surveyed the presence of *E. sakazakii* in 120 dried infant milk samples (five manufacturers) obtained from Canadian retail market and reported that the prevalence of this bacterium ranged between 0 and 12% of the samples/manufacturer. Iversen and Forsythe (2004) isolated *E. sakazakii* from 24% of 82 powdered infant milk formulas.

Many studies have focused on the infant formula as the main source of this serious pathogen (Postupa and Aldova, 1984; Muytjens *et al*., 1988; Nazarowec-White and Farber, 1997b; Van Acker *et al*., 2001; Block *et al*., 2002). Despite the fact that formulas are exposed to heat treatment during processing, *E. sakazakii* was still isolated from these products. Post-processing contamination of the infant formula from food production environments may be responsible for the presence of this pathogen in infant formula since standard pasteurization practices are effective for the inactivation of the organism (Iversen *et al*., 2004).

Nazarowec-White and Farber (1997a) stated that *E. sakazakii* can gain access to the powder from the environment or from the addition of the ingredients at the powder stage, especially using the Dry-mix process of production. Iversen and Forsythe (2003) reported that the presence of *E. sakazakii* in powdered infant milk formula depends on the process conditions and nature of the product. However, the prevalence of the organism following the drying stage and survival in powdered foods for a long time may be due (in part) to the organism's ability to resist desiccation and osmotic stress (Arku *et al*., 2008).

Unlike commercially available ready-to-feed liquid infant formula, which is sterile, powdered infant formula (including dried bovine milk and milk products) is not a sterile product (FDA, 2002). Powdered infant formula has been known to be contaminated, on occasion, with bacterial pathogens, including *Bacillus* species, *Clostridium* species, *Staphylococcus* species and *Enterobacteriaceae*, notably *Cronobacter* (Forsythe, 2005). Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process.

For the case of the milk-based product, the organism was not detected in Bournvita, *E. sakazakii* could not be ruled from being a possible pathogen of this product, putting into consideration that each sample represented itself only. This result is unusual as the organism has been isolated from a wide range of foods including chocolate, spices, rice seeds, cereals, potato flour and pasta (Shaker *et al*., 2007). Also, it could that the organism is unequally distributed in the sample or its presence escaped detection (Muytjens *et al*., 1988).

From the results obtained from the antibiotic sensitivity test (Table 3), streptomycin (94.7%) seems to be a better drug for treatment. Others include Ofloxacin (92.1%), levofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%). Streptomycin and gentamycin are aminoglycosides; while Ofloxacin, levofloxacin, ciprofloxacin and pefloxacin are quinolones.

These results were consistent with the findings by Stock and Wiedemann (2002) that *E. sakazakii* is susceptible to tetracyclines, aminoglycosides, quinolones, antifolates and chloramphenicol. Aminoglycosides and quinolones inhibit protein synthesis and DNA replication respectively in the organism (McKane and Kandel, 1996).

Enterobacter sakazakii like other *Enterobacter* species have acquired resistance by inactivating broad spectrum beta-lactam antibiotics due to the production of beta-lactamases (Drudy *et al*., 2006). Other mechanisms

Table 3: Number (percentage) of *E. sakazakii* isolates sensitive to antibiotics

Sample brands	No. of isolates tested	Antibiotics							
		S	NB	CH	CPX	LEV	RD	CN	AML
SMA	4	4(100)	1(25)	0(0)	3(75)	3(75)	0(0)	3(75)	0(0)
NAN	3	3(100)	1(33.3)	1(33.3)	3(100)	3(100)	0(0)	2(66.7)	0(0)
Peak 123	3	3(100)	0(0)	0(0)	2(66.7)	3(100)	0(0)	2(66.7)	0(0)
Nutrend	2	2(100)	1(50)	1(50)	2(100)	2(100)	0(0)	1(50)	0(0)
Soya	3	2(66.7)	1(33.3)	0(0)	1(33.3)	3(100)	0(0)	1(33.3)	0(0)
Cerelac	3	2(66.7)	2(66.7)	0(0)	1(33.3)	2(66.7)	0(0)	2(66.7)	0(0)
Frisocrem	2	2(100)	1(50)	0(0)	1(50)	1(50)	0(0)	2(100)	0(0)
Peak	3	3(100)	1(33.3)	0(0)	3(100)	1(33.3)	0(0)	1(33.3)	0(0)
Cowbell	3	3(100)	1(33.3)	0(0)	2(66.7)	3(100)	0(0)	3(100)	0(0)
Nunu	2	2(100)	0(0)	0(0)	2(100)	1(50)	0(0)	2(100)	0(0)
Coast	1	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	0(0)	0(0)
Blue boat	2	2(100)	1(50)	0(0)	2(100)	2(100)	0(0)	2(100)	0(0)
Jago	2	2(100)	0(0)	0(0)	2(100)	2(100)	0(0)	1(50)	0(0)
MBP	5	5(100)	1(20)	0(0)	5(100)	5(100)	0(0)	3(60)	0(0)
Total (%)	38	94.7	31.6	7.9	79.0	84.2	0.0	65.8	0.0

Sample brands	No. of isolates tested	Antibiotics						
		OFX	PEF	AU	CEP	NA	SXT	PN
SMA	4	4(100)	4(100)	1(25)	1(25)	0(0)	1(25)	0(0)
NAN	3	3(100)	2(66.7)	1(33.3)	1(33.3)	0(0)	0(0)	0(0)
Peak 123	3	3(100)	1(33.3)	2(66.7)	0(0)	0(0)	0(0)	1(33.3)
Nutrend	2	2(100)	1(50)	1(50)	1(50)	0(0)	0(0)	0(0)
Soya	3	3(100)	3(100)	1(33.3)	2(66.7)	1(33.3)	0(0)	1(33.3)
Cerelac	3	2(66.7)	2(66.7)	0(0)	1(33.3)	0(0)	0(0)	0(0)
Frisocrem	2	2(100)	2(100)	0(0)	2(100)	0(0)	1(50)	0(0)
Peak	3	2(66.7)	2(66.7)	0(0)	2(66.7)	1(33.3)	1(33.3)	0(0)
Cowbell	3	3(100)	2(66.7)	0(0)	2(66.7)	0(0)	0(0)	0(0)
Nunu	2	2(100)	1(50)	0(0)	1(50)	1(50)	0(0)	0(0)
Coast	1	1(00)	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)
Blue boat	2	2(100)	2(100)	1(50)	1(50)	0(0)	0(0)	0(0)
Jago	2	2(100)	2(100)	1(50)	1(50)	0(0)	0(0)	0(0)
MBP	5	4(80)	5(100)	4(80)	1(20)	1(20)	0(0)	0(0)
Total (%)	38	92.1	79.0	31.6	42.1	10.5	10.5	5.3

S = Streptomycin, CN = Norfloxacin, CH = Chloramphenicol, CPX = Ciprofloxacin, LEV = Levofloxacin, RD = Rifampicin, CN = Gentamycin, AML = Amoxicillin, OFX = Ofloxacin, PEF = Pefloxacin, AU = Amoxicillin-clavulanate, CEP = Cephalexin, NA = Nalidixic Acid, SXT = Trimethoprim-sulfamethoxazole, PN = Ampicillin. NZI = No Zone of Inhibition

include: decreased cell permeability, active efflux, modification of drug receptor site, synthesis of resistant metabolic pathway and acquisition of plasmids and transposons (Chao *et al*., 2007). These could be the reasons for the strong resistance against cephalexin (42.1%), amoxicillin-clavulanate (31.6%), ampicillin (5.3%) and amoxicillin (0%) as observed in this research. Other studies have also shown rifampicin, macrolides and fusidic acid to be resistant (Stock and Wiedemann, 2002).

Conclusion: *E. sakazakii* is an emerging pathogen, often transmitted through the consumption of powdered infant foods and its products. It is responsible for series of infections with potential fatal outcomes in infants. The findings from this study showed that *E. sakazakii* was detected in powdered infant foods, powdered milk and milk-based products. The organism was found to be resistant to some antibiotics; the quinolones, in combination with an aminoglycosides would be a better choice drug for treatment of infections caused by this organism.

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Current Position of Sanitation in Nigerian Food Industries

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Abstract: Evaluation of conformity of major commodity food industries to sanitation standards was carried out. Major fourteen food industries were investigated include dairy industry, bakery, beverage factory, fish processing industry, brewery, cereal processing and fruit canning industry. The survey shows that most Nigerian food industries were designed, constructed and operated without sufficient consideration for many important sanitary factors and without adequate facilities of the right kind or the type of conditions which would promote good personal hygiene and impeccable plant sanitation. Few of the industries performed satisfactorily in some of the area investigated while four companies attained the minimum standard specified in this survey on all overall basis. Much more attention needs to be paid to rodent and pest control, water treatment, waste disposal and utilization.

Key words: Sanitation standard, food industries, food quality

INTRODUCTION

Food meant for human and animal consumption should be produced under conditions of cleanliness and sanitary decency. No consumer would knowingly wish to consume food products that has been canned, dried or processed in a rodent infested, insect ridden, filthy or bacteriologically unclean factory. Clean food shouldn't be processed in dirty equipment and surroundings.

Good plant sanitation often reduces loss due to bacterial spoilage, mould, fermentation, insect infestation and rodent contamination. G.W. Harrison defined food sanitation as "A system or a set of system for ensuring that food is prepared, manufactured, stored, distributed and sold under the cleanest possible conditions and with an absence of avoidable contamination (Harrison, 1976).

According to the definition given by the National Canner Association Research Laboratory of America, sanitation has been defined as "The planned maintenance of the work and product environment to prevent or minimize hazard of product contamination and conditions aesthetically offensive to the consumer and to provide healthful and safe working conditions" (NCARL, 1968).

Parker and Litchfield have broadly defined sanitary practice in food industry as "the systematic control of environmental conditions during the transportation, storage and processing of food in such a manner that their contamination by micro organisms, insects, rodents or other animals pests and by foreign chemical materials can be prevented" (Parker and Litchfield).

There seems to be so many specific sanitation problems in food business such as insanitary methods of transporting perishable foods (e.g. meat and fresh vegetables), inadequate and inappropriate storage facilities, filthy markets, squalid abattoirs, exposure of raw and cooked foods to surroundings of deplorable sanitation and the commercial processing of food in unhygienic plants, give much concern for responsible food scientists, food technologist, health authorities and discriminating consumers.

The National Agency for Food and Drug Administration and control (NAFDAC) is the legal Agency in Nigeria that enforces the Food and Drugs Decree of 1974 which made the following provisions for the regulation of the manufacture, sale and advertisement of food:

It prohibits the sale of any food which:

(i) Has in it or upon it any poisonous or harmful substance not being a food additive or contaminant of a type and within the level permitted by regulation/make under this Decree.
(ii) Is unfit for human consumption.
(iii) Consists in whole or in part of any filthy, disgusting, rotten or diseased substance.

The penalties for successful prosecution are severe and legal action can sometimes be taken (Harrison, 1973).

MATERIALS AND METHODS

Investigation: An investigation was carried out on many aspects of plant sanitation using the inspection technique recommended by Parker and Litchfield (Ossai *et al*., 1984-85). Sanitation evaluation format for the purpose of the investigation was prepared in order to avoid overlooking pertinent details.

Major commodity food industries such as meat processing factories, dairy industries, bakeries, soft drink factories, cereal processing factories, beverage factories, fish processing factories and fruit canning industries were evaluated.

Codes (C.1, C.2, C.3, etc) rather than actual company names have been used for known facts.

Numerical scores were given to sanitary observations on a 9 point hedonic.
Scales: 9-Extremely good, 8-Very good, 7-Good, 6-Fairly good, 5-Neither good nor bad, 4-Fairly good, 3-Bad, 2-Very good, 1-Extremely bad.
Great pain was also taken to make sure the score was as accurate as possible. Percentage score on each main items evaluated was calculated. A score below 75% was considered unsatisfactory for a food industry because the maintenance of a sound sanitary practice is important to the production of high quality and safe products.

RESULTS AND DISCUSSION

Table 1 shows the percentage scores on all the plants evaluated for sanitation and indicates the degree to which the selected food industries conform to standard sanitary practice.

Factory surroundings: Most of the industries visited were not located in swampy or heavily wooded areas or near centers of pollution. The plant surroundings were generally clean. All the industries visited were either located close to residential areas or office complexes which often harbored a large number of rodents. Refuse dumps, swamps and woods are detrimental to the sanitary interests of food industries; Insects, rodents and reptiles normally comes from such environments which ought to have been avoided at the early stage of the search for a site.
Normal drainage facilities were inadequate in C5 and C7 which were water logged during my visit. All the industries effected a measure of dust control by properly cementing land surrounding process areas but C1,C2, C5, C7, C8 and C11 kept empty drums, metal pipes and unused planks in their premises which constitute an eye sore for the factory surroundings.

Factory buildings: The industries were mostly of single storey construction and high enough to allow the use of fork lift trucks. This facilitates free and direct flow of raw materials and equipments. The breweries and flour mills were in necessity, multi storey. There were no false ceilings except in C7 which can harbor rodents and reptiles and also allows for mould growth. The walls had smooth surfaces. Five of the industries had their walls tiled a reasonable distance from the floor to facilitate cleaning. Some of the walls were painted in light colour which would aid the reflection of light.
If the humidity was high in such factories the paints could peel off (The Federal Military Government of Nigeria, 1974) as was observed in two of the industries. Except for C5, cracks and crevices, which tend to harbor insects and encourage microbial growth, were generally absent. The factory floors were constructed of impervious materials. The floors of seven of the industries showed evidence of wear. This was were prominent in the fruit processing and baking industries due to the effect of fruit acids and dragging of baking pan on the floor. Floor slopes were generally adequate and led to drains, most of which were covered. However, exit of the drains to the outside were unscreened in all cases. The junction between wall and floors were not coved. Pipes were laid in such a manner as to make dismantling easy.
Ventilation was largely adequate except in C7 and C11. This would help minimize heat stress on workers and reduce, moisture condensation, thereby reducing mould growth. All the industries had windows, the sills of which were not sloped to minimize dust accumulation. Only C7 had fly screens on its windows and doors to protect the process areas from insects. Lighting was generally adequate in the process areas but not so in the storage areas. Adequate and appropriate lighting in food plant is essential for the efficient cleaning and sanitizing of food equipment and machinery. Adequate lighting is also necessary for good general cleaning, for measurement, reading, color comparisons, labeling and for the inspection of raw materials and finished products (Parker and Litchfield, 1962).

Water supply: In the food industries investigated water obtained either from the river, boreholes or municipal water sources. There was an encouraging awareness in most of the industries of the need to use potable water in their processing. Ten of the industries had water treatment units except C2, C3, C7 and C11 had none.

Ware house: All the industries had ware houses where finished products were kept. Most of the industries ware houses were well illuminated and kept well clean while C5 and C14 did not keep their ware houses clean and tidy. The untidiness of these ware houses might cause contamination of products through rodents and insects as well as reptiles. On a general note, the ware houses were kept in good conditions for most of the industries.

Waste disposal: Waste disposal as well as its utilization, received sufficient and necessary attention in ten industries except in C2, C3, C7 and C11. Liquid wastes were channeled out of the factory premises without treatment into streams or into public drainage system. Most of the industries employed the services of waste disposal contractors. Only a few of the industries put their wastes into profitable use, for instance, a vegetable oil processing company sold all its solid waste as livestock feed.

Facilities for employees: None of the industries had all the essential facilities recommended for workers in the food industry. None of the industries had hand-washing

Table 1: Sanitary status of some major food industries in Nigeria

Food factory	FS	FB	DRR	C	FA	WH	WD	FE	T	RIC	CP	PA	WS	EPH	OA
C1	52	63	69	40	30	69	75	51	59	57	80	75	70	50	60.35
C2	49	69	64	85	75	70	65	60	62	62	67	77	35	60	64.29
C3	57	60	59	70	75	70	54	71	76	48	59	70	40	65	62.29
C4	78	75	69	71	79	77	79	69	75	56	77	76	64	69	72.43
C5	45	49	65	75	79	60	78	54	82	35	58	40	75	57	60.71
C6	82	77	74	79	80	69	78	65	79	67	75	74	76	76	75.07
C7	40	45	20	75	75	62	59	69	80	67	62	42	45	41	56.57
C8	65	80	69	77	82	73	80	81	81	74	62	85	73	72	75.21
C9	77	81	79	84	81	75	77	70	84	72	69	80	65	74	76.29
C10	76	79	71	78	84	75	80	69	80	79	70	67	75	69	75.14
C11	54	45	20	54	35	69	56	45	77	45	62	54	40	47	50.21
C12	78	76	60	72	70	74	84	76	75	42	64	54	75	60	68.57
C13	75	75	70	76	82	72	76	68	77	62	78	76	77	65	73.5
C14	65	77	64	78	38	62	74	55	76	37	59	67	70	69	63.64

FS = Factory Surroundings FB = Factory Building DRR = Dressing and Rest Rooms C = Cafetaria
FA = First Aid WH = Ware House WD = Waste Disposal FE = Facilities for Employees
T = Toilet RIC = Rodent and Insect Control CP = Clean up Procedures PA = Processing Area
WS = Water Supply EPH = Employer's Personal Hygiene OA = Overall Acore

facilities in the process area except C6 and none provided soap for hand-washing and no towels for hand drying.

Toilet: All the industries provided toilet facilities for their staff. Some of the toilets had their floors and walls tile. The toilets in C1 and C2 were not so clean but all others were clean. No soap was provided in the industries except in C6 and means of drying their hands was also not provided. Liquid and hot air drying device can be substituted for tablet soap and hand towels as the excuse given by most of the company's management is that the tablet soap was been taken away by employees. During normal operations hands become soiled with a wide variety of contaminants. Hand is probably one of the most common vehicles for transmitting contamination of food and food contact surfaces. Harwood and Minch (1951), investigated the number and types of bacteria found on the hands of food handlers. They identified a large number of coliform organisms in addition to other bacteria. It is therefore essential that hands be washed thoroughly at frequent intervals during the working day, most especially after each visit to the toilet.

First aid: First aid facilities were provided in all the companies except C1, C7, C11 and C14. Most of the companies' first aid facilities were handled by untrained attendant except C6 and C10.

Dressing and rest rooms: C7 and C11 had no dressing rooms for their employees. In all the other factories dressing rooms were close to the toilets and washrooms and equipped with fairly clean ventilated lockers. Personal clothing can be a source of food contamination. It is therefore, desirable to have workers routinely change into clean factory uniforms at the beginning of each day's operation.

Cafeteria: Most of the industries had cafeteria and cleaning was unsatisfactory. None used the three stages cleaning procedure of soaking and sterilization. Impeccable hygiene should be maintained in all aspects of cafeteria services. Dirty uniforms and habits must not be tolerated.

Employee's personal hygiene: All the industries provided uniforms, some provided caps in addition. However C7 and C11 did not provide uniform for their employees. Nose picking and smoking were not noticed in any of the fourteen industries investigated. There was also no evidence of illness. It should be noted that insanitary and unsightly habits, apart from their contamination potentials, may adversely affect confidence in the industry.

Rodent and insect control: Premises which are badly designed and managed are ideal for the development of infestations of many types. C5, C7, C10, C11, C12 and C14 did not maintain their premises well. Their surroundings were bushy which gives room for rodent infestation. No infestation should be tolerated in food premises. It is not possible to comply with the guide and to produce a consistently uncontaminated product if insects, rodents or birds are present.

Clean-up procedures: Most of the companies visited had a good cleaning procedure. C1, C4, C6, C8, C10, C13 use clean-in-place system. This makes for efficient cleaning of their equipment both internally and externally.

Sanitary condition of process areas: The processing area of C1, C2, C4, C8, C9 and C13 were sparklingly

clean, most of them are either producing dairy related products or breweries. C5, C7 and C12 were particularly dirty. C5 was not well planned and raw materials were stored in the process area.

Conclusion: From Table 1, only about four companies (C6, C8, C9 and C10) maintained high sanitary standard. Six out of the fourteen industries maintained their factory surrounding very well. Eight of the industries also designed and build their factory in conformity with hygienic standards.

In particular, none of the companies satisfied the minimum standard of sanitation in the area of facilities for employees while only one company satisfy minimum standard in the area of rodent and insect control. Also in the area of personal hygiene of employees, only C6 which is a dairy industry satisfy the minimum sanitary standard. The food factory workers in Nigeria therefore appear to be neglected in terms of facilities and their level of personal hygiene is very low. It is evident that the entire company's except one make little effort to control rodents and insects. This may have undesirable or, perhaps, deleterious consequences on the quality of their products and the health of the consumers (Olunlade *et al*., 2008).

Finally, much more attention need to be paid to rodent and insect pest control, water treatment, waste disposal and utilization. The condition of sanitation in most food industries is therefore very bad and demands considerable and urgent improvement.

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Evaluation of Yam Starch (*Discorea rotundata*) as Aquatic Feed Binder

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Abstract: This experiment was conducted to evaluate the suitability of yam starch as a local alternative binding agent in aquatic feed, which is effective and nutritive. The binding property of yam starch in feed pellet, increased significantly with the levels of inclusion in fish feed production. Five percent (5%), inclusion level was found to be appropriate in producing desirable water stable pellet that is also firm to handling during transportation and storage.

Key words: Yam starch, binder, aquatic feed

INTRODUCTION

Nigeria is abundantly endowed with the root crop-Yam (*Dioscorea rotundata*) of which is obtained the bidding agent yam starch. The bidding capability of yam starch discovered has the potential of boosting aquacultural sub-sector of the economy.

On-Farm aqua feed unlike livestock feed requires adequate level of processing to guarantee optimum availability to and utilization of compounded feed by the target fish (Misra *et al*., 2003). Such a feed should be firm to handling as well as maintain reasonable degree of stability in the aquatic medium, long enough for fish to consume it (Wood, 1993; Pigott and Tuker, 1989; Pigott *et al*., 1982; Fagbenro and Jauncey, 1995; Misra *et al*., 2003).

Such a stable pellet feed will allow for wholesome delivery and utilization of feed materials for the fish unlike as experienced in the broadcast method, leaching of nutrients will be minimized (Sadiku and Jauncey, 1995; 1998; Tiamiyu *et al*., 2003 a, b). The role of binder in aqua-feed is significant but the high cost of conventional synthetic binders make pellet feed production at on-farm level a difficult exercise especially for small and medium scale aqua culturists, thus, most farmers result to arbitrary inclusion of any available unconventional binding agents in the production of the pellet feed at any level of inclusion of binder. Aside from establishing the range of natural binders from 0-20% (Jauncey, 1992), there has not been any significant research into such natural binders as yam.

Moreover, the establishment of the appropriate level of yam starch inclusion, in addition to other local binders such as cassava starch (Asiedu, 1992), arrow root, potato starch (Wood, 1993), corn starch (Orire *et al*., 2001), etc will go a along way in addressing the problem confronting fish farmers at on-farm level of aquatic feed production. The binder level that will give desirable pellets (Hastings, 1980) as well as that will ensure wholesome delivery of nutrients to fish with minimum wastage (Viola *et al*., 1986; Natividad, 1994).

In addition, the availability of yam starch at on farm level will be an appropriate local alternative to fish-farmers. The finding would enhance aquacultural production by rendering feed production affordable and attainable at on farm level thus, reducing existing pressure on the stressed fresh and marine water bodies resources that are presently seriously threatened with extinction due to over exploitation and over-dependence on them for fish supply to teeming population of Nigerians and for export.

MATERIALS AND METHODS

Starch processing: A yam tuber of 2kg size of the variety *Dioscorea rotundata* (white yam) was peeled, washed and grated. The paste (4 litres) was then mixed with sufficient quantity of water to allow for proper exudation of the starch from the fiber. The solute was the poured into cheesecloth and squeezed to obtain the starch solution filtrate. This was then allowed to stand overnight for thorough separation. The supernatant was decanted to obtain the starch, which was dried in the sun at about 36°C for 6 hours and then packaged in 5, 10, 15 and 20 grams for incorporation in the five (5) diets.

Feed preparation and pelleting: Binder level in an existing isonitrogenous diet formula of 30% crude protein was reconstituted, with the use of Yam starch at inclusion levels of 0, 5, 10, 15 and 20% as diets 1, 2, 3, 4 and 5 respectively (Table 1).

For effective inclusion of the starch, the Yam starch was mixed in its powdery form with other feed ingredients and on which a 120% v/w boiled water was added and stirred thoroughly to obtain a good dough. The formed dough was then fed into an Atlas motorized Bohr miller (Pelleter) with a 3mm die for pellet size. The pellet strands were then cut at 5mm length; sun dried (36°C) for 6hrs and packed.

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Table 1: Percentage composition of diets with varying levels of yam starch as feed binder

	Diets				
Feedstuff	1	2	3	4	5
Soybean meal	37.26	37.26	37.26	37.26	37.26
Fish meal[1]	3.64	13.64	13.64	13.64	13.64
Corn bran	16.37	16.37	16.37	16.37	16.37
Guinea corn bran	32.74	32.74	32.74	32.74	32.74
Yam starch	0	5	10	15	20
Vit.-Min-Premis*	20	15	10	5	0
Proximate composition					
moisture	7.43	13.40	16.81	20.21	23.61
Crude protein	28.91	28.91	28.91	28.91	28.91
Ether Extract	9.9	9.9	9.9	9.9	9.9
Ash	26.19	21.19	16.19	11.19	6.19

*Vitamin-mineral premix is as contained in Sadiku and Jauncey (1995 and 1998)

Evaluation of physical properties of the pellets: The following physical tests were conducted on the pellets; pellet ability, dust level, hardness, friability and water stability.

i) **Pellet ability:** The pelleted feed was sifted to separate the well formed from the unformed. The percentage pellet ability was obtained by expressing the pellet weight to the total weight.
ii) **Hardness:** The procedure used was to determine the force required to cause a pelleted feed particle to fragment. This gives an indication of pellet's degree of hardness. To do this an improvised pentagon nut was used, by placing a pellet sample of 5mm in length longitudinally between two rods and gently tighten the grip. The pentagon nut was then turned and the calibration read against when the pellet gets broken. This was repeated for 24 more pellets and average number of turn was taken for each. The average number of was then taken for the pellets sample to ascertain its hardness.
iii) **Friability:** Fifty grams (50g) of pellet sample was put in a container and adapted into a rotary machine at different preset speed levels of rotation per minute (rpm) of 40, 63, 80 and 100 rpm at 20 minutes. The dust generated from the agitated pellets was then collected through 2mm sieve and was weighed and expressed as a percentage of the sample weight.
iv) **Water stability:** Fifty grams (50g) of pellet sample was placed in a beaker into which 200 mL of tap water was added. It was then allowed to stand with occasional gentle shaking for 20 seconds every 2 minutes. It was then passed over 2mm sieve, the particles were allowed to settled and decanted which was then sun-dried. The retained dry weight was then expressed as a percentage of the sample dry weight.
v) **Dust:** Sample pellets of fifty grams (50g) by weight and was placed under normal stress-condition, such as handling, packaging and transportation for a period of two (2) weeks. The dust particles produced was collected through a 2mm sieve and was measured as a percentage of the original weight.

Experimental design: Completely randomized block design was used to analyze parameters such as pellet ability, hardness, dust and water stability while a 5×4 factorial design i.e. 5 starch levels of inclusion in the feed×4 levels of rpm was adopted for pellet friability.

Statistical analysis: The data was analyzed using a one-way Analysis of variance (ANOVA). Also Arc-sine data transformation was done according to Zar (1984). Means comparison was done using multiple range of test (Steele and Torrie, 1960).

RESULTS

In Table 2, the pelleted yam starch (binder) exhibited significant differences among the parameters measured; the percent Dust level shows a significant ($p<0.05$) difference among different level of starch inclusion. Highest percent of dust was recorded at 0% level of starch and lowest in pellets with 20% level of inclusion. The pellet ability among starch level also showed significant ($p<0.05$) difference, with highest percent pellet ability in 0% level of starch inclusion and lowest in 15% level of inclusion. Also there was a significant ($p<0.05$) difference in level of starch inclusion for hardness, pellets with 5% and 10% starch level respectively displayed high resistance to pressure of pentagon nut while pellets with 20% starch level offered the least resistance to pressure of the pentagon nut. There was a significant ($p<0.05$) difference in the water stability as per the level of starch inclusion. Pellets with 5% level of starch inclusion displayed greatest degree of stability while those with 20% level of starch demonstrated the least stability in the water medium. Measurement for the friability indicated no significant ($p>0.05$) difference among starch levels irrespective of the variations in the rpm. However there was a significant ($p<0.05$) difference among rpms irrespective of starch levels. Moreover, friability percentage was found to be highest at 100 rpm) Table 2 and lowest at 40 rpm, which was also significant ($p<0.05$).

DISCUSSION

From the result obtained, it was observed that the feed stability in the pelleted form with respect to its physical characteristics vis; Pellet ability, Hardness, Friability, Dust and water stability, all exhibited high degree of variability regarding different starch levels of inclusion.

As for pellet hardness it exhibited the high degree of hardness (Table 2) at 0% level of starch inclusion, which would be assumed as okay and perhaps the best pellet. But the Dust level was the highest (Table 2), which is an indication for insufficient binder in the feed that resulted In the softness of the pellets (Church and Pond, 1988).

Table 2: Physical parameters of yam starch based feed

	Starch level				
Parameter	0%	5%	10%	15%	20%
Dust	0.12^{c}	0.09^{bc}	0.09^{bc}	0.06^{ab}	0.02^{a}
Pellet ability	90.11^{e}	84.96^{d}	80.91^{c}	65.75^{a}	79.11^{b}
Hardness	5.16^{c}	5.24^{d}	4.16^{b}	6.52^{d}	3.68^{a}
Water stability	46.03^{bc}	47.74^{d}	46.31^{c}	45.74^{b}	41.71^{a}

Data on the same row carrying different letters differ significantly from each other ($p<0.05$)

Table 3: Friability of yam starch based feed

Rpm	0%	5%	10%	15%	20%
40	0.04^{a}	0.05^{ab}	0.05^{ab}	0.05^{ab}	0.05^{ab}
63	0.07^{abc}	0.07^{abc}	0.09^{acbdf}	0.09^{abcd}	0.10^{bcd}
80	0.09^{abcd}	0.10^{bcd}	0.09^{abcd}	0.09^{abcd}	0.13^{d}
100	0.14^{d}	0.12^{cd}	0.11^{cd}	0.13^{d}	0.13^{d}

Data on the same row carrying different letters differ significantly from each other ($p<0.05$)

Moreover, at 20% level of starch inclusion, the Dust level was the lowest but lowest percentage of pellet ability was obtained (Table 2). This is due to gumming together of the pellets, which is an indication of over binding, a disadvantage of over starching. The gumming together of the pellet strands is a function of the adhesive property of the binder (Somsveb, 1993; Lim and Dominy *et al*., 1991; Akiyama *et al*., 1989; Stivers, 1970). However, at 5% level of starch inclusion, the best obtainable pellets were achieved. The pellet displayed good pelletablity, hardness, water stability and minimum dust level of 0.9% (Table 2) that would ensure stable pellets that will not easily disintegrate in the aquatic medium (Hardy, 1989; Jobling, 1994; De Silva and Anderson, 1995) and friability was best at 40 rpm and at most 63 rpm but could not withstands rpm as high as 100 rpm (Table 3). Therefore, it can be concluded that the use of Yam starch as binder for fish feed is best at 5% level of inclusion that would ensure the desirable pellets.

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Nutritional and Sensory Quality of Cookies Supplemented with Defatted Pumpkin (*Cucurbita pepo*) Seed Flour

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Abstract: Pumpkin (*Cucurbita pepo*) seed was processed into defatted flour (DCPF) and evaluated for nutritional and sensory attributes. The potential of the flour as composite with wheat flour in cookie production was also evaluated. The crude protein content of DCPF was as high as 57.50% with highly valuable amino acid profile, rich in essential amino acids and minerals. DCPF was highly digestible (77.91%) and has a Protein Efficiency Ratio (PER) of 1.80. The anti-nutrients were below allowable limits. Cookie diameter negatively correlated with alkaline water retention capacity of *Cucurbita pepo* seed/wheat flour blends with correlation coefficient of -0.89. The physico-chemical and sensory evaluation of cookies revealed that up to 10% substitution of wheat flour with DCPF produced acceptable cookies similar to the control (100% wheat flour).

Key words: Pumpkin seed, defatted flour, amino acid, biological value, cookies

INTRODUCTION

Ways of expanding the use of available local food sources are increasingly pursued but knowledge of the nutritive value of such local ingredients and foodstuffs is necessary in order to encourage the increased cultivation and consumption. Knowledge of the nutritive value is essential in supplementing staple carbohydrate foods. Worldwide, much research has focused on various sources of plant proteins (El-Adawy *et al*., 2001; Rangel *et al*., 2003) that may help in increasing the nutritional value of food products at low cost. Pumpkin (*Cucurbita pepo)* has received considerable attention in recent years because of the nutritional and health protective values of the seeds. The seed is an excellent source of protein and also has pharmacological activities such as anti-diabetic (Quanhong *et al*., 2003), antifungal (Wang and Ng, 2003), antibacterial and anti-inflammation activities (Caili *et al*., 2006) and antioxidant effects (Nkosi *et al*., 2006). In addition to good health benefits, pumpkin seeds are less expensive and are widely distributed. The present study examined the chemical, nutritional and supplementary potential of defatted pumpkin seed flour in biscuit making.

MATERIALS AND METHODS

Defatted *Cucurbita pepo* seed flour production: Pumpkin seeds were extracted, washed, sundried and manually decorticated. The seeds were crushed using a household mill (Super intermet blender SI-462 model) and defatted by soaking in n-hexane for 36h with change of solvent every 8 h. The defatted flour was filtered, dried at room temperature (27°C±1°C) and ground to pass through a 355MICS sieve. The flour was packaged in an air-tight plastic container and kept in a refrigerator until analyzed.

Chemical analysis: Crude protein, fibre, moisture and vitamin C were determined by methods described by AOAC (1990). Fat, ash and mineral content were determined as described by James (1995). Carbohydrate was determined by difference and calorific value was obtained using the method of Onyeike *et al.* (1995). Thiamin and riboflavin were determined as described by Onwuka (2005). Vitamin A was determined using the method described by Martin and Ruberte (1976). Tannin, phytic acid and trypsin inhibitor were determined by the methods of Pearson (1976); Hang and Lantzsch (1983) and Arntifield *et al.* (1985), respectively. Saponin was determined by the method of Harborne (1973) and cyanogenic glycoside as described by Onwuka (2005). The oligosaccharides (stachyose and raffinose) were determined by the method of Ojiako and Akubugwo (1997).

Amino acids analysis: Amino acids were determined using a Technicon Sequential Multi-Sample Analyzer (TSM) according to the method of Speckman *et al.* (1958). *In-vitro* protein digestibility was determined using trypsin-pepsin enzyme system according to the method of Saunders *et al.* (1973). *In-vitro* digestibility

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was expressed as percentage enzymatic digestion as shown below:

$$\text{Enzymatic digestion (\%)} = \frac{\text{Nitrogen released by enzyme}}{\text{Total nitrogen content of undigested sample}}$$

Protein Digestibility Corrected Amino Acid Score (PDCAAS) was determined using the method of Henley and Kuster (1974).

Biological values of defatted *Cucurbita pepo* seed flour was determined on the basis of the amino acid profiles. Amino acid score was calculated for each essential amino acid in a given test protein using the (FAO/WHO, 1990) reference pattern:

$$\text{Amino acid score} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in 1 g reference}}$$

The method described by Rasco (2002) was used in calculating the Essential Amino Acid Index of protein in the flour using the amino acid composition of whole egg protein as standard (Hidvégi and Békes, 1984).

$$\text{EAA I (\%)} = 100 \times \sqrt[10]{\frac{a_i}{a_{i\ ref}}}$$

Where a_i and $a_{i\ ref}$ represent the concentration of essential amino acids (lysine, tryptophan, isoleucine, valine, arginine, threonine, leucine, phenylalanine, histidine and the sum of methionine and cystine) in test sample and the reference - the egg protein, respectively. Protein Efficiency Ratio (PER) was estimated according to the regression equation proposed by Alsmeyer *et al.* (1974).

$$\text{PER} = -0.468 + 0.454\ (\text{Leucine}) - 0.105\ (\text{Tyrosine})$$

Flour blend formulation: Defatted pumpkin seed-wheat flour blends were prepared by replacing wheat flour at 10, 20, 30 and 40% by weight.

Alkaline water retention capacity: The method described by Sathe *et al.* (1982) was used to evaluate the alkaline water retention capacity of the flour blends.

Preparation of cookies: The recipe used for cookie preparation included flour 60g, vegetable shortening 50g, granulated sugar 25g, baking powder 0.6g, nutmeg 200 mg, salt 700 mg, and water 5 ml. Cookies were prepared by replacing the all purpose wheat flour with *Cucurbita pepo* seed flour at 10, 20, 30, and 40 % (by weight). The dough was allowed to equilibrate for 1 h at 4°C and divided into 15 g portions, shaped round and baked on a greased tray at 149°C for 25 min. Baked cookies were cooled to room temperature and evaluated for physical parameters and sensory properties (Sathe *et al.*, 1982).

Plate 1: Cookies from 0:100 DCPF-wheat flour blend

Plate 2: Cookies from 10:90 DCPF-wheat flour blend

Plate 3: Cookies from 20-80 DCPF-wheat flour blend

Physical evaluation of cookies: Weight, height and diameter measurements were performed in triplicate on

Plate 4: Cookies from 30:70 DCPF-wheat flour blend

Plate 5: Cookies from 40:60 DCPF-wheat flour blend

Plate 6: Cookies from 100:0 DCPF-wheat flour blend

five representative cookies in each batch. The cookie spread ratio (%) was calculated as the increase in volume of the unbaked stamped out dough:

$$\text{Spread ratio (\%)} = \frac{\text{Increase in volume of cookie dough}}{\text{Original volume of cookie dough}} \times 100$$

Cookie break strength was determined (Okaka and Isieh, 1990).

Sensory evaluation: Twenty untrained judges comprising of staff and students of Michael Okpara University of Agriculture, Umudike were used for the evaluation of the quality parameters (colour, taste, texture, flavor and general acceptability) of the cookies. The panelist were asked to indicate their preference using a nine-point Hedonic scale with 1 and 9 representing liked extremely and disliked extremely, respectively.

Statistical analysis: Correlation analysis was performed on duplicate determination for alkaline water retention capacity and the data obtained from sensory evaluation were subjected to Analysis of Variance (ANOVA) the SPSS statistical package (Version 13.0). Means were separated with Duncan's Multiple Range Test (DMRT). Significant differences were determined at $p<0.05$ level and results were expressed as the mean value ± standard deviation of duplicate determinations.

Table 1: Anti-nutritional factors in pumpkin flour

Tannin (%)	0.69
Saponin (%)	0.56
Hydrogen cyanide (mg/100 g)	4.08
Trypsin Inhibitor (TIU/g)	2.07
Phytate (%)	0.44
Stachyose (%)	3.00
Raffinose (%)	0.80

RESULTS AND DISCUSSION

Nutritional properties: Hydrogen cyanide was found to be 4.08 mg (Table 1). HCN values from this study were below the safety level for cyanide poisoning in man. The lethal dose range of ingested HCN for humans is estimated to be 50-60 mg/kg body weight/day as reported by Balagopalan *et al.* (1988). Stachyose and raffinose were high compared to Udensi *et al.* (2008) who recorded lower values for stachyose (1.29%) and raffinose (0.32%) in *Mucuna flagellipes*. Raffinose and stachyose have been identified as flatulence inducers and when ingested cause accumulation of gas, discomfort, diarrhea, pain and cramps (Liew and Buckle, 1990), a factor which tends to render legumes less acceptable.

Amino acid: The amino acid composition of *Cucurbita pepo* seed flour is presented in Table 2. *Cucurbita pepo* seed flour exhibited lower amino acid content compared to chickpea flour which ranged between 1.6-19.5 g/100 g protein (El-Adawy *et al.*, 2001). Cystine and tryptophan showed the lowest values 0.79 and 0.99 g/100 g protein,

Table 2: Amino acid profile of pumpkin (*Cucurbita pepo*) seed flour

Amino acid	Composition g/100 protein	FAO / WHO / UNU (1985) pre-school child (2-5 yrs) reference pattern (g/100 g protein)	Uncorrected amino acid score	PDCAAS
Isoleucine	2.98	2.80	1.06	0.82
Leucine	5.71	6.60	0.87	0.67
Lysine	4.30	5.80	0.74	0.58
Cystine[a]	0.79	-	-	-
Methionine[a]	1.43	Methionine + cystine = 2.50	0.89	0.69
Total sulphur amino acid	2.22	-	-	-
Tyrosine	3.06	-	-	-
Phenylalanine	3.47	Phenylalanine + tyrosine =6.30	1.04	0.81
Total aromatic amino acids	6.53	-	-	-
Threonine	2.11	3.40	0.62	0.48[a]
Tryptophan[a]	0.99	1.10	0.90	0.70
Valine	4.50	3.50	1.29	1.01
Histidine	2.33	1.90	1.23	0.96
Arginine	4.85			
Aspartic acid	8.91	-	-	-
Glutamic acid	9.50	-	-	-
Serine	2.19	-	-	-
Proline	2.12	-	-	-
Glycine	3.80	-	-	-
Alanine	4.76	-	-	-
E/T (%)	43.27	-	-	-

[a] = Limiting Amino Acid, E/T = Essential to Total Amino Acid, PDCAAS = Protein Digestibility Corrected Amino Acid Score

Table 3: Protein nutritional quality of *Cucurbita pepo* seed flour

Samples	Chemical score (%)	Limiting amino acids			EAAI (%)	*In-vitro* protein digestibility (%)	PER
		First	Second	Third			
DCPF	62.0	Threonine	Lysine	Leucine	57.31	77.91	1.80

DCPF = *Cucurbita pepo* Seed Flour; EAAI = Essential Amino Acid Index; PER = Protein Efficiency Ratio

respectively. Percentage ratios of essential to total amino acids (E/T, %) for *Cucurbita pepo* seed flour (43.27) was above 36% which is considered adequate for an ideal protein (FAO/WHO, 1973). The protein nutritional quality of *Cucurbita pepo* seed flour is presented in Table 3. The first, second, and third limiting amino acids were threonine, lysine and leucine, respectively. The present observation is similar to other legumes (Sathe *et al*., 1982; Akobundu *et al*., 1982). The *in-vitro* protein digestibility exhibited by the flour was high and may be attributed to its low fat and protease inhibitor content which usually hinder the action of digestive enzymes when present in large amount (Sánchez-Vioque *et al*., 1999). It could also be attributed to the low tannin content (Table 1). Tannins cause reduction in digestibility of dietary protein (Barroga *et al*., 1985).

Alkaline water retention capacity of *Cucurbita pepo* seed composite flour: The alkaline water retention capacity predicts the baking behaviour of flour in cookies based on the established inverse relationship between alkaline water retention and baked cookie diameter. The cookie diameter was negatively correlated to the alkaline water retention capacity of the blends (Table 4) with correlation coefficient of -0.89. Similar correlation between alkaline water retention capacity and cookie diameter for several wheat flours and Great Northern Bean (*Phaseolus vulgaris L.*) were reported by Yamazaki *et al*. (1977) and Sathe *et al*. (1982), respectively. Twenty percent wheat flour substitution had the highest alkaline water retention capacity with reduced cookie diameter while 100% wheat flour gave the least alkaline water retention with high cookie diameter (Table 4).

Table 4: Correlation between the alkaline water retention capacity of defatted *Cucurbita pepo* seed flour blends and cookie diameter

DCPF-wheat flour blends	Alkaline water retention capacity[a] (g/g blend)	Cookie diameter[a] (cm)	Correlation coefficient
cpw (control)	0.95±0.28	6.22±0.01	
cpw_1	1.05±0.35	6.21±0.06	
cpw_2	1.18±0.18	6.19±0.01	-0.89
cpw_3	1.00±0.21	6.20±0.03	
cpw_4	1.15±0.07	6.15±0.07	

[a]Mean±standard deviation of duplicate determinations.
cpw_1= Defatted *Cucurbita pepo* seed-wheat flour (10:90)
cpw_3= Defatted *Cucurbita pepo* seed-wheat flour (30:70)
cpw (control) = Defatted *Cucurbita pepo* seed-wheat flour (0:100)
cpw_2= Defatted *Cucurbita pepo* seed-wheat flour (20:80)
cpw_4= Defatted *Cucurbita pepo* seed-wheat flour (40:60)

Physicochemical properties of cookies: Physical parameters and protein content of cookies are

Table 5: Physicochemical properties of cookies

DCPF-wheat flour blends	Protein content (%)	Increase in protein content[a] (%)	Weight (g)	Diameter (cm)	Height (cm)	Spread ratio (%)	Break strength (Kg)
cpw (control)	6.80	0.00	13.30	6.22	0.60	66.03	0.46
cpw_1	6.92	1.76	13.39	6.21	0.53	51.22	0.46
cpw_2	7.00	2.94	13.34	6.19	0.53	46.83	0.51
cpw_3	8.59	26.32	13.31	6.20	0.53	47.29	0.68
cpw_4	10.00	47.06	13.53	6.15	0.47	28.52	0.62

[a]Increase (%) with reference to protein content of the control cookies.
cpw (control) = Defatted *Cucurbita pepo* seed-wheat flour (0:100)
cpw_1= Defatted *Cucurbita pepo* seed-wheat flour (10:90)
cpw_2= Defatted *Cucurbita pepo* seed-wheat flour (20:80)
cpw_3= Defatted *Cucurbita pepo* seed-wheat flour (30:70)
cpw_4= Defatted *Cucurbita pepo* seed-wheat flour (40:60)

Table 6: Sensory evaluation[e] of cookies made from DCPF-wheat flour blends

DCPF-wheat flour blend	Colour	Taste	Texture	Flavour	General acceptability
cpw (control)	$1.65^{a}\pm0.7452$	$2.30^{ab}\pm0.4702$	$2.45^{ab}\pm0.6863$	$1.90^{a}\pm0.7182$	$2.20^{ab}\pm0.6156$
cpw_1	$2.10^{ab}\pm0.5525$	$2.05^{a}\pm0.5104$	$2.15^{a}\pm0.8127$	$2.10^{a}\pm0.7182$	$1.90^{a}\pm0.7881$
cpw_2	$2.25^{ab}\pm0.7164$	$3.05^{c}\pm1.0990$	$2.95^{b}\pm0.6863$	$2.80^{b}\pm0.7678$	$3.00^{c}\pm1.0260$
cpw_3	$2.20^{ab}\pm0.8944$	$2.80^{bc}\pm0.7678$	$2.50^{ab}\pm0.6883$	$2.90^{b}\pm1.0208$	$2.90^{bc}\pm0.7182$
cpw_4	$2.60^{b}\pm1.0955$	$3.30^{c}\pm0.9234$	$2.95^{b}\pm1.1459$	$3.75^{c}\pm1.3328$	$3.05^{c}\pm1.3169$

[e]Mean± standard deviation of duplicate determinations.
[a,b,c,d]Means with the same superscripts within the same column are not significantly different ($p<0.05$).
cpw (control) = Defatted *Cucurbita pepo* seed-wheat flour (0:100)
cpw_1= Defatted *Cucurbita pepo* seed-wheat flour (10:90)
cpw_2= Defatted *Cucurbita pepo* seed-wheat flour (20:80)
cpw_3= Defatted *Cucurbita pepo* seed-wheat flour (30:70)
cpw_4= Defatted *Cucurbita pepo* seed-wheat flour (40:60)

presented in Table 5. Increase in wheat flour substitution with *Cucurbita pepo* seed flour increased the protein content of the cookies. Generally, the cookie spread ratio decreased with increase in protein content of the cookies. It was reported that rapid partitioning of free water to hydrophilic sites during mixing increased dough viscosity and limited cookie spread ratio during baking (McWatters, 1978). The reduction in spread ratio was more pronounced in 40% wheat flour substitution. Similar results were recorded by Sathe *et al.* (1982) for cookies made with Northern Bean (*Phaseolus vulgaris L.*) flour. The break strength of the cookies increased with increase in protein content. *Cucurbita pepo* seed flour-wheat blend of 30% had the highest break strength while 10% wheat flour substitution emerged the least and had the same value as 0% substitution (Control).

Sensory properties of cookies: The sensory evaluation of cookies made from *Cucurbita pepo* seed-wheat flour blends is presented in Table 6. The cookies were not significantly different in colour at 10-30% wheat flour substitution. There was no significant difference in texture at all levels of wheat flour substitution. Apart from the control, cookies made from 10% wheat flour substitution had the best flavour. This result revealed that up to 10% substitution of wheat flour with defatted *cucurbita pepo* seed flour produced acceptable cookies which were not significantly different from the control (100% wheat flour).

Conclusion: In addition to good chemical and nutritional values, *Cucurbita pepo* seed flour performed well as composite in cookie production. Wheat flour substitution at 10% is recommended to produce acceptable cookies. Observations in this study further support high correlation between alkaline water retention capacity and cookie diameter and that *Cucurbita pepo* seed flour has the potential of being used as a nutritional supplement.

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Impact of Maternal Copper and Zinc Status on Pregnancy Outcomes in a Population of Pregnant Nigerians

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Abstract: Micronutrient deficiencies, especially during pregnancy have been identified as important public health problem especially in economically disadvantaged settings. To determine the effect of maternal deficiencies of copper and zinc on pregnancy outcomes in a population of pregnant Nigerians, 349 pregnant women aged 15-40 years (mean; 27.04±2.75 years) recruited at gestational age of ≤25 week (mean; 21.8±3.14 wks) were evaluated for plasma copper and zinc using Atomic Absorption Spectrophotometer. The women were followed-up till delivery during which maternal morbidity and foetal outcomes were recorded. Both maternal sociodemographic and obstetric data were obtained by questionnaire. One hundred and sixty (45.8%) women were zinc deficient (mean = 2.65±1.16 µmol/l), 58.2% were deficient in copper (mean = 3.26±1.80 µmol/l), 23.8% were deficient in both copper and zinc while 18.6% were not deficient in either copper or zinc. There was comparative prevalence of illness in copper-deficient and copper-adequate mothers, except for hypertension which was significantly ($p = 0.021$) higher in the former. Significantly ($p = 0.026$) higher proportion of zinc adequate pregnant women suffered upper respiratory tract infections and malaria when compared with their zinc deficient counterparts. However, the prevalence of diabetes mellitus was found to be significantly ($p<0.05$) higher in mothers who were zinc deficient. Both plasma copper and zinc status had no significant effect on foetal outcome. The reason for the lack of effect of copper and zinc deficiencies on foetal outcomes in the presence of adverse maternal outcomes remained unknown.

Key words: copper and zinc deficiencies, pregnant Nigerian women, public health

INTRODUCTION

Micronutrient deficiencies are common during pregnancy, especially in pregnant women from economically disadvantaged settings where diets with low content of minerals and vitamins are consumed. Zinc is an essential trace element with wide range of functions in the body including the synthesis of enzymes and nucleic acids (WHO, 1996). Studies of pregnant women in African countries such as Nigeria, Egypt, Zaire and Malawi have shown lower plasma or hair zinc concentrations than in pregnant women from developed countries (Okonofua *et al*., 1990; Kirksey *et al*., 1994; Anaud *et al*., 1994; Gibson and Huddle, 1998).

Also, several studies have reported that maternal plasma zinc decreases during pregnancy from 24-33 week of gestation (Perveen *et al*., 2002; Ajose *et al*., 2001; Izquierdo *et al*., 2007; Martin-Lagos *et al*., 1998). However, Meran *et al*. (2003) while reporting comparable plasma zinc in pregnant and non pregnant women documented decreased plasma zinc in pregnant women greater than 35 years old.

Like zinc, copper is involved in the functions of several cuproenzymes that are essential for life (Goel and Misra, 1982). Copper plays a role in the mobilization of iron to plasma from the tissue stores (Raman and Leela, 1992) and copper deficiency during embryonic and foetal development has been found to cause numerous gross structural and biochemical abnormalities. It has been reported that more than 50% of human conception fail to implant and of those implanted, approximately 30% fail to reach term due to copper deficiency (Ebbs *et al*., 1984). Significantly higher mean serum copper had been reported in healthy pregnant Nigerian women than their non-pregnant counterparts (Ajose *et al*., 2001). Similar increase in serum copper had been reported in Spanish (Izquierdo *et al*., 2007) and Turkish women (Meran *et al*., 2003).

Also deficiencies of trace elements; copper and zinc have been implicated in various reproductive events like infertility, pregnancy wastage, congenital abnormalities (Black, 2001), pregnancy induced hypertension, placental abruption, premature rupture of membranes,

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still birth and low birth weight (Pathak and Kapil, 2004). In Nigeria, there is paucity of data on the impact of plasma zinc and copper levels on pregnancy outcomes. Therefore the present study is aimed at evaluating the effect of maternal plasma levels of copper and zinc during pregnancy on pregnancy outcomes.

MATERIALS AND METHODS

The study was carried out at the Department of Obstetrics and Gynaecology of the Federal Medical Centre, Abakaliki, one of the referral tertiary health institutions in the South eastern part of Nigeria. Abakaliki and the environs are inhabited mainly by subsistence population whose main occupation is farming (mainly, yam and cassava) with some animal husbandry and other professions and/or activities such as civil service, trading, artisan and stone quarrying. Malaria transmission is intense and occurs throughout the year (perennial).

Three hundred and fifty-one (351) consecutive women aged 15-40 years (Gestational age ≤25 weeks) who gave their consent to participate in the study were recruited between July 2007 and September 2008. Those excluded from the study were women with chronic disease, women that were HIV-seropositive and those with multiple pregnancies. The protocol for this study was approved by the Ethics and Research Committee of the Federal Medical Centre, Abakaliki. The sociodemographic data of the participants were collected by structured questionnaires. Height and weight were measured with the subject in light clothes without shoes and BMI (Kg/m^2) was calculated.

Five millilitres (5.0 ml) of non-fasting venous blood collected between 08.00-10.00 hours were dispensed into trace element-free heparinized plastic bottle (3.0 ml) and EDTA bottle (2.0 ml) for biochemical and haematological analyses respectively. Plasma was separated by centrifugation at 2000 g for five minute. The plasma samples were frozen until they were analyzed.

Participants were followed-up till delivery. At every follow-up, participants were evaluated by the attending Obstetricians for anaemia (Hb < 11.0 g/dl), hypertension (blood pressure > 140/90 mmHg), diabetes (fasting plasma glucose > 7.8 mmol/l), *H. pylori* infection (Seropositive to *H. pylori* antibody) and concomitant illness such as malaria (positive thin or thick film), upper respiratory tract infection (cough and catarrh), urinary tract infection (positive urine protein, nitrite and leucocytes).

At delivery, baby's birth outcomes such as weight, length, head circumference as well as still birth, mode of delivery, gestation age at delivery was recorded. Birth weight was determined using electronic weighing balance and recorded to the nearest 0.05 Kg with the scale checked periodically throughout the study for accuracy while birth length and head circumference was determined by a measuring tape to the nearest 0.1 cm. Baby was considered underweight if the birth weight was ≤2.5 Kg (Fawzi *et al*., 2007), preterm if delivered at ≤37 weeks and post-term if delivered at > 42 weeks.

Plasma copper and zinc were determined in duplicates using flame Atomic Absorption Spectrophotometer and the mean was recorded as the absolute value of the elements while plasma albumin was determined by bromocresol green method as previously described (Hill, 1985).

Maternal haemoglobin concentration was determined by Cyanmethaemoglobin method as described previously (Dacie and Lewis, 1994). The World Health Organisation (1992) criteria for typing anaemia in pregnancy were adopted in this study. Plasma copper < 8.0 mmol/l and zinc < 5.0 mmol/l were considered deficiencies (Robets *et al*., 2006).

Statistical analysis: The data obtained were analyzed using Statistical Package for Social Science (SPSS) version 7.5. The results were expressed and mean ± S.D or proportion. Mean plasma levels of zinc and copper were compared among groups by Chi-square and the level of significance set and $p<0.05$.

RESULTS

Although three hundred and fifty one (351) pregnant women were recruited, one (0.3%) died early into the study remaining three hundred and fifty (99.7) of which data was available but samples were obtained from 349 participants as one participant declined participation. At delivery, data was available for three hundred and nineteen (91.4%) women and their neonates. Data was incomplete or not available for the remaining thirty (8.6%).

Table 1 shows the general characteristics of pregnant women recruited at ≤25 weeks gestation. Although, in general, the women were deficient in either of the two trace elements (Mean: 9.59±9.42 and 9.19±9.16 mmol/l for copper and zinc respectively) evaluated, the ranges of the elements vary from very low levels to very high concentrations, with copper and zinc concentrations from 0.89 and 0.70 mmol/l respectively to values as high as 45.36 and 67.32 mmol/l respectively. However, the participants were generally anaemic with mean haemoglobin concentration of 10.21±1.26 g/dl.

One hundred and sixty (45.8%) women were zinc deficient (mean = 2.65±1.16 µmol/l) and 203 (58.2%) were deficient in copper (mean = 3.26±1.80 µmol/l), 83 (23.8%) were deficient in both copper and zinc while 65 (18.6%) were not deficient in either copper or zinc.

Figure 1 and 2 shows the prevalence of maternal concomitant illnesses in relation to copper and zinc status. There was comparative prevalence of illness in copper-deficient and copper-adequate mothers, except for hypertension which was significantly ($p = 0.021$) higher in the former. However, there appeared to be generally non-significant higher prevalence of concomitant illness in the copper deficient groups.

Table 1: General characteristics of pregnant women at ≤25 weeks gestation

Parameters	N	Mean	SD	Range
Age (yrs)	350	27.04	4.75	15-40
BMI (Kg/m^2)	350	27.3	4.3	17.8-42.6
Parity (n)	350	1.41	1.46	0-4
Haemoglobin (g/dl)	349	10.21	1.26	6.5-13.3
Albumin (g/dl)	349	3.45	0.80	1.80-5.50
Copper (mmol/l)	349	9.59	9.42	0.89-45.36
Zinc (mmol/l)	349	9.19	9.16	0.7-67.32
Antenatal attendance (n)	343	7.01	2.52	1-14

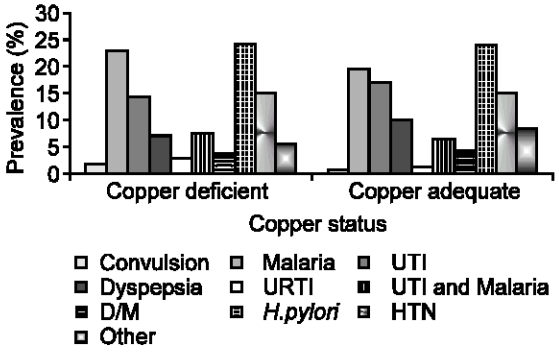

Fig. 1: Maternal morbidity in relation to copper status. UTI: Urinary tract infection; D/M: Diabetes Mellitus; URTI: Upper Respiratory Tract Infections; HTN: Hypertension. P<0.05 for Hypertension

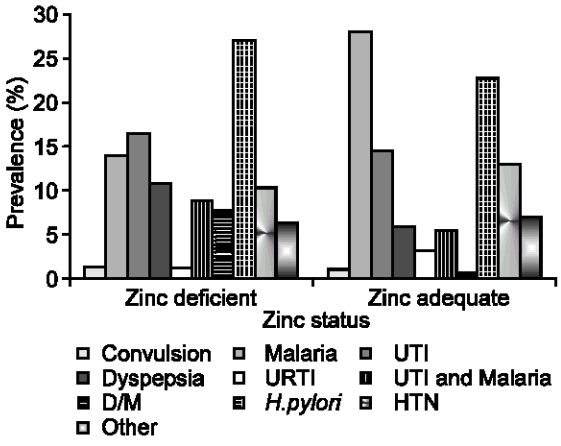

Fig. 2: Maternal morbidity in relation to zinc status. UTI: Urinary Tract Infection; D/M: Diabetes Mellitus; URTI: Upper Respiratory Tract Infections; HTN: Hypertension. P<0.05 for URTI, Malaria and D/M

For plasma zinc, significantly (p = 0.026) higher proportion of zinc adequate pregnant women suffered upper respiratory tract infections and malaria when compared with their zinc deficient counterparts. However, the prevalence of diabetes mellitus was found to be significantly (p<0.05) higher in mothers who were zinc deficient than in mothers who had adequate plasma zinc concentrations.

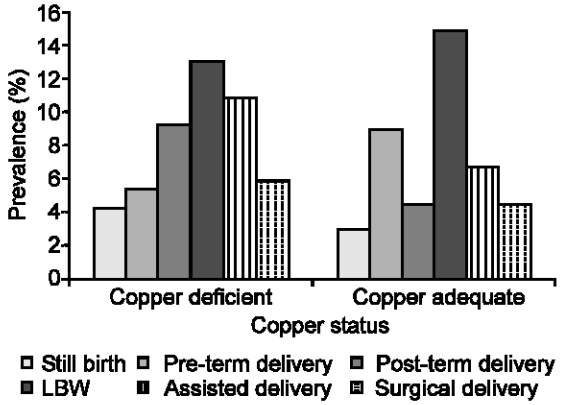

Fig. 3: Foetal outcomes relative to maternal copper status. LBW: Low Birth Weight

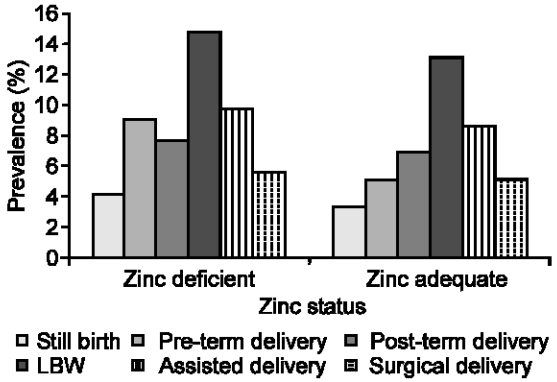

Fig. 4: Foetal outcomes relative to maternal zinc status. LBW: Low Birth Weight

Plasma copper status was found to have non-significant effect on foetal outcome (Fig. 3). Although while lower proportions of pre-term and LBW infants were delivered by copper deficient pregnant women, higher proportions of post-term infants, still birth infants and infants delivered through instrument assisted/or caesarean sections were recorded in women who were not copper deficient, although these were found to be statistically non-significant.

From Fig. 4, both zinc-deficient and zinc-adequate pregnant women had comparable foetal outcomes, although non-significantly (p>0.05) higher proportions of infants with these outcomes were delivered by zinc deficient mothers when compared with zinc-adequate mothers.

DISCUSSION

Pregnancy has been associated with increased demands of all nutrients including copper and zinc and deficiency of any of these could affect the course and outcomes of pregnancy (Upadhyaya *et al*., 2004). Although no study has identified the role of copper

deficiency in pregnancy-induced hypertension, significantly higher prevalence of hypertension in pregnant women deficient in copper when compared to their copper-adequate counterparts in the present study needs further investigation.

While copper-containing protein, ceruloplasmin has been found to promote LDL oxidation *in vitro* (Fox *et al*., 2000) leading some researchers to propose that increased plasma copper rather than deficiency could increase coronary heart disease, another cuproenzyme, Superoxide Dismutase (SOD) and ceruloplasmin possesses antioxidant activities, leading experts to believe that copper deficiency rather than excess copper increased the risk of CHD (Jones *et al*., 1997). However, both epidemiological (Malek *et al*., 2006; Leone *et al*., 2006) and experimental studies (Turley *et al*., 2000; Rock *et al*., 2000) have failed to unequivocally confirm the role of copper in the aetiology of pregnancy-induced hypertension.

Zinc is an important trace element involved in a number of metabolic reactions where they acts as cofactor and deficiency of zinc has been associated with many diseases. However, in the present study, the prevalence of both URTI and malaria were found to be higher in zinc-adequate than in zinc-deficient pregnant women. This is in contrast to the role of zinc in the maintenance of the immune system (Baum *et al*., 2000). Studies have shown that zinc supplementation reduced the incidence of respiratory tract infections such as pneumonia (Bhutta *et al*., 1999), diarrhoea (Fischer and Black, 2007) and malaria (Black, 1998) in children. However, a randomized controlled trial in over 42,000 children showed that zinc supplementation did not significantly reduce the mortality associated with malaria and other infectious diseases (Sazawal *et al*., 2007). Nevertheless, the role of zinc in the prevention of malaria and URTI during pregnancy is yet to be ascertained.

The prevalence of Diabetes Mellitus (D/M) was also found to be significantly ($p<0.05$) higher in zinc-deficient than in zinc-adequate pregnant women. Although results from micronutrients (including vitamins and minerals) studies in diabetes mellitus have been conflicting (O'Connel, 2001; Bo *et al*., 2008; Hussain *et al*., 2009) and data is scarce on the study of zinc metabolism during pregnancy complicated with diabetes mellitus, the present finding requires further investigations. However, *in vitro* studies have found that zinc enhances the effectiveness of insulin in non-insulin dependent D/M (Arquilla *et al*., 1978). Again, the development of glucose intolerance in animals deprived of zinc together with the occurrence of zinc deficiency in type 2 D/M suggests a role for zinc deficiency in the pathogenesis of gestational D/M.

Comparable foetal outcomes observed in pregnant women deficient in copper and zinc in the present study contrasts several studies where independent deficiency of copper or zinc was associated with adverse pregnancy outcomes. The reason for the present observation is not clear but it may not be unconnected to the fact that the deficiencies were of the mild type. In the presence of such mild deficiencies, the foetal plasma copper and zinc levels as well as their functions may not be affected as these may be maintained at the expense of maternal plasma levels (Balai *et al*., 1992).

Therefore it is concluded that maternal trace elements status was associated with maternal morbidity such as hypertension (copper), infections and D/M (zinc), without a significant effect on foetal outcomes. The reasons for the non-significant impact on foetal outcomes of these trace elements deficiencies in the presence of significant effect on maternal outcomes need to be investigated further.

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The Level of Heavy Metals in Selected Vegetables Crops Collected from Baghdad City Markets

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Abstract: Nineteen raw vegetable crops were collected from major markets in Baghdad areas uncontaminated by human activities other than normal agricultural practice. Six hundred samples were prepared and analyzed under carefully controlled conditions for cadmium, lead, iron, copper and Zinc. The Levels of these Heavy Metals in the vegetables were relatively low with respect to the proposed maximum acceptable concentrations for human consumption. In lettuce, spinach and parsley relatively high level of lead and cadmium were noticed compared with tomato, eggplant and onion. Iron was the only element that showed statistical variation among the different of vegetables.

Key words: Heavy metals, vegetable, crops, dietary toxicity, nitric acid, perichoric acid, atomic absorption spectrophotometer

INTRODUCTION

In recent years much public interest focused on the subject of environmental contamination with toxic elements such as cadmium and lead. Intake of relatively low dose of these elements over a long period of time can lead to malfunction of certain organs such as kidney and chronic toxicity to human (Pier and Bang, 1980; Sherlock and Walter, 1983). Many of these metals have contaminated water, soil and entered the food chain (Wolink and Fricke, 1985; Reilly, 1991; Sanchez-Camazao *et al*., 1994). Cadmium and lead partially ingested with edible parts of agricultural and horticultural crops or derived products (Reilly, 1991; Sanchez-Camazao *et al*., 1994). Application of fertilizer and sewage sludge increase the level of Pb and Cd in crops (Wong, 1996). Vegetables constitute essential component of the diet. However, these plants may contain both essential and toxic elements, such as heavy metals, at a wide range of concentrations (Afshin and Masoud, 2008).

In a number of countries a survey of trace metals in various crops have been carried out e.g. the major agricultural crops of united state of America were analyzed for Pb and Cd. These extensive studies lead to established a maximum acceptable levels of heavy metals in edible parts of crops or processed food in several countries (Wolink and Fricke, 1985; Sanchez-Camazao *et al*., 1994; Wong, 1996; Afshin and Masoud, 2008; Khairiah *et al*., 2004; Kursad *et al*., 2002).

Little information is available in Iraq concerning the levels of Heavy Metals in different type of agricultures products. Therefore the aim of this study is to obtain some information concerting the levels of Heavy Metals in vegetables available in Iraqi markets.

Vegetables selected for this study based on the market volume.

MATERIALS AND METHODS

A wide selection of vegetables such as tomato, onion, leafy vegetables…etc were collected from different markets in Baghdad city in sufficient quantities to provide a representative samples. More than 600 samples of different types of vegetables were analyzed. All samples were put in plastic bags to avoid contamination and taken to laboratory for analyses. The edible parts of the vegetables used for human consumption were washed with double distilled water, dried in air oven at 75°C for one to two days until it reached constant weight, then ground in special mills with provision to prevent contamination. About (1 to 2) gram of samples were wet digestion with nitric-per choreic acid according to method (Reilly, 1991). Certain precautions were taken to avoid possible contamination of samples, reagents and equipments during digestion. Concentrations of Pb, Cd, Fe, Cu and Zn were determined using flame atomic absorption spectrophotometer. Acetified standard reference material was used to ensure accuracy. Data processing, mean ($\bar{x}$), standard error ($S\bar{x}$), F-test with critical probability p 0.01 (**) and LSD were determined.

RESULTS AND DISCUSSION

Table 1 and 2 clearly list the concentrations of lead, cadmium, iron, copper and Zn in different types of vegetables. Relatively high concentration of Fe was found in spinach and lettuce. Leafy vegetables accumulate higher concentration of iron than other crops. The levels of iron in nearly all the samples examined were lower or within the proposed maximum level (0.15 mg/kg) fresh weight (Khairiah *et al*., 2004; Kursad *et al*., 2002; Parveen *et al*., 2003). However, tomato and onion contained relatively higher

Table 1: The concentration of Pb, Cd, Fe, Zn and Cu in vegetables (ppm)

	Pb		Cd		Fe		Zn		Cu	
Samples	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$
Okra	0.034	0.005	0.081	0.037	4.873	0.329	0.625	0.042	0.182	0.09
Marrow	0.037	0.024	0.043	0.007	5.259	0.342	0.599	0.051	0.158	0.039
Egg plant	0.026	0.019	0.046	0.005	5.733	0.474	0.576	0.037	0.186	0.03
Radish	0.036	0.025	0.042	0.006	8.788	0.665	0.550	0.033	0.188	0.03
Red radish	0.035	0.024	0.048	0.007	8.113	0.616	0.568	0.043	0.213	0.043
Cucumber	0.030	0.021	0.046	0.005	4.539	0.423	0.667	0.035	0.167	0.033
Tomato	0.024	0.017	0.044	0.007	5.388	0.399	0.519	0.049	0.195	0.039
Pepper	0.031	0.020	0.051	0.006	9.717	0.778	0.545	0.046	0.205	0.050
Green pepper	0.032	0.020	0.046	0.006	12.994	1.209	0.582	0.044	0.200	0.034
Onion	0.028	0.019	0.047	0.007	7.325	0.944	0.525	0.052	0.209	0.045
Calculated F-test	$0.779^{n.s}$		$0.245^{n.s}$		15.767**		$0.783^{n.s}$		$0.190^{n.s}$	
LSD (Last significant difference)	1.429		0.018		1.874		0.127		0.115	

$\bar{x}$ = Mean, S $\bar{x}$ = Standard Error, n.s = not significant. **Significant at 0.01 probability levels

Table 2: The concentration of Pb, Cd, Fe, Zn and Cu leafy vegetables (ppm)

	Pb		Cd		Fe		Zn		Cu	
Samples	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$	$\bar{x}$	S $\bar{x}$
Parsley	0.038	0.003	0.048	0.006	16.50	0.088	0.573	0.060	0.197	0.041
Celery	0.033	0.005	0.043	0.006	15.54	0.626	0.515	0.055	0.204	0.034
White radish	0.032	0.003	0.048	0.006	15.31	0.638	0.595	0.054	0.211	0.053
Leek	0.031	0.003	0.044	0.007	16.47	0.549	0.548	0.052	0.1730	0.029
Mantis	0.035	0.004	0.049	0.007	11.50	0.702	0.594	0.047	0.208	0.038
Beet	0.037	0.004	0.043	0.004	14.15	0.802	0.543	0.037	0.144	0.032
Spinach	0.041	0.004	0.076	0.006	17.66	0.641	0.595	0.045	0.158	0.032
Lettuce	0.031	0.004	0.065	0.004	6.81	0.813	0.533	0.035	0.148	0.025
Green Onion	0.036	0.006	0.046	0.006	9.30	0.645	0.542	0.043	0.209	0.046
Calculation F-Test	$1.134^{n.s}$		$0.550^{n.s}$		26.693**		$0.320^{n.s}$		$0.532^{n.s}$	
LSD (least significant difference)	0.744		1.636		1.977		0.135		0.110	

concentration of iron than that reported in the literatures. A variety of factors may be responsible for such variation e.g. sampling, handling, harvesting.....etc.

Statistical analysis (F-test and LSD) indicated no significant differences between different types of vegetables in their lead concentration.

The level of cadmium varied according to the type of vegetables. In okra, spinach and lettuce the level of cadmium were relatively high. On the other hand, the cadmium level in eggplant, onion and tomato were quiet low.

It has been reported that spinach, lettuce and carrot are likely to accumulate cadmium at higher levels compared to other vegetables (Wong, 1996; Afshin and Masoud, 2008; Radwan and Salama, 2006). Radish and onion do not normally accumulate elevated concentration of cadmium. However; soil which normally contains significant amount of cadmium could be the sources of cadmium in vegetables (Sanchez-Camazao *et al*., 1994; Sherlock and Walter, 1983). It has been reported that soil amended with sewage sludge has higher level of cadmium which subscuntly absorbed by vegetables roots (Sanchez-Camazao *et al*., 1994). Apparently the levels of cadmium found in all the sample of vegetables examined were below the proposed maximum acceptable daily intake (Sherlock and Walter 1983; WHO, 1992). Provisional tolerable human intake of cadmium have been established at 57-71 microgram per day or 1 microgram per day kilogram body weight (Sherlock and Walter, 1983). However, although the levels of cadmium were below the acceptable level (Radwan and Salama, 2006), the effect of chronic low level exposure to cadmium and lead are not well studied in human because of the difficulties in assessing of such exposure accurately.

Statistical analysis (F-test) showed no significant difference between the different types of vegetables. However, LSD showed significant differences only for okra (Table 1) when comparison was made with the other types of vegetables.

Levels of zinc were quite low in all the sample of vegetables examined, even lower than that reported in the literatures (Parveen *et al*., 2003).

A wide variation between the types of vegetables and the level of iron were observed Table (1-3). Green pepper contained the higher level of iron compared with okra and cucumber which contained the lowest levels.

It is obvious from the results obtained that leafy vegetables that leafy vegetables such as spinach, manties and parsley contained a high levels of iron compared with fruity types (Table 1, 2). Statistical

Table 3: Statistical analysis (F-Test and LSD) of tested metals concentration for the different types of vegetables

Samples	Zn	Fe	Pb	Cd
	($\bar{x}$)			
Parsley	0.573	16.500	0.038	0.048
Opium	0.595	15.540	0.033	0.043
Beet	0.543	14.150	0.037	0.043
Spinach	0.595	17.660	0.033	0.046
Lettuce	0.533	6.810	0.027	0.035
Eggplant	0.576	5.730	0.026	0.046
Tomato	0.599	5.388	0.024	0.044
Cucumber	0.667	5.539	0.030	0.046
Okra	0.625	4.879	0.028	0.081
Onion	0.625	4.873	0.028	0.081
Calculation F-Test	0.903n.s	69.23**	1.10n.s	0.30n.s
LSD (least significant difference)	0.041	0.574	0.005	0.0036

$\bar{x}$ = Mean, S $\bar{x}$ = Standard Error, n.s = not significant. **Significant at 0.01 probability levels

analysis indicated a significant differences (F-test) between the different types of vegetables (Table 1-3). Even such differences were observed between individual samples (LSD). The possible sources of such variation in the levels of the iron are plant it self soil and condition of the crops during harvesting and processing (Afshin and Masoud, 2008; Khairiah *et al*., 2004). Although human are generally well protected from over doses of iron, children age 1-2 years are vulnerable to high level of iron in diet (WHO, 1992).

The levels of copper found in samples of vegetables examined were lower than that reported in the literatures (Sherlock and Walter, 1983; WHO, 1992, 1995; Jarup, 2003). Even some of the samples examined were below the detected limited.

Conclusion: The present study have shown that the levels of heavy metals in the major vegetables crops found in Baghdad are within the acceptable levels, although lead were reported high in some of the samples examined especially in leafy vegetables. It is Therefore, suggested that regular monitoring of Heavy Metals in plant tissues is essential in order to prevent excessive build-up of these metals in the human food chain.

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Optimized Conditions of Steeping and Germination and Their Effect on Sorghum [*Sorghum bicolor (L.) Moench*] Composition

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Abstract: The work consisted in optimizing steeping and germination condition and their effect on sorghum grain in term of malt loss, Soluble Solid (SS) yield, cold paste viscosity, amylase activity, tannin and protein content. The factors studied included steeping time and temperature with temperature and time of germination. Germination significantly affected the increase in malt loss, SS yield, amylase activity and protein content with a decrease in cold paste viscosity and tannin content of sorghum. Optimum conditions for sorghum were: steeping time for 24 h at 31°C and 4.5 d of germination at 30°C. Values predicted at optimum conditions by the response surface model for all responses were experimentally tested and close agreement between experimental and predicted values was observed.

Key words: RSM, sorghum, steeping, malting

INTRODUCTION

Recently, there has been increased interest in sorghum as a gluten-free cereal to substitute the gluten-rich cereals in the diet of people suffering from celiac disease (Elkhalifa *et al*., 2005). The physiological maturity of sorghum grain generally occurs 50 days after anthesis and marks the end of nutrient delivery and the beginning of senescence and caryopsis desiccation (Waniska, 2000). The mature grain is then harvested and stored. In a dormant stage, it is characterized by dehydration and a dramatic decrease of metabolic activity. Germination is induced by rehydration of the seed, which increases both respiration and metabolic activity thus allowing the mobilization of primary and secondary metabolites (Limami *et al*., 2002). Germination induces the synthesis of hydrolytic enzymes. Significant changes occur in seed during germination in biochemical and physical aspects (Obatolu, 2002) and the total nutrition value is improved (Badau *et al*., 2005; Jingjun *et al*., 2010).

Germination is a process used to make malt for the brewing industry, although germinated or malted flour can also be used in bakery products (Selvaraj *et al*., 1986), nonalcoholic drinks and weaning food formulations (Wahed *et al*., 1994; Malleshi *et al*., 1989; Malleshi *et al*., 1986; Marero *et al*., 1988). Flour from germinated seeds has been reported to have better nutritional properties than flours from nongerminated cereals (Finney, 1983; Lorenz, 1980). Supplementary foods made from germinated flours have low viscosity and high nutrient density (Wahed *et al*., 1994) and have acceptable properties to weaning and infants foods in developing countries (Wahed *et al*., 1994; Malleshi *et al*., 1989; Malleshi *et al*., 1986; Marero *et al*., 1988).

RSM is an effective statistical technique for optimizing complex processes. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions with less laborious and time-consuming (Irakoze *et al*., 2010). RSM is widely used in optimizing the extraction process variables, such as polysaccharides, anthocyanins, vitamin E, phenolic compounds and protein from varied materials (Chandrika and Fereidoon, 2005; Lee *et al*., 2005; Li and Fu, 2005; Qiao *et al*., 2009).

The objective of this study was to determine the optimum condition of steeping and germination and their effect on sorghum composition. Since very few reports have been carried out in this area, the present investigation was further conducted by using RSM and the model could develop an equation that could predict the quality of the germinated sorghum flour. This work highlighted the different biochemical modifications that occurred in sorghum grain during steeping and germination and presented an improvement of the nutritional properties of sorghum flour acceptable to brewers and infant foods.

MATERIALS AND METHODS

Sorghum (Sorghum bicolor (L.) Moench) was grown in Shandong, a coastal province East of China and known to have average January temperature of 0°C and July

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28°C. The average annual rainfall is about 500 mm, most of which falls in the summer. Red sorghum was obtained from 2008 and 2009 harvest. The length/breadth ratio of sorghum kernel was 1.12/1.23 and the density (g/l) was 691.40. The average weight of 1000 kernels was 26.80 g.

All the chemicals used were of analytical grade and purchased from Sinopharm Chemicals Reagent Company (SCRC), Shanghai, China.

Germination and preparation of sorghum flour: After removing chaff and unviable grain, sorghum grains (1000 g) were thoroughly cleaned by washing with tap water and then soaked in 0.20 ppm wooden ash water (1:2, w/v) for 24 h at ambient temperature with the soaking water being changed at 8 h interval. After soaking, the grains were evenly spread on jute bags and covered with the same material, in a secluded and dark area and allowed to germinate. The grains were wetted with water at regular interval of 24 h. The Steeping and germination conditions are stated in Table 1. The withered rootless were gently brushed off and dried grain were milled using a bench-top attrition mill (Dade, DFT-600, 25000 rpm, Zhejiang Linda Mechanic Ltd Co, China). The resultant flour was sieved into a particle size of 70-mesh. The flour was then packaged in a low density polyethylene bag and stored using plastic containers with lids in a refrigerator at 4°C for later use.

Analysis of sorghum composition: Malt loss was calculated as the total of leaching, metabolic and vegetative losses as described by Malleshi and Desikachar (1986a,b). Percentage yield or recovery was the proportion of soluble solids in malted sorghum flour. The flour slurry (15 g/L) was prepared by mixing flour with distilled water in a glass beaker, heating to 95°C within 7-10 min, holding at 95°C for 5-10 min then cooling to 30°C. The viscosity of the gruel was measured after cooling the hot porridge to 50°C using a Rapid Visco Analyser (RVA, Brabender, Duisburg, Germany) (Mosha and Svanberg, 1983). Amylase activity was analyzed following the method of Bernfeld (1987). The flour sample was extracted with acetate buffer (pH 4.8) for 1 h at ambient temperature (about 20°C). Amylase activity of the extract was expressed as maltose units and defined as the amount of maltose (mg) released by the action of malt enzyme extracted from 1 g malt flour in acetate buffer (pH 4.8) on soluble starch at 37°C for 30 min (Malleshi and Desikachar, 1986a,b).

Quantitative estimation of tannin, as catechin equivalent, was carried out using the modified vanillin-HCl method of Price *et al.* (1978). Total protein content was measured using AOAC standard methods (AOAC, 1984) and % N was multiplied by a factor of 6.25. The moisture content was determined following the AACC (1991) method (Price, 1991). All chemical and physical analyses were carried out in duplicate and expressed on a dry weight basis unless otherwise stated.

Experimental design and statistical analysis: A central composite second-order design was chosen to determine the influence of four independent variables and the optimum steeping and germination conditions. The effect of the variables, steeping time (X1), steeping temperature (X2), germination time (X3) and germination temperature (X4) in sorghum composition was investigated. Each variable was coded at five levels: -2, -1, 0, 1, 2 (Table 1). The process variables and the responses were defined from published data (Mizubuti *et al.*, 2000; Moure *et al.*, 2002; Quanhang and Caili, 2005).

The quality of the malted sorghum flour needed for lowbulk, high-energy supplementary food formulations was chosen as the dependent variables, namely: malt loss (y1), SS yield (y2), viscosity (y3), amylase activity (y4), tannin (y5) and protein (y6) contents. These variables were expressed individually as a function of the independent variables.

The data were fitted to a Taylor second-order approximating function:

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_1^2 + \sum_{\substack{i=1 \\ i<j}}^{k} B_{ij} X_i X_j \qquad (1)$$

Where Y is the response function, *B*o the centre point of the system, Bi, Bii and Bij represent the coefficients of the linear, quadratic and interactive effects, respectively, and Xi, Xii and XiXj represent the linear, quadratic and interactive effects of the independent variables (steeping time, steeping temperature, germination time and germination temperature), respectively.

Optimization and verification: Optimum processing conditions for steeping time and temperature and germination time and temperature were determined by superimposing the plots for all response variables

Table 1: Process variables and their levels in the four-factor, five-level response surface design

		Coded variables levels				
Variable	Symbol	-2	-1	0	1	2
Steeping time (St) (h)	X1	0	8	16	24	32
Steeping temperatures (ST) (c)	X2	5	15	25	35	45
Germination time (Gt) (d)	X3	0	1.5	3	4.5	6
Germination temperature (GT) (c)	X4	10	20	30	40	50

(Floros and Chinnan, 1988; Henika, 1982; Henika, 1972). The region that fulfils the requirements for formulating low-bulk, high-energy supplementary food, such as low viscosity and tannin and high protein content, was considered and shaded. The optimum germination conditions were selected and used for calculating the predicted properties of germinated sorghum flour using the prediction equations derived by RSM.

Verification of the optimum conditions for germinating sorghum was performed by germinating the seed. The germinated sorghum flour obtained was experimentally analyzed and the results statistically compared to those predicted by the mathematical model.

RESULTS AND DISCUSSION

Fitting the models: The effects of steeping and germination on viscosity, malt loss, enzyme activity, SS yield, tannin and protein contents are shown in Table 2. The independent and dependent variables were fitted to the second order model equation and examined for the goodness of fit. In general, exploration and optimization of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking of the model adequacy (Liyana-Pathirana and Shahidi, 2005). Several indicators were used to evaluate the adequacy of the fitted model. A test for the lack of fit was used wherein a low F-value indicates that the model equation is an adequate approximation to the data. The R^2 values, Coefficients of Variation (CV) and model significance (F-value) were also used to judge the adequacy of the model. The significance of the F-value depends on the number of Degrees of Freedom (DF) in the model (Cai *et al*., 2008; Qiao *et al*., 2009).

The analysis of variance of the effect of germination conditions as a linear term, quadratic term and interaction on the response variables is shown in Table 3. The results indicated that the model is highly adequate because responses have satisfactory levels of R^2, CV and model significance. The SS yield and protein, however, showed a rather high CV and could be due to the experimental region covered in the study. However, the model was highly significant and possesses 88.03% of R^2 for SS yield and 88.57% for protein. Considering the high value of R^2, the model for SS yield and protein can be accepted.

Table 2: Central composite design arrangement and responses for sorghum seed germination process

	Variable levels[b]				Responses[c]					
Run[a]	X1	X2	X3	X4	Y1	Y2	Y3	Y4	Y5	Y6
1	-1(8)	-1(15)	-1(1.5)	-1(20)	2.04	8.55	4.87	2.6	1.39	8.98
2	1(24)	-1(15)	-1(1.5)	-1(20)	3.48	8.56	5.40	3.5	1.4	8.68
3	-1(8)	1(35)	-1(1.5)	-1(20)	2.12	8.44	4.34	2.4	1.43	8.81
4	1(24)	1(35)	-1(1.5)	-1(20)	4.06	16.41	8.45	4.2	1.22	16.80
5	-1(8)	-1(15)	1(4.5)	-1(20)	2.45	9.39	3.50	2.6	1.17	9.63
6	1(24)	-1(15)	1(4.5)	-1(20)	4.23	16.70	4.60	4.2	1.24	16.48
7	-1(8)	1(35)	1(4.5)	-1(20)	5.24	21.80	12.76	5.7	1.36	21.27
8	1(24)	1(35)	1(4.5)	-1(20)	6.69	26.10	12.94	6.8	1.22	26.38
9	-1(8)	-1(15)	-1(1.5)	1(40)	2.52	9.50	4.60	2.1	1.37	9.82
10	1(24)	-1(15)	-1(1.5)	1(40)	2.57	8.81	4.20	2.5	1.44	8.77
11	-1(8)	1(15)	-1(1.5)	1(40)	2.56	8.75	4.10	2.9	1.32	8.55
12	1(24)	1(35)	-1(1.5)	1(40)	4.56	17.86	8.80	4.7	1.15	17.65
13	-1(8)	-1(15)	1(4.5)	1(40)	5.67	23.90	20.55	5.2	1.09	23.51
14	1(24)	-1(15)	1(4.5)	1(40)	8.25	32.50	20.85	8.8	1.25	32.70
15	-1(8)	1(35)	1(4.5)	1(40)	9.69	39.30	22.24	9.3	1.26	39.69
16	1(24)	1(35)	1(4.5)	1(40)	14.46	56.80	34.34	14.3	1.15	56.93
17	-2(0)	0(25)	0(3)	0(30)	2.67	10.16	4.40	2.3	1.29	10.37
18	2(32)	0(25)	0(3)	0(30)	4.68	18.40	8.56	4.7	1.24	18.31
19	0(16)	-2(5)	0(3)	0(30)	8.93	34.40	12.56	8.5	1.23	34.07
20	0(16)	2(45)	0(3)	0(30)	10.37	41.20	20.65	10.7	1.22	41.4
21	0(16)	0(25)	-2(0)	0(30)	1.47	6.80	4.40	1.6	1.46	6.53
22	0(16)	0(25)	2(6)	0(30)	8.52	33.20	36.78	8.5	1.2	33.16
23	0(16)	0(25)	0(3)	-2(10)	2.35	9.60	4.43	2.6	1.37	9.57
24	0(16)	0(25)	0(3)	2(50)	4.47	20.70	11.42	4.5	1.21	19.52
25	0(16)	0(25)	0(3)	0(30)	2.56	9.10	4.50	2.4	1.39	9.64
26	0(16)	0(25)	0(3)	0(30)	2.22	8.40	4.80	2.2	1.41	8.34
27	0(16)	0(25)	0(3)	0(30)	2.68	10.81	4.50	2.4	1.4	10.61
28	0(16)	0(25)	0(3)	0(30)	5.47	25.83	8.50	5.5	1.41	25.36
29	0(16)	0(25)	0(3)	0(30)	5.76	22.84	9.21	5.4	1.39	22.56
30	0(16)	0(25)	0(3)	0(30)	5.93	23.75	8.56	5.4	1.41	23.60

[a]Experimental runs were performed in random order. [b]Based on coded variables. [c]y_1 = malt loss (%); y_2 = SS yield (%); y_3 = cold paste viscosity (poise); y_4 = amylase activity (maltose unit); y_5 = tannin (µg/g); y_6 = protein (%)

Table 3: Analyses of variance on the effect of germination conditions (Xk) as linear, quadratic and interaction (cross product) terms on the response variables (Yk)

		Sum of squares					
		Malt loss	SS Yield	Viscosity	Amylase	Tannin	Protein
Source	Degree of freedom	Y1	Y2	Y3	Y4	Y5	Y6
Model	14	0.49***	3908.79***	12.61***	244.14**	0.31***	3922.60***
Linear	4	0.37***	2544.18***	8.62***	147.64***	0.13**	2511.60***
Quadratic	4	0.09**	780.11***	1.79***	58.12***	0.085*	795.07***
Cross product	8	0.026	166.59	0.55	8.73	0.00348	168.60
Residual	7	0.073	364.72	0.76	15.09	0.0045	337.99
Lack of fit	10	0.026	199.26	0.68	9.37	0.0035	192.23
Pure error	5	0.073	332.05	0.63	14.45	0.00483	313.77
R squares		83.14	88.03	90.63	93.89	98.71	88.57
CV (%)		19.97	30.34	13.98	25.46	1.26	29.65

*Significant at 10% level: **Significant at 5% level; ***Significant at 1% level.

$Y_1 = 1/y1^{1/2}$; $Y_2 = y2$; $Y_3 = \ln y3$; $Y_4 = \ln y4$; $Y_5 = y5$; $Y_6 = y6$

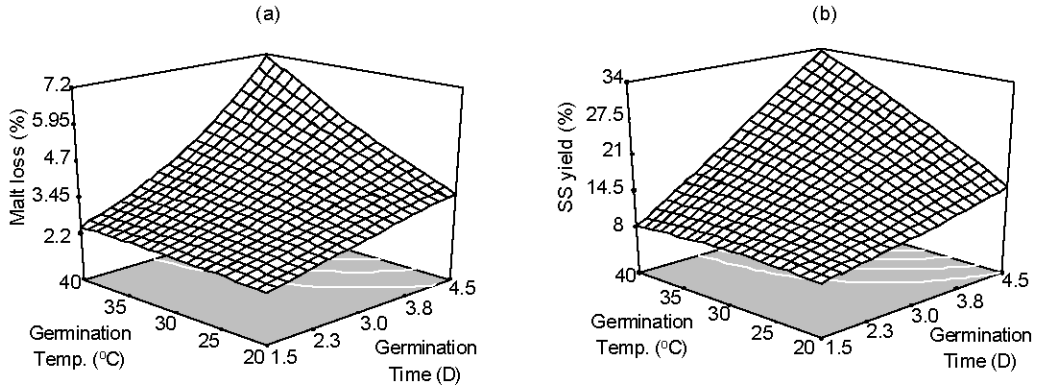

Fig. 1: Response surface plots of the effect of germination conditions on malting loss and SS yield of sorghum

Malting loss and SS yield: The viability of germination can be realized if high SS yield and low malting loss can be maintained. Malting loss includes losses due to leaching of solids during steeping and losses due to increased metabolic growth during germination. Increase in malting loss may subsequently decrease the level of water soluble nutrients in the germinated flour. However, during seed germination, adequate moisture must be attained to hasten metabolic development of the roots and shoots.

The seed should attain suitable moisture content by steeping for the optimum time and temperature. The effects of steeping and germination conditions on malting loss (Y1) are shown in Table 4 and 5. The mathematical model clearly suggests that any increase in germination time and temperature will significantly increase malting loss (Fig. 1a).

The germination conditions that affected the SS yield (Y2) are presented in Table 4 and 5. Steeping time and temperature, as well as germination time and temperature, significantly influenced the SS yield. There was no significant interaction between the independent variables.

The regression coefficient of the model suggested that any increase in germination time (bk3 = 8.02) will increase the SS yield. The significant quadratic and linear effect of germination time (bk33 = 4.73) further accelerated the increase in SS yield. These findings clearly support the hypothesis that the development of roots and shoots contributes soluble sugars from starch and protein. In general, during malting the reserves of nutrients like starch and protein are respectively degraded to soluble sugars and amino acids to meet the seedling requirements during the germination process. Depression of starch and protein degradations indicated the interference with metabolic systems operating on reserve starch and protein, mainly enzymes such as amylases and proteases acting in malting system (Dalvi, 1974). Fig. 1b illustrates the influence of the process variables on the SS yield of malted sorghum flour.

Table 4: Regression coefficients of the second-order polynomials showing the relationship between response variables (Yk) and independent variables (Xk)

	Malt loss	SS Yield	Viscosity	Amylase	Tannin	Protein
Coefficients	Y1	Y2	Y3	Y4	Y5	Y6
Bk0	0.53***	16.7883***	1.8473***	3.8833***	1.4016***	16.685***
Bk1	-0.048**	2.9412**	0.1462*	0.875***	-0.0175***	2.9170**
Bk2	-0.031*	3.7979***	0.2023***	0.9666***	-0.0108***	3.8404***
Bk3	-0.1***	8.0170***	0.4755***	1.9083***	-0.0625***	7.9912***
Bk4	-0.043**	4.3195***	0.2659***	0.9***	-0.03***	4.1870***
Bk11	-0.0038	1.4781	0.0986	0.2	-0.0587***	1.5468
Bk21	0.012	1.3318	-0.0441	0.4	0.0175***	1.4156
Bk22	0.017	0.9331	0.0040	0.3375	0.0137***	0.9268
Bk31	-0.017	2.8418*	0.1217	0.7375**	0.045***	2.8993*
Bk32	0.0046	1.1531	-0.0911	0.4	-0.0187***	1.1581
Bk33	-0.033	4.7218***	0.3211***	1.175***	-0.005***	4.8468***
Bk41	0.0029	-0.9974	-0.0331	-0.1479	-0.0339***	-0.9113
Bk42	-0.051***	4.8826***	0.2080***	1.3770***	-0.0439***	4.9373***
Bk43	0.0150	0.4326	0.1490**	0.2395	-0.0177***	0.4648
Bk44	0.0093	-0.7799	0.0037	-0.1354	-0.0277***	-0.8601

*Significant at 10% level: **Significant at 5% level; ***Significant at 1% level. Y1 = 1/y1 ½; Y2 = y2; Y3 = ln y3; Y4 = ln y4; Y5 = y5; Y6 = y6.

Table 5: Analysis of variance showing the significance of the overall effect of germination conditions (Xk) on each of the response variables (Yk)

			Sum of squares					
			Malt loss	SS Yield	Viscosity	Amylase	Tannin	Protein
Germination condition	Code	DF	Y1	Y2	Y3	Y4	Y5	Y6
Steeping time (St) (h)	X1	14	0.0550**	207.6228**	0.5132**	18.375***	0.0073***	204.225**
Steeping temperatures (ST) (c)	X2	14	0.0232*	346.1801***	0.9830***	22.4266***	0.0028***	353.9712***
Germination time (d)	X3	14	0.2459***	1542.567***	5.4265***	87.4016***	0.0937***	1532.642***
Germination temperature (Gt) (GT) (c)	X4	14	0.0439**	447.8112***	1.6972***	19.44***	0.0216***	420.76****

Significant at 10% level: **Significant at 5% level; ***Significant at 1% level. $Y_1 = 1/y1^{1/2}$; $Y_2 = y2$; $Y_3 = \ln y3$; $Y_4 = \ln y4$; $Y_5 = y5$; $Y_6 = y6$. DF = Degree of Freedom

Viscosity and amylase activity: Germination is one of the methods that can be used to reduce the viscosity of the grain extracts (Wahed *et al*., 1994; Malleshi *et al*., 1989; Malleshi *et al*., 1986; Marero *et al*., 1988). During germination, the starch is degraded by the action of enzymes present in the seed. Amylases break down the amylose and amylopectin components of the starch producing smaller dextrins, maltose and glucose (Bewley and Black, 1985; Allen and Spradlin, 1974), thus reducing the viscosity. A good correlation between viscosity or falling number and amylase activities in cereals has been reported (Raschke *et al*., 1995). The factors affecting the viscosity of malted sorghum flour during germination were investigated and are presented in a model equation (Y3) in Table 4. The coefficients of determination indicated a strong dependence of viscosity to the linear and quadratic terms. The viscosity decreased significantly by time and temperature of germination increased. Likewise, increase in steeping time contributed to a decrease in cold paste viscosity. Significant linear effects were contributed by germination time (bk33 = 0.3211). Analysis of variance shown in Table 5 further validates this result and can be clearly seen in Fig. 2a.

The relationship between amylase activity and the factors that contribute to its change during germination are presented in Table 4. The Ln transformation of the model equation (Y4) revealed that not only the time and temperature of steeping with germination contributed significantly to the increase in enzyme activity, but also the linear terms of germination time (bk33 = 1.175). Thus enzyme activity increased dramatically and alpha- with ß-amylase require sufficient moisture and temperature to hydrolyze starch in the sorghum grain. The ranges of steeping and germination temperatures to which the sorghum seeds have been subjected favorably increase their enzyme activity at different rates. Similar observations were reported in germinated millets (Malleshi and Desikachar, 1986a,b), kaffircorn (Morrall *et al*., 1986, Novellie, 1962), barley (Pal *et al*., 1976) and wheat (Reddy *et al*., 1984). The relationship of the independent variables to amylase activity is shown in Fig. 2b.

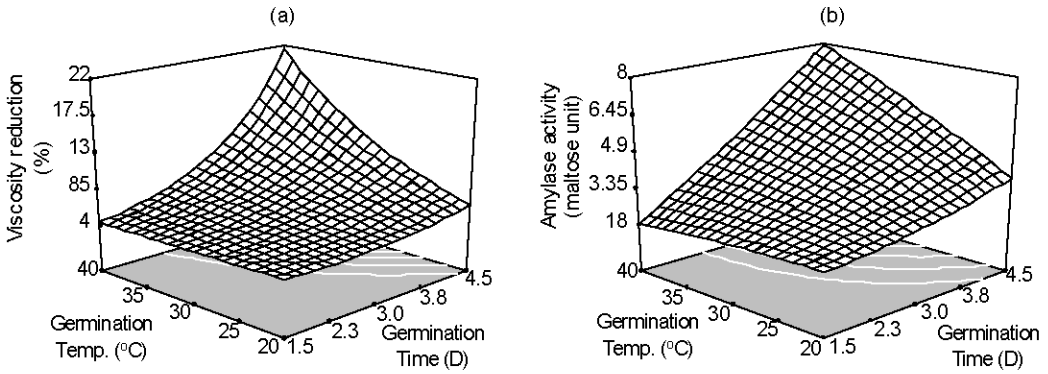

Fig. 2: Response surface plots of the effect of germination conditions on cold paste viscosity and amylase activity of sorghum

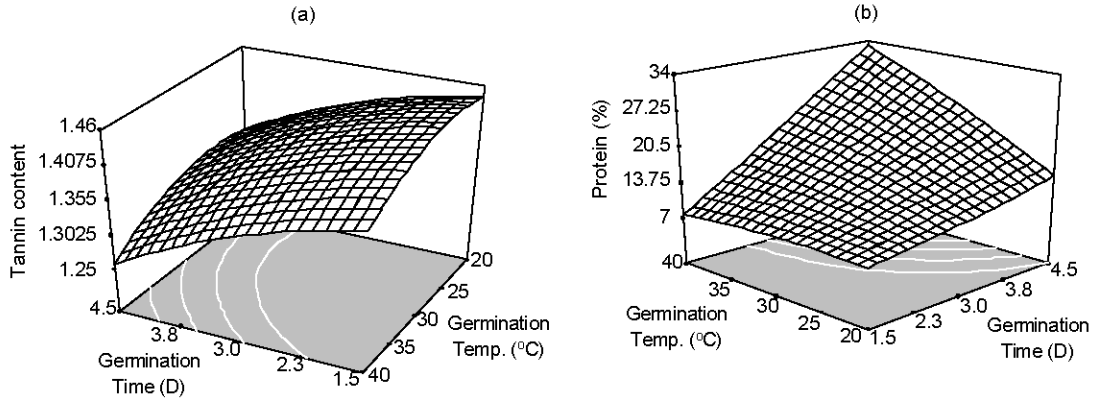

Fig. 3: Response surface plots of the effect of germination conditions on tannin (µm/g) and protein content of sorghum

Tannin and protein contents: Tannins are located in the seed coat (Jambunathan and Mertz, 1973) and are reported to form complexes with hydrolytic enzymes and to inactivate them (Milic *et al*., 1972). The changes in the seed coat permeability may be much greater and rapid thus allowing higher solid losses. Part of the tannins may enter into the endosperm along with the imbibed water. Such tannins are likely to form complexes with reserve seed protein and enzymes and to inactivate them (Price *et al*., 1978). The loss of tannins, therefore, can be attributed to leaching of tannins into the growth medium during malting (Capanzana and Malleshi, 1989). During germination sorghum seed is steeped in water which may decrease some water soluble nutrients, including tannin. The influence of this operation was investigated and the result of the mathematical model and analysis of variance showing the effect of process variables are presented in Table 4 and 5, respectively.

The regression coefficients (Y5) indicated that increasing in germination time (bk3 = -0.0625) and temperature (bk4 = -0.03) subsequently decreased the tannin content. Steeping time and temperature showed also a significant effect (Table 5). Interaction effects between independent variables were noted and implied that the effect of one variable depends on the specific levels of other variables. From these findings it can be concluded that steeping and germination can be used as a process for reducing the level of tannin. The decrease in viscosity and tannin content after stepping and germination of sorghum flour can be considered as an advantage, particularly if the flour is to be used for formulating supplementary weaning food. Fig. 3a illustrates the effect of the independent variables on the tannin content of sorghum flour.

The protein content of sorghum flour during germination is shown in Fig. 3b. Germination time and temperature and steeping temperature influenced the amount of protein present in the germinated sorghum. This is further confirmed in the analysis of variance presented in Table 5. Several researchers reported improvement in the protein quantity as well as quality during germination of maize (Tsai *et al*., 1975), millet (Malleshi *et al*., 1986), sorghum (Obizoba, 1988) and wheat (Dalby and Tsai, 1976).

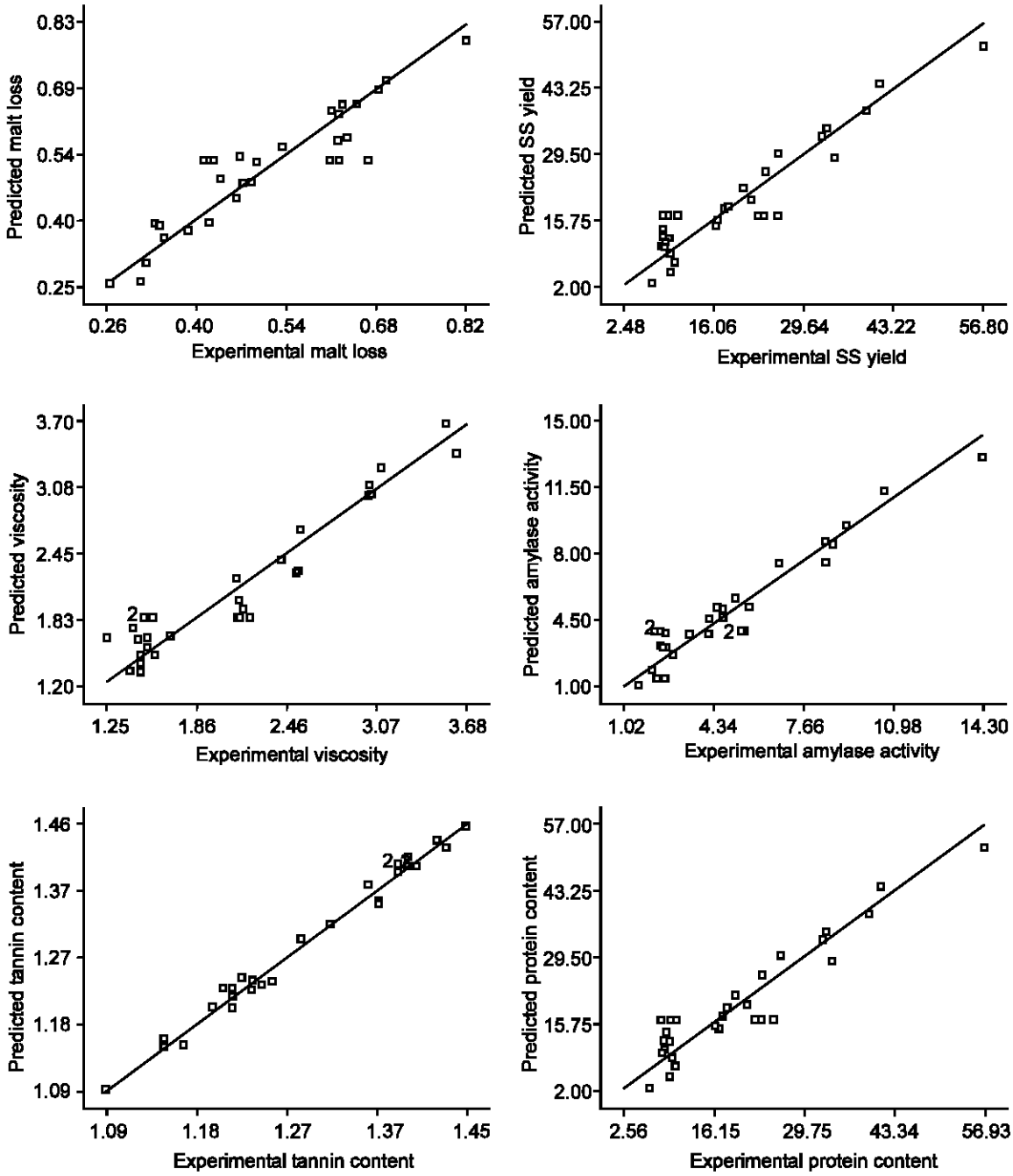

Fig. 4: Response trace plots of experimental data against predicted values of malt loss; SS yield; cold paste viscosity; amylase activity; tannin content and protein

Adequacy of the model: The coefficient of determination (R^2) was calculated to examine the amount of the variation in the response that is explained by the model. A relatively high R^2 value is a sign that the regression model can be used with confidence for the purpose of the predicting response values (Hu, 1999). RSM optimization approach was also used to validate experimentally and predict the values of the responses using the model equation. The experimental and predicted values were within the range and were found to be not statistically different at 5% level of significance (Table 6).

Furthermore, we plotted the experimental data against the predicted values by the model (Fig. 4). Overall, the points are scattered favorably around the straight line, which indicates that the model fits the data. Thus, the

Table 6: Predicted and experimental values of the responses at optimum conditions

		Experimental value[a]		
Response variable	Code	Predicted value	Mean	Range
Malt loss (%)	Y1	6.84	7.96±0.79	1.47-14.46
Yield (%)	Y2	34.28	31.8±1.75	6.8-56.8
Viscosity (poise)	Y3	14.03	17.26±2.28	33.11-15.9
Amylase activity (maltose unit)	Y4	8.59	7.95±0.45	1.6-14.3
Tannin (%)	Y5	1.17	1.27±0.07	1.09-1.46
Protein	Y6	34.19	31.73±1.73	6.53-56.93

[a]Mean value of five determinations. Optimum conditions are: steeping time, 24 h; steeping temperature, 35°C; germination time 30 h germination temperature, 40°C. $Y_1 = 1/y1^{1/2}$; $Y_2 = y2$; $Y_3 = \ln y3$; $Y_4 = \ln y4$; $Y_5 = y5$; $Y_6 = y6$

model can be used to predict the quality of the germinated sorghum flour and can be applied between a steeping time of 0-32 h, steeping temperatures of 5-45°C, germination time of 0-6 d and germination temperature of 10-50°C.

Conclusion: Introduction of germination in the processing of sorghum brought significant improvements in the nutritional quality and functional properties of malted sorghum flour. Germination induces important desirable nutritional modifications, and the low level of tannin achieved would make the flour suitable as a carrier for micronutrient fortification. The model equation developed can be used for predicting the quality of malted sorghum flour. The optimum germination conditions for sorghum suitable for supplementary food formulations with low bulk but with high nutrient density were established to be steeping for 24 h at 31°C with 4.5 d of germination at 30°C.

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The Effect of Green and Oolong Tea Extracts Supplementation on Body Composition in Wrestlers

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Abstract: We examined the effect of both Green and Oolong Tea Extracts (GTE, OTE) with different Epigallocatechin Gallate (EGCG) and Caffeine (C) levels on body weight and composition in Wrestlers (W). A randomized, controlled trial was conducted. 30 male wrestlers were randomly assigned to 1 of 3 treatments group: GTE, OTE, or Placebo (P). Athletes daily ingested 509.9 mg EGCG, 36.9 mg C and 172.0 mg EGCG, 138.2 mg C from GTE and OTE, respectively. After 6 weeks supplementation period the athletes lost mean 0.6±0.1 kg (0.8%); $p<0.05$ and 1.4±0.3 kg (1.6%); $p<0.01$ their body weight in GTE and OTE group respectively. Body weight loss in both GTE and OTE group was primarily due to absolute fat loss, but only in the OTE group this result was significant (GTE: 1.3±0.6 kg, 8.6%; OTE: 1.9±0.3 kg, 8.0%; $p<0.05$). No significant differences in mean energy balance values were observed after supplementation period in groups ingested GTE, OTE or P. We conclude that for wrestlers during the training period, for healthy body weight reduction, accompanied with fat mass loss, it seems reasonable to recommend supplementation of 2.4 g/day of OTE where EGCG content is similar to C.

Key words: Body weight, fat mass, green tea extract, oolong tea extract, wrestlers

INTRODUCTION

It has been established that many athletes from weight-sensitive disciplines (e.g. wrestlers, boxers, judokas and rowers) strive for a rapid weight loss just before weight-in time to qualify for a lower weight division (Oppliger *et al*., 2003; Rankin, 2002). The major motivation for those athletes to lose weight is the hope of achieving greater success by competing in a lower weight class (Oppliger *et al*., 2003; Fogelholm *et al*., 1993). Traditionally many athletes from those disciplines have used aggressive methods such as food restriction, dehydration, vomiting, diuretics and exercise in thermal environments to accomplish weight loss (Rankin, 2002; Wagner, 1996; Horswill *et al*., 1990). Athletes who use drastic food or fluid restriction to lose weight may experience negative consequences, including loss of lean tissue accompanied by a decrease in metabolic rate and hormonal disturbances (Rankin, 2002; Horswill *et al*., 1990). Maffulli (1992); Dale and Landers (1999) indicated that rapid weight loss was strongly associated with reduced physical performance of athletes, e.g. in wrestlers was connected with reduced strength endurance and anaerobic capacity or muscle weakness, thermoregulatory problems, renal system dysfunctions and blood pressure abnormalities. The study conducted by Fogelholm *et al*. (1993) on wrestlers also confirmed that rapid weight loss was associated with an increased risk of stress fractures and impaired physical performance. Moreover Tsai *et al*. (2009) demonstrated that prolonged intensive training and rapid weight reduction significantly increased of upper respiratory tract infections incidence-URTI (by suppressed mucosal immunity) after competition in elite male Taiwanese Taekwondo.

In this context, new dietary supplements have been searched for, which could enable athletes to promote the body fat loss while maintaining the content of fat-free mass. During the last decade, numerous studies have focused on the identification of food supplements of plant origin, which would support a reduction of body weight. Based on the research data such benefits seem to be found in a non-fermented green tea and/or partially-fermented oolong tea (Cabrera *et al*., 2006; McKay and Blumberg, 2002; Sato and Miyata, 2000; Yang and Landau, 2000). Green and oolong tea, widely consumed by the Japanese and Chinese population as one of the most popular and traditional non-alcoholic beverages, may retain a considerable amount of the original bioactive compounds, such as caffeine and catechines, in particular Epicatechin (EC), Epicatechin Gallate (ECG), Epigallocatechin (EGC) and Epigallocatechin Gallate (EGCG) (Graham, 1992; Zheng *et al*., 2004). Moreover, obese people are seldom found in populations of long term tea drinking individuals group (Wu *et al*., 2003). Chen *et al*. (1998) observed that Chinese women who drank four cups of 2 g Oolong tea infusion per day lost over a kilogram of body weight during a six-week period. In turn, several reports have

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shown that the administration of polyphenols resulted in a significant reduction in body weight gain and body fat accumulation induced by high-fat diet in both animal and human subjects (Hsu *et al*., 2006, Han *et al*., 1999; Bose *et al*., 2008; Ito *et al*., 2008; Yang *et al*., 2001). For example, the results of the study conducted by Hsu *et al*. (2006) indicated that polyphenol-enriched oolong tea could increase lipid excretion into feces when subjects consumed a high-fat diet. Other research showed that oolong tea prevented body weight increase and parametrial adipose tissue in mice fed with a diet containing 40% beef tallow for 10 weeks (Han *et al*., 1999). Bose *et al*. (2008) indicated that long-term EGCG treatment attenuated the development of obesity in mice fed a high-fat diet (60% energy from fat) as shown in the supplementation with dietary EGCG (3.2 g/kg diet) for 16 weeks.Only Hsu *et al*. (2008) showed in a double-blind, placebo-controlled one-year clinical trial conducted at a Taipei Hospital no statistical differences in percentage reduction in body weight, BMI and waist circumflex between treated GTE (491 mg catechins containing 302 mg EGCG) vs. placebo. It was showed that dietary GTE supplementation, combined with regular exercise, stimulates fat reduction and attenuates obesity induced by a high-fat diet in mice (Shimotoyodome *et al*., 2005). In spite of great interest, there is scant information concerning the effect of tea extract supplementation on body weight and body fat mass in non-obese subjects obligated to control body fat content as, for example, professional athletes from "weight sensitive" disciplines. Therefore we examined the effect of GTE and OTE with different EGCG and C levels on body weight and its composition in wrestlers.

MATERIALS AND METHODS

Subjects: A total of 35 Greco-Roman wrestlers aged between 18-24 years were recruited for the present study from the "Sobiesky Poznan" wrestling team. All of the athletes competed at the national level. They were subjected to medical examination and were screened for supplement use and the length of their training period. This process resulted in the selection of 30 wrestlers who were in good health, not using medication/supplements decreasing their body mass. The experiment was performed during the preseason conditioning program. Before the supplementation period athletes were matched for age, body weight, height and percentage fat mass. The enrolled subjects were randomly allocated to one of the three treatments: a total of 10 wrestlers were supplemented with GTE, 10 with OTE and 10 athletes received P for 6 weeks. A random numbers were generated by the computer for each subject. All of the subjects gave their written information consent. The study was approved by the head of wrestling coaches and the Poznan Medical Ethics Committee.

Experimental design: The subjects were asked to take two capsules three times per day for 6 weeks. Each capsule (400 mg) containing 60% of GTE or 40% OTE or 100% cellulose as placebo. The capsules were taken 30 min after meals (breakfast, lunch and dinner). The GTE and OTE and P capsules were indistinguishable in color, size and appearance. During the supplementation period athletes had a regular diet containing 55% calories from carbohydrates, 25% form lipids and 15% from proteins. Athletes received guidelines from a dietician, listing allowed and prohibited foods with recommended serving sizes and possible combinations. Athletes reported to the laboratory on 4 occasions-baseline (week 0), 2, 4 and 6 week, when they were weighed only in essential clothing. Body composition was determined using bioelectric impedance during each visit. Measurements at baseline and up to week 6 were taken at approximately the same time and day of the week and by the same researcher.

Preparation of sample and treatment: The GTE was obtained by water extraction from dry tea leaves of unfermented *Camellia sinensis*, according to the standard procedures with certificate of analysis given and commercially prepared in capsular form. The manufacturer was Olimp Green Tea®, Sportatut, Debica, Poland. The OTE was obtained by water extraction from dry leaves of semifermented *Camellia sinensis* (Oolong Formosa tea, purchased from Akso® Krakow Company from Poland), according to the standard procedures with certificate of analysis given and commercially prepared in capsular form by the Synteza®, Poznan, Poland. The placebo given to the control group comprised cellulose. GTE in two capsules-800 mg contained 16.4 mg of C and 226.6 mg of EGCG, in this supplement the level of C was lower than EGCG. OTE in two capsules (800 mg) contained 46.0 mg of C and 57.2 mg of EGCG-in this supplement C and EGCG contents were similar. Placebo in two capsules (800 mg) contained cellulose. Consequently, the ingestion of capsules containing the green or oolong tea extract provides daily a total 49.2 mg and 679.8 mg; 138.0 mg and 171.6 mg of C and EGCG respectively. Extraction and HPLC analysis were performed according to the standard procedures (Gramza and Regula, 2007; Zuo *et al*., 2002). Greater than 15% of the products were randomly tested and their components were differed by < 5%. Results of HPLC analysis of each treatment are presented in Table 1.

Measurements: Height of the subjects was measured by using anthropometer (model WPT 200.0 by Rad Wag, Poland), at the time entry into the study. Body weight was measured with an electronic scale (model WPT 200.0 by Rad Wag, Poland and weighing accuracy of 0.1 kg) morning, before breakfast and after defecated, in

Table 1: Components of polyphenols and caffeine in GTE and OTE (400 mg each capsule)

	mg (% weight)		
Components (mg)	Placebo (P)	Green Tea Extracts (GTE)	Oolong Tea Extracts (OTE)
EGCG	0	113.3 (28.3)	28.6 (7.2)
Caffeine	0	8.2 (2.1)	23.0 (5.8)
Other polyphenols substances*	0	118.4 (29.6)	148.0 (37.0)
Cellulose	400.0 (100.0)	160.0 (40.0)	200.0 (50.0)

*Rest of green and oolong tea extract components comprised: catechines fraction, phenolic acids, polymerized polyphenols etc

subjects dressed in their underwear only. Body composition in terms of the adipose tissue (FM) and lean body mass (FFM) was determined immediately after body weight measurements, by the bioelectric impedance technique using a BIA 101S, (AKERN–RJL) bioanalyser, according to recommendations by Lukaski (1987). The Total Energy Expenditure (TEE) was evaluated by the HR-Flex method (Jeszka *et al*., 2001). The TEE in athletes was measured twice in each weeks of treatment period. The evaluation of energy, macro and micro nutrients intake was carried out by 24-h dietary recalled with the use of "Album of Photographs of Food Products and Dishes". The energy value of individually Daily Food Rations (DFR) was estimated with the use of "Dietetyk" software. TEE and net energy balance were approximated and monitored throughout the training.

Statistical analysis: All data were analyzed using StatSoft Software (Version 8.0). Differences in changes of body weight, percentage of fat mass and energy balance over time and between the treatments (GTE, OTE and P) were determined using two-factors ANOVA with repeated measures. If a significant F ratio was obtained, Tukey's HSD was used to locate differences between means. The t-test was used to test differences in means (between baseline in each of treatment group) between baseline and end of treatment in GTE; OTE and P. Statistical significance was set at 0.05.

RESULTS

The characteristics of the subjects before the intervention are presented in Table 2. The variables used to match subjects were not significantly different and thus balanced treatment groups were achieved. By week 6, treatment resulted in a significant total weight loss of 0.6±0.1 kg (0.8%) in the GTE group ($p<0.05$) and of 1.4±0.3 kg (1.6%) in the OTE group ($p<0.01$). In the P group after a 6 week supplementation period body weight decreased non-significantly (P: 0.1±0.2 kg (0.1%, Table 3). There was a significant difference ($p<0.05$) in body weight changes only between the OTE and P groups at weeks 4 and 6 (Fig. 1).

The total body weight reduction observed during the six week GTE or OTE treatment was accompanied by a significant decrease in absolute FM, but only in the OTE group (FM: -1.9±0.3 kg (8.0%); $p<0.05$, Table 3). There was a significant difference ($p<0.05$) in the %FM

Table 2: Baseline characteristics of 3 treatment groups[1]

Parameters	P Group (n = 10)	GTE treatment Group (n = 10)	OTE treatment Group (n = 10)
Age (years)	21.0±0.3	21.0±0.5	20.5±0.2
Height (cm)	176.0±3.0	179.0±3.0	179.0±1.5
Body weight (kg)	83.1±4.4	79.6±4.7	86.8±2.5
Body fat (%)	18.4±2.4	17.5±1.6	21.5±1.9

[1]Mean±SEM; subjects stratified at baseline for age, height, body weight, body fat %. Subjects matched for characteristic; no differences between groups were statistically significant by one factor ANOVA ($p<0.05$)

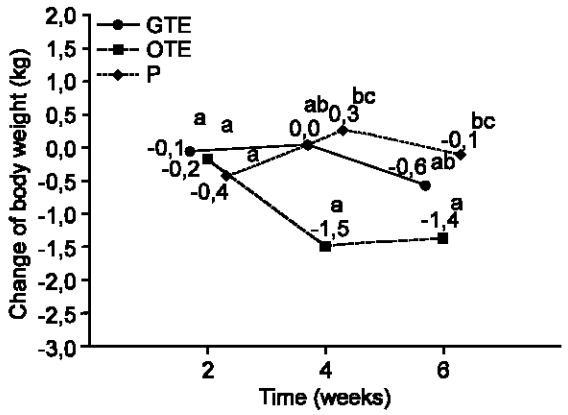

Fig. 1: The changes of body weight of subjects in green tea extracts group (●; n = 10), Oolong tea extracts group (■; n = 10) and Placebo group (◆; n = 10) after 2, 4 and 6 weeks. Different letters indicate significantly different ($p<0.05$) between groups (repeated-measures ANOVA)

changes between the OTE and P groups at weeks 2, 4 and 6 and between GTE and P, but only at week 6 (Fig. 2).

Neither group experienced a significant change in FFM in the treatment period (Table 3), but in both supplemented groups (GTE and OTE) the FFM increased (GTE: 0.7±0.6 kg (1.1%); OTE: 0.5±0.7 kg (0.7%), while in the P group this body component insignificant decreased (P: -0.4±0.2 kg (0.6%).

No significant differences in mean energy balance values were observed after supplementation period in group of athletes ingested GTE, OTE or P. However in groups of athletes ingested both GTE and OTE were

Table 3: The effect of 6 week supplementation with green tea extracts, oolong tea extracts and matching placebo on body weight and body composition[1]

	Placebo (n = 10)			GTE (n = 10)			OTE (n = 10)		
Parameters	Week 0	Week 6	P vs. Baseline	Week 0	Week 6	P vs. Baseline	Week 0	Week 6	P vs. Baseline
Body weight (kg)	83.1±4.4	83.0±4.1	-0.1±0.2 (0.1±0.3%)	79.6±4.7	79.0±4.8*	-0.6±0.1 (0.8±0.2%)	86.8±2.7	85.4±2.5**	-1.4±0.3 (1.6±0.3%)
FFM (kg)	67.2±2.8	66.8±2.8	-0.4±0.2 (0.6±0.3%)	65.3±3.2	66.0±3.4	0.7±0.6 (1.1±0.9%)	67.8±1.4	68.3±1.5	0.5±0.7 (0.7±1.1%)
FM (kg)	15.9±2.8	16.2±2.7	0.3±0.2 (3.0±1.9%)	14.3±2.1	13.0±1.9	-1.3±0.6 (8.6±5.0%)	19.0±2.2	17.1±1.7	-1.9±0.3* (8.0±2.5%)

[1]Mean±SE, *Mean value was significantly different to that at baseline ($p<0.05$) (t-student test), **Mean value was significantly different to that at baseline ($p<0.01$, t-student test), FFM = Fat Free Mass, FM = body fat

Table 4: Energy balance value of green tea extract, oolong tea extract and placebo group at baseline and after treatment[1]

	GTE (n = 10)		OTE (n = 10)		P (n = 10)	
Parameters	Week 0	Week 6	Week 0	Week 6	Week 0	Week 6
Energy balance [MJ/day^{-1}]	-0.16±1.9	-0.98±1.6	0.74±2.4	-1.08±1.9	0.50±1.9	0.50±2.6

[1]Mean±SE

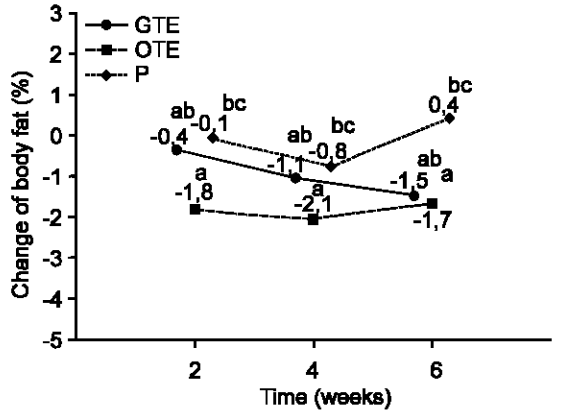

Fig. 2: The changes of body fat % of subjects in Green tea extracts group (●; n = 10), Oolong tea extracts group (■; n = 10) and Placebo group (◆; n = 10) after 2, 4 and 6 week. Different letters indicate significantly different ($p<0.05$) between groups (repeated-measures ANOVA)

observed tendency to decrease of negative energy balance (Table 4).

DISCUSSION

The present experiment showed that a six week supplementation of GTE and OTE, in which content of EGCG and caffeine was different resulted in a significant reduction of wrestlers body weight: 0.6±0.1 kg ($p<0.05$) in the GTE and 1.4±0.3 kg ($p<0.01$) in OTE group. Weight loss in both GTE and OTE group of athletes was primarily due to the absolute fat loss, but only in the OTE supplemented group this result was significant (FM: 1.9±0.3 kg; $p<0.05$). Numerous studies confirm a positive effect of GTE and OTE treatment on body weight reduction but only in obese people or animals (Chantre and Lairon, 2002; Chan *et al*., 2006; Nagao *et al*., 2005). For example, research conducted in France showed that a three month consumption of GTE at 270 mg of EGCG effectively reduced body weight by 4.6% in obese men and women (Chantre and Lairon, 2002). Nagao *et al*. (2005) in their experiment with overweight persons showed that ingestion of about 690 mg catechins, including 136 mg EGCG and 75 mg caffeine stimulated a significant loss in body weight (2.4 kg) and fat mass. Chan *et al*. (2006) reported that body weight of obese volunteers was reduced by 2.4% after a three-month green tea treatment, although the difference was not statistically significant. Our results were confirmed only in animal studies, where non-obese mice were fed a normal diet, in which a combination of catechines and caffeine was reported to induce a strong suppression of body fat accumulation. This effect was correlated with catechines content in the diet. A higher catechines (0.5%) level was connected with significantly lower body weight gain in rodents (in comparison with the control, Ito *et al*., 2008). Simultaneously, the authors of this study explained that GTE usually contains a smaller amount of caffeine, thus their results are more attributable to the effect of the catechins and not caffeine (Ito *et al*., 2008). Murase *et al*. (2002) using an obese mice model demonstrated that tea catechins at identical dosages as those applied by Ito *et al*. (2008, from 0.1-0.5% catechins) caused a meaningful increment in lipid catabolism in the liver. However, in their study Zheng *et al*. (2004) indicated that catechins and caffeine were synergistic in anti-obesity activities. The findings described by Ito *et al*. (2008) and Murase *et al*. (2002) confirm that catechins (much more than caffeine) at clinically appropriate doses, affect lipid metabolism in non-obese and obese subjects. However, in our results a higher body weight reduction, accompanied with fat mass reduction, was observed in subjects treated with oolong tea powder, where EGCG level was similar to C. Based on biochemical and pharmacological studies, the

mechanisms of action of both types of teas (non-fermented and semi-fermented) in preventing obesity may be obtain through stimulating hepatic lipid metabolism, inhibiting gastric and pancreatic lipases (Hsu *et al*., 2006; Han *et al*., 1999; Bose *et al*., 2008; Ito *et al*., 2008; Yang *et al*., 2001), stimulating thermogenesis (Dulloo *et al*., 1999; Dulloo *et al*., 2000; Komatsu *et al*., 2003), modulating appetite (Kao *et al*., 2000) synergism with caffeine and theanine and finally suppressing Fatty Acid Synthase (FAS) (Lin and Lin-Shiau, 2006; Yeh *et al*., 2003). Lin and Lin-Shiau (2006) studied the mechanisms of hypolipidemic and antiobesity effects of tea and tea polyphenols found that the molecular mechanisms of fatty acid synthase gene suppression by tea polyphenols (EGCG and theaflavins) may incite downregulation of the EGFR/PI3K/Akt/Sp-1 signal transduction pathways. Yeh *et al.* (2003) studied the suppression of fatty acid synthase in MCF-7 breast cancer cells by tea and tea polyphenols found a possible mechanism for their hypolipidemic effects and suggested that tea polyphenols may induce hypolipidemic and antiproliferative effects by suppressing fatty acid synthase. Therefore, antiobesity effect of tea polyphenols could be also observed in the normal (non-obese) people. Described by Lin and Lin-Shiau (2006); Yeh *et al.* (2003) mechanism of action of tea polyphenols can by use to explain reductions in fat mass among the athletes treated of OTE, because contents of theaflavins formed from polymerization of catechins at the semi fermentation stage during the manufacture of oolong is high and amount to ~2 g/100 g of the dried water extract of oolong tea (Subramanian *et al*., 1999). More research is needed to determine whether oolong tea consumption has an unequivocal hypolipidemic effects benefit and especially which components in tea may be responsible for this effect.

The first thermogenic effect of green tea was attributed to its caffeine content (Astrup *et al*., 1990; Borchardt and Huber, 1975). However, Dulloo *et al.* (2000) observed that green tea could stimulate thermogenesis to a much greater extent than that which can be attributed to the caffeine content alone. In another study the same researchers found that GTE ingestion increased 24 h energy expenditure by 4% (328 kJ) in 10 health men, reflecting green tea stimulatory effect on thermogenesis (Dulloo *et al*., 1999). Dulloo *et al.* (1999) explained that catechins in green tea and oolong tea may stimulate thermogenesis and fat oxidation through an inhibition of catechol O-methyl-transferase, an enzyme that degrades noradrenaline. In our research, we only observed tendency to negative energy imbalance, supported by increase daily energy expenditure between athletes supplemented both green and oolong tea extract. Komatsu *et al.* (2003) stated that consumption of oolong and green tea beverages increased TEE in healthy non obese Japanese females, by 10 and 4% respectively. Also Auvichayapat *et al.* (2008) indicated that green tea capsules in dosage of 100 mg/day EGCG can increase energy expenditure and fat oxidation in Obese Thai subjects in 12 weeks period. In turn, Hsu *et al.* (2006) indicated that polyphenol-enriched oolong tea beverages (27.2 mg EGCG and 134 mg C in 750 ml of beverages) consumed at three meals could increase lipid excretion into feces when subjects took a high-lipid diet (polyphenol-enriched oolong tea beverages: 19.3+/-12.9 g/3 day vs. placebo: 9.4+/-7.3 g/3 day). Wrisez and Lambert (2001) reported also that other polyphenolic compounds, such as tannins and tannic acids, exerted an influence on lipid metabolism in rats. Kao *et al.* (2000) found that rats in a 7-day experiment of daily intraperitoneal injections of EGCG consumed up to 60 percent less food in comparison to the control.

Conclusion: Two important findings may be presented regarding tea extract supplementation. Firstly, supplementation of oolong tea (EGCG content similar to C) caused a statistically significant decrease in fat mass of non-obese subjects in comparison to than following the administration of green tea (C content higher than EGCG). Secondly, the C to EGCG ratio should be in balance to both body weight and fat loss. This study showed also that for athletes from weight-sensitive disciplines, during the training period, for the purpose of healthy body weight reduction, accompanied by FM loss and FFM maintenance, it seems reasonable to recommend the OTE supplementation of 2.4 g/day.

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Effects of Biological and Mineral Fertilization on Yield, Chemical Composition and Physical Characteristics of Faba Bean (*Vicia faba* L.) Cultivar Seleim

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Abstract: A field experiment was conducted at Dongola University Farm at Al-Seleim - Dongola University Farm, Northern State to study the effects of *Rhizobium leguminosarum biovar viceae* and *Bacillus megatherium* var. *Phosphaticum* (BMP) inoculation and phosphorus and nitrogen fertilization on the yield and seed quality of faba bean (*Vicia faba* L.) cultivar seleim. *Rhizobium* inoculation significantly ($p \leq 0.05$) increased yield, seed ash, fat, crude protein and 100-seed weight. BMP inoculation significantly ($p \leq 0.05$) increased seed moisture, fat, crude fiber and crude protein content. On the other hand, *Rhizobium* and BMP Co-inoculation significantly ($p \leq 0.05$) increased yield, seed ash, protein content and 100-seed weight. Application of chemical fertilizers increased yield, seed ash, fat, crude protein content and 100-seed weight. In addition to that, nitrogen fertilization significantly ($p \leq 0.05$) increased the hydration coefficient. Nitrogen fertilization in the presence of *Rhizobium* significantly ($p \leq 0.05$) increased the cookability.

Key words: *Rhizobium*, *Bacillus megatherium* var. *Phosphaticum*, inoculation, faba bean

INTRODUCTION

Legumes are the major direct source of proteins for both man and livestock, especially in poor countries, where animal protein is expensive (Hubbell and Gerald, 2003). Grain legumes play an essential role in human nutrition balancing the deficiencies of cereal-based diet (Dart and Krantz, 1977). The importance of legumes is that they can fix nitrogen in symbiotic association with rhizobia, and so they increase the soil nitrogen content (Poth *et al*., 1986). This association enables legumes to benefit from an augmented nitrogen supply and can grow well on relatively poor soils (Heywood *et al*., 1985).

Faba bean (*Vicia faba* L.) is one of the major legume crops cultivated in the Northern and the River Nile States of Sudan, produced in an average area of 69720 ha, with an average yield of 1896 kg/ha (AOAD, 2007). It is the main food for millions of people and the source of protein for the middle and low income groups (Salih, 1981). Faba bean is an important cash crop for farmers (Watson, 1981).

Crop productivity can be increased by the application of chemical, organic and biological fertilizers (Elsheikh *et al*., 2009). Efforts throughout the world are directed towards improving the quality of food crops by increasing the nutritional value of the grains and decreasing the antinutrients level. The objectives of this study is to determine the effects of biological and mineral fertilization on yield, chemical composition and physical characteristics of faba Bean.

MATERIALS AND METHODS

A field experiment was conducted at Dongola University Farm at Al-Seleim, Northern State, Sudan for two consecutive winter seasons 2004/2005 and 2006/2007 in a factorial design with four replicates.

In these experiments, plants were fertilized with either nitrogen or phosphorus or with both of them. Plants were either inoculated with the introduced *Rhizobium* strain (TAL 1399) which was obtained from NifTAL project, University of Hawaii USA or with *Bacillus megatherium var phosphaticum* strain (BMP) which was locally isolated, in addition to combination of each (TAL 1399 + BMP).Control plants were kept for comparison. The land was prepared by deep ploughing, harrowing then leveling and ridging. The land was then divided into plots 6 x 3.8 m each. In treatments with nitrogen, 43 kg N/ha as urea was broadcasted immediately after sowing. Phosphorus was applied at a rate of 43 kg P/ha as triple super phosphate broadcasted before sowing.

Certified seeds of faba bean (*Vicia faba* L.) cultivar (Seleim-SM-L) were obtained from Dongola Research Station, Agricultural Research Corporation, Sudan.

Two to three seeds were placed in a hole on the top of the ridge with 20 cm spacing (between holes) and 70 cm (between ridges). Plots were immediately irrigated after sowing and then subsequently irrigated at 10 days intervals. Harvest was done at 13 weeks after sowing. Each plot was harvested separately by cutting the plants just above soil level. Plants were then threshed on

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a large mat, then collected and weighed to determine yield of each plot. 100 seeds from the collected samples from each plot were counted in 4 replicates randomly then weighted.

Samples were taken from seeds of each plot, ground and used for proximate analysis which were conducted according to the methods of AOAC (1984), except for *In vitro* Protein Digestibility (IVPD) which was determined using the method of Maliwal (1983) as modified by Manjula and John (1991), Tannin content which was estimated using the modified vanillin HCl method (Price *et al*., 1978) and Hydration coefficient and Cookability which were determined using the methods described by Elsheikh *et al*., 2009.

Multifactor Analysis of Variance (ANOVA) was used to determine the effect of different treatments on the measured parameters.

Least significance difference was used to compare between means (Gomez and Gomez, 1984). Significance was accepted at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effects of treatments on faba bean yield: Inoculation of faba bean plants with *Rhizobium* strain TAL 1399 significantly ($p \leq 0.05$) increased seed yield in both seasons (Table 1). In previous studies similar results were reported by Osman and Mohamed (1994); Rugheim and Abdelgani, (2009). However, Co-inoculation with *Rhizobium* and BMP significantly ($p \leq 0.05$) increased faba bean seed yield in both seasons as was previously found by Rugheim and Abdelgani (2009).

Effects of treatments on the proximate analysis of faba bean seeds moisture content: The average moisture content of faba bean seeds was found to range from 2.26-5.65%. None of the treatments had a significant effect on the moisture content of faba bean seeds except inoculation with BMP as phosphobacterin (Table 2).

Ahmed (1998) reported that there is no effect on moisture content for faba bean following *Rhizobium* inoculation. However, BMP inoculation with phosphobacterin significantly increased moisture content of faba bean seeds (Rugheim and Abdelgani, 2009).

Generally, the moisture content of legume seeds was found to be affected by the relative humidity of the surrounding atmosphere at the time of harvest and during storage (Elsayed, 1994).

Table 1: Effects of treatments on faba bean yield (kg/f)*

	Yield	
Treatments	First season	Second season
No inoculation		
Control	283.40	112.80
Nitrogen (43 kg N/h)	346.50	167.08
Phosphorous (43 kg P_2O_5/h)	298.45	122.68
Nitrogen + Phosphorous	341.40	19750
Mean	317.43	150.02
Inoculation with *Rhizobium* (TAL 1399)		
Control	348.00	139.39
Nitrogen	358.50	143.32
Phosphorous	323.60	131.67
Nitrogen + Phosphorous	386.10	176.98
Mean	354.05	147.84
Inoculation with phosphobacterin (BMP)		
Control	294.90	134.47
Nitrogen	372.70	110.00
Phosphorous	300.90	204.59
Nitrogen + Phosphorous	332.60	130.86
Mean	325.27	144.98
Inoculation with *Rhizobium* + phosphobacterin		
Control	377.50	167.41
Nitrogen	397.50	113.00
Phosphorous	340.90	141.09
Nitrogen + Phosphorous	228.00	126.73
0 Mean	335.97	137.06
LSD for *Rhizobium*	46.24	25.6685
LSD for phosphobacterin	8.1746	4.5376
LSD for *Rhizobium* x phosphobacterin	92.49	51.3371
LSD for nitrogen	8.1746	4.5376
LSD for phosphorous	8.1746	4.5376
LSD for nitrogen x phosphorous	16.3492	9.0753
LSD for *Rhizobium* x phosphobacterin x nitrogen x phosphorous	184.97	102.6742

*f(feddan) = 0, 42 ha

Table 2: Effects of treatments on moisture, ash and fat and crude fiber content of faba bean seeds

Treatment	Moisture (%)	Ash (%)	Fat content (%)	Crude fiber (%)
No inoculation				
Control	5.07	2.90	1.17	7.52
Nitrogen (43 kg N/h)	4.28	7.45	1.12	7.43
Phosphorous (43 kg P_2O_5/h)	4.39	7.23	1.28	7.03
Nitrogen + Phosphorous	5.19	2.48	1.40	7.08
Mean	4.73	5.02	1.24	7.26
Inoculation with *Rhizobium* (TAL 1399)				
Control	2.26	5.28	1.39	7.04
Nitrogen	3.64	4.63	1.31	8.12
Phosphorous	5.52	5.39	1.39	7.73
Nitrogen + Phosphorous	3.67	16.95	1.20	7.93
Mean	3.77	8.69	1.32	7.55
Inoculation with phosphobacterin (BMP)				
Control	5.65	2.73	1.30	7.99
Nitrogen	5.42	3.50	1.27	7.87
Phosphorous	4.75	6.07	1.32	7.17
Nitrogen + Phosphorous	4.57	2.49	1.21	7.19
Mean	5.09	3.96	1.27	7.73
Inoculation with *Rhizobium* + phosphobacterin				
Control	5.03	4.37	1.26	7.16
Nitrogen	4.79	2.74	1.31	7.12
Phosphorous	3.98	5.34	1.20	7.28
Nitrogen + Phosphorous	4.40	2.68	1.19	8.33
Mean	4.55	3.78	1.24	7.47
LSD for *Rhizobium*	0.132	0.186	0.083	0.201
LSD for phosphobacterin	0.132	0.186	0.083	0.201
LSD for *Rhizobium* x phosphobacterin	0.260	0.372	0.167	0.402
LSD for nitrogen	0.132	0.186	0.083	0.201
LSD for phosphorous	0.132	0.186	0.083	0.201
LSD for nitrogen x phosphorous	0.260	0.372	0.167	0.402
LSD for *Rhizobium* x phosphobacterin x nitrogen x phosphorous	2.08	1.488	0.664	1.608

Ash content: *Rhizobium* inoculation and co-inoculation with *Rhizobium* and BMP significantly ($p \leq 0.05$) increased ash content of faba bean seeds (Table 2). Rugheim and Abdelgani (2009) reported that inoculation and co-inoculation significantly increased the ash content of faba bean seeds. BMP inoculation didn't affect the ash content of faba bean seeds. However Rugheim and Abdelgani (2009) found that BMP inoculation significantly increased ash content of faba bean seeds. Application of nitrogen and phosphorus chemical fertilizers separately significantly increased ash content of faba bean seeds. This result is in accord with the observations of Rugheim and Abdelgani (2009).

Fat content: *Rhizobium* and BMP inoculation and application of chemical fertilizers separately significantly ($p \leq 0.05$) increased fat content of faba bean seeds (Table 2). The increase in fat content of faba bean due to biological, chemical and organic fertilization was reported by Elsheikh (1998), Elsheikh and Ahmed (2000) and Elsheikh (2001).

Fiber content: None of the treatments had significant effect on the crude fiber content of faba bean seeds except, inoculation with BMP as phosphobacterin (Table 2). *Rhizobium* inoculation didn't affect the crude fiber content of faba bean seeds. This result is in accord with the findings of Elsheikh (1998) and Elsheikh and Ahmed (2000) and contradictory to the findings of Rugheim and Abdelgani (2009). BMP inoculation significantly ($p \leq 0.05$) increased crude fiber content of faba bean seeds. This result is in accord with observations of Rugheim and Abdelgani (2009). Co-inoculation, chemical fertilization and the 4-way interaction didn't affect crude fiber content of faba bean seeds.

Protein content: Faba bean contains a high protein content compared to other legumes amounting to 33% (Elskeikh *et al*., 2000). The individual or combined inoculation of *Rhizobium* and BMP significantly ($p \leq 0.05$) increased crude protein content in faba bean seeds compared to uninoculated control plants (Table 3). This finding prove the results of Lucas-Garcia *et al*. (2004) and Rugheim and Abdelgani (2009). Application of nitrogen and phosphorus separately and their combination significantly ($p \leq 0.05$) increased crude protein in faba bean seeds, as was previously found by Rugheim and Abdelgani (2009). The 4-way interaction reduced crude protein content in faba bean seeds.

Carbohydrates content: *Rhizobium* and BMP inoculation, co-inoculation with *Rhizobium* and BMP and

Table 3: Effects of treatments on crude protein, carbohydrates, *in vitro* protein digestibility and tannin content of faba bean seeds

Treatments	Crude protein	Carbohydrates	IVPD	Tannin
	(%)			
No inoculation				
Control	29.16	54.18	59.74	0.188
Nitrogen (43 kg N/h)	30.04	49.68	57.96	0.138
Phosphorous (43 kg P_2O/h)	30.91	49.16	52.92	0.363
Nitrogen + Phosphorous	31.20	52.65	55.76	0.063
Mean	30.32	51.41	56.59	0.188
Inoculation with *Rhizobium* (TAL 1399)				
Control	32.44	51.59	49.22	0.124
Nitrogen	29.61	52.71	51.65	0.119
Phosphorous	32.13	47.84	58.51	0.033
Nitrogen + Phosphorous	32.95	37.3	49.59	0.266
Mean	31.78	47.36	52.24	0.136
Inoculation with phosphobacterin (BMP)				
Control	32.66	49.67	47.07	0.119
Nitrogen	33.25	48.69	52.56	0.041
Phosphorous	31.5	49.19	51.79	0.075
Nitrogen + Phosphorous	28.0	55.82	54.35	0.044
Mean	31.35	50.84	51.44	0.096
Inoculation with *Rhizobium* + phosphobacterin				
Control	31.58	50.60	56.97	0.099
Nitrogen	32.43	51.69	50.83	0.091
Phosphorous	30.91	51.29	57.42	0.053
Nitrogen + Phosphorous	28.79	54.61	52.96	0.049
Mean	30.92	52.04	54.54	0.073
LSD for *Rhizobium*	0.8573	0.9749	0.4972	0.0538
LSD for phosphobacterin	0.8573	0.9749	0.4972	0.0538
LSD for *Rhizobium* x phosphobacterin	1.7146	1.9498	0.9945	0.1077
LSD for nitrogen	0.8573	0.9749	0.4972	0.0538
LSD for phosphorous	0.8573	0.9749	0.4972	0.0538
LSD for nitrogen x phosphorous	1.7146	1.9498	0.9945	0.1077
LSD for *Rhizobium* x phosphobacterin x nitrogen x phosphorous	6.8584	7.7993	3.9780	0.086

application of chemical fertilizers decreased carbohydrates content in faba bean seeds (Table 3). This result is in accord with observation of Rugheim and Abdelgani (2009).

Generally the carbohydrates content in the seeds of leguminous crops was found to decrease with *Rhizobium* inoculation (Elsheikh, 2001).

***In vitro* protein digestibility:** None of the treatments had a significant effect on the *in vitro* protein digestibility of faba bean (Table 3). Similar results were reported by Elsheikh *et al.* (2009) and Rugheim and Abdelgani (2009). However Elskeikh and Ahmed (2000) found that *Rhizobium* inoculation gave significant increment in the *in vitro* protein digestibility of faba bean seeds (Elskeikh and Ahmed, 2000). The *in vitro* protein digestibility has been reported to be affected by many factors such as genotype and tannin content (Babiker *et al.*, 1995).

Tannin content: None of the treatments had a significant effect on the tannin content of faba bean seeds, except phosphorus chemical fertilization (Table 3).

Rhizobium inoculation and chicken manure fertilization had no significant effect on tannin content of soybean seeds Elsheikh *et al.* (2009). However, *Rhizobium* inoculation significantly increased the tannin content of groundnut and faba bean seeds (Elsheikh and Mohamedzein, 1998; Babiker *et al.*, 1995).

100-Seed weight: *Rhizobium* inoculation and co-inoculation with *Rhizobium* and BMP significantly ($p \leq 0.05$) increased 100-seed weight compared to uninoculated control (Table 4). Application of 43 kg N/ha + 43 kg P_2O_5/ha and phosphorus chemical fertilizers significantly ($p \leq 0.05$) increased 100-seed weight.

Nitrogen chemical fertilizer, BMP inoculation and the 4-way interaction didn't affect 100-seed weight. The increase in 100-seed weight resulted from *Rhizobium* inoculation was observed in faba bean earlier by Mohamed Ahmed (2000). The increase in 100-seed weight due to co-inoculation with *Rhizobium* and BMP or phosphorus chemical fertilization was previously observed by Barea *et al.* (2005). However, Rugheim and Abdelgani (2009) found that chemical fertilizers didn't affect 100-seed weight when interacted with *Rhizobium* and BMP.

Hydration coefficient: None of the treatments had a significant effect on the hydration coefficient of faba bean seeds except fertilization with nitrogen as urea in comparison with control (Table 4). Elsheikh *et al.* (2009) reported that hydration coefficient of soybean seeds was

Table 4: Effects of treatments on 100-seed weight, hydration coefficient and cookability of faba bean seeds

Treatments	100-seed weight (g)	Hydration coefficient (%)	Cookability (%)
No inoculation			
Control	52.73	207,74	15,48
Nitrogen (43 kg N/h)	52.82	223,39	14,21
Phosphorous (43 kg P_2O/h)	57.97	193,23	19,53
Nitrogen + Phosphorous	56.94	204,14	19,55
Mean	55.10	207.13	17.19
Inoculation with *Rhizobium* (TAL 1399)			
Control	57.91	209,53	12.46
Nitrogen	56.19	206,80	25.46
Phosphorous	52.85	201,39	18.72
Nitrogen + Phosphorous	50.20	204,14	12.30
Mean	54.29	205.47	17.24
Inoculation with phosphobacterin (BMP)			
Control	51.81	201,12	11.67
Nitrogen	50.62	206,58	17.79
Phosphorous	57.37	206,99	9.52
Nitrogen + Phosphorous	54.60	213,88	12.34
Mean	53.6	207.14	12.83
Inoculation with *Rhizobium* + phosphobacterin			
Control	58.91	199,74	19.11
Nitrogen	53.15	197,78	18.95
Phosphorous	56.39	207,81	20.90
Nitrogen + Phosphorous	50.39	197,34	22.01
Mean	54.71	200.72	20.24
LSD for *Rhizobium*	1.91	2,06	1.64
LSD for phosphobacterin	1.91	2,06	1.64
LSD for *Rhizobium* x phosphobacterin	3.82	4,12	3.28
LSD for nitrogen	1.91	2,06	1.64
LSD for phosphorous	1.91	2,06	1.64
LSD for nitrogen x phosphorous	3.82	4,12	3.28
LSD for *Rhizobium* x phosphobacterin x nitrogen x phosphorous	15.31	16,47	13.13

significantly increased by inoculation with *Rhizobium* isolate-2 and not significantly with *Rhizobium* strain TAL-377.

Cookability: None of the treatments had a significant effect on the cookability of faba bean seeds except fertilization with nitrogen in the presence of *Rhizobium* inoculation in comparison with control (Table 4). Elsheikh *et al.* (2009) found that inoculation and chicken manure fertilization insignificantly affected the cookability of soybean seeds. It was previously reported that chicken manure significantly increased the cookability in the presence or absence of *Rhizobium* inoculation (Elsheikh and Elzidany, 1997).

Conclusion: Inoculation with *Rhizobium* indicated an increase in faba bean seed yield, ash, fat, crude protein, and 100-seed weight. BMP inoculation increased moisture, fat, crude fiber and crude protein content. Co-inoculation with *Rhizobium* and BMP increased seed yield, ash, protein content and 100-seed weight. Application of chemical fertilizers increased seed yield, ash, fat, crude protein content and 100-seed weight. None of the treatments had a significant effect on the hydration coefficient of faba bean seeds except fertilization with nitrogen as urea in comparison with control and also none of the treatments had a significant effect on the cookability of faba bean seeds except fertilization with nitrogen in the presence of *Rhizobium* inoculation in comparison with control.

From all these results we can conclude that inoculation with *Rhizobium* and co-inoculation with *Rhizobium* strain TAL 1399 and BMP as phosphobacterin was efficient to give significant yield, with good quality.

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The Phytochemical Composition and Some Biochemical Effects of Nigerian Tigernut (*Cyperus esculentus L.*) Tuber

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Abstract: The phytochemical composition of the tigernut tuber and the effect of the aqueous extract on some biochemical parameters such as blood glucose, serum protein, albumin and cholesterol, white blood cells, red blood cells, haemoglobin, erythrocyte sedimentation rate and packed cell volume were determined in rats administered different concentrations of the extract. From the result of the phytochemical analysis, the presence of alkaloids, cyanogenic glycosides, resins, tannins, sterols and saponins were observed in the raw tuber, however only alkaloids, sterols and resins were observed in the roasted tuber. Analysis of the antinutrient composition yielded oxalates (0.25±0.65 g/100 g), phytate (1.97±0.81 mg/100 g), saponins (0.88±0.02/100 g), tannins (9.50±0.46 mg/100 g) and cyanogenic glycosides (1.80±0.69 mg/100 g). Roasting numerically decreased the levels of the anti-nutritive factors analyzed. At the end of the treatment period, the mean weights of the animals increased. The blood glucose level decreased significantly in concentration dependent manner ($p<0.05$) and serum albumin level increased significantly in a concentration dependent manner ($p<0.05$) in the groups administered the different concentrations of the extract. There was no significant effect ($p>0.05$) on serum cholesterol and protein and on total and differential white blood cell, red blood cell, haemoglobin, packed cell volume and erythrocyte sedimentation rate. The results therefore indicate the absence of undesirable effect in the use of the tigernut tuber even in the raw form at least at the administered concentration and for the duration of feeding. The findings are of nutritional, health and industrial relevance since the tuber is currently being used as food in many homes in Nigeria.

Key words: *Cyperus esculentus*, tigernut, phytochemicals, biochemical effects

INTRODUCTION

The worsening food crisis and the consequent wide spread prevalence of malnutrition in developing and underdeveloped countries have resulted in high mortality and morbidity rates, especially among infants and children in low income groups (Enujuigba and Akanbi, 2005). The reliance on starchy roots and tubers and protein deficient cereals as main staples results in consumption of non-nutritious foods. The insufficient availability of nutrient rich diets and high cost of available ones have prompted an intense research into harnessing the potentials of the lesser known and underutilized crops, which are potentially valuable for human and animal foods to maintain a balance between population and agricultural productivity, particularly the tropical and subtropical areas of the world.

Cyperus esculentus (Tigernut) is an underutilized plant of the family *Cyperaceae*, which produces rhizomes from the base and tubers that are some what spherical (Cortes *et al*., 2005). The plant is not really a nut but a tuber first discovered some 4000 years ago (Lowe and Whitewell, 2000). It has other names like yellow nutsedge, chufa, flatsedge, rush nut, water grass, earth almond, northern nut grass and nut grass (Shilenko *et al*., 1979). *Cyperus esculentus* is known in Nigeria as aya in Hausa, ofio in Yoruba and akihausa in Ibo. *Cyperus esculentus* grows mainly in the middle belt and northern regions of Nigeria (Okafor *et al*., 2003), where three varieties (black, brown and yellow) are cultivated (Umerie *et al*., 1997). Among these, only two varieties, yellow and brown are readily available in the market. The yellow variety is preferred to all other varieties because of its inherent properties like its bigger size, attractive colour and fleshier body (Belewu and Abodurin, 2006). *Cyperus esculentus* can be eaten raw, roasted, dried, baked or be made into a refreshing beverage called kuunu (Oladele and Aina, 2007).

Cyperus esculentus was reported as healthy and helps in preventing heart, thrombosis and activates blood circulation. It helps in preventing cancer, due to high content of soluble glucose. It was also found to assist in reducing the risk of colon cancer (Adejuyitan *et al*., 2009). The nut is rich in energy content (starch, fat, sugars and protein), mineral (phosphorus, potassium)

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and vitamins E and C (Belewu and Belewu, 2007). *Cyperus esculentus* is suitable for diabetic persons and also helps in loosing weight (Borges *et al*., 2008).

Food contains various compositions of nutrients and antinutrients and could have important or deleterious effects in the body when consumed. The composition of the nutrients and antinutrients, usually leads to side effects found in most plants which may lead to toxicity, hyperlipidaemia, excessive weight gain, hyperglycaemia, carotenemia, constipation, kidney stones, body odour, bad breath, allergies, diarrhoea, frequent urination and acne (Anonymous, 2009). In most of these side effects, the biochemical and haematological parameters are usually altered. For a food to be considered safe for human and animal health, its effect on these parameters need to be investigated to understand the nutritional potentials and safety of such foods with a view to determining their acceptability.

The aim of the present study is to determine the phytochemical composition of the tuber and to ascertain if the tuber could have beneficial effect on biochemical parameters such as blood glucose, serum albumin, protein, cholesterol, red blood cell, haemoglobin, erythrocyte sedimentation rate, packed cell volume and total and differential white blood cell of the rats as our model for the research.

MATERIALS AND METHODS

Collection and preparation of tigernut tuber flour and the aqueous extract: Fresh tigernut tuber was purchased from a local market in Katsina, Katsina state, Nigeria. The tuber was identified and authenticated by Mr A. Ozougwu of Botany department, university of Nigeria, Nsukka, Enugu state. The tigernut tubers were cleaned, sorted and washed. The fresh tubers were dried in an oven (GallenKamp, England) at 37°C for one hour, milled separately using a laboratory electric mill (Retsch, 5657, GmbH, Germany) to pass through a 40-mesh sieve, packaged in glass jars and stored at 4°C in a refrigerator until analysis. A Quantity, 400 g of the fresh milled tubers was extracted by shaking it with 3 litres of n-hexane for one hour, three times to remove the oil. The defatted milled tubers were dried in a desiccator under vacuum. The water extract was obtained by stirring the dry defatted milled tubers with seven (7) litres of distilled water at room temperature (27±1°C) for twelve hours. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was filtered through white muslin cloth and then whatman filter paper No.1 under vacuum. The extract was concentrated using water bath at an optimum temperature of 65°C to avoid the denaturation of the bioactive compounds. The weight of the dry extract was determined. The different concentrations (500, 1000, 1500 and 2000 mg/kg) of the extract were prepared.

Table 1: The phytochemical composition of the tigernut tuber

Phytochemical	Raw	Roasted
Alkaloids	+++	+
Glycosides	-	-
Cyanogenic glycosides	+	-
Resins	+++	+++
Flavonoids	-	-
Cardiac glycosides	-	-
Tannins	+	-
Sterols	+++	+++
Saponins	+	-

+++ = Present in very high concentration, ++ = Present in moderately high concentration, + = Present in trace concentration, - = Not detected

Experimental animals: Adult male Wistar albino rats were purchased from the faculty of biological sciences animal house, University of Nigeria, Nsukka, Enugu state, Nigeria. The animals were about 12 weeks with average weight of 112.37±11.7 g. The animals were kept under standard conditions for 7 days with free access to water and food before starting the experiment. Albino mice, 20.50±4.27 g weights were used for the acute toxicity tests. The animals were housed in standard cages with food and water *ad libitum* at room temperature and provided with pelletized feed.

Experimental design: An acute toxicity study of the aqueous extract of tigernut was done by the method of Lorke (1983). Twenty five (25) male Wistar albino rats of 12 weeks were divided into five groups of five rats each of average weight were randomly assigned to five (5) cages labelled I, II, III, IV and V respectively and kept at room temperature (25°C). All the rats were allowed free access to water and feed *ad libitum* for a week to acclimatize them to laboratory conditions. After this period, the control animals (group I) were administered 0.2 ml of normal saline (0.9% NaCl) while groups II, III, IV and V were administered different concentrations of the extract. The extracts were administered for 30 days to the animals using the oral route by means of polythene cannula. The weights of the animals were taken before commencement of the feeding experiment and then later every six days interval. At the end of the 30 days, blood samples from each rat were collected through the orbital technique for analysis of haematological parameters like total and differential white blood cells, red blood cell, haemoglobin, packed cell volume, erythrocyte sedimentation rate and biochemical parameters like blood glucose, serum protein, albumin and cholesterol.

Phytochemical analysis: The phytochemical test for the presence and absence of saponins, alkaloids, flavonoids, cyanogenic glycosides, tannins, glycosides, and sterols were carried out according to the method described by Harbone (1984).

Antinutrient analysis: Percentage compositions of some antinutrients like oxalates, phytates, cyanogenic glycosides, saponins and tannins were determined by the method described by AOAC (1990). All determinations were done in triplicate determination.

Biochemical studies: Serum cholesterol was determined by the method of Meiatini *et al.* (1978), serum total protein by the method of Wooten (1964), blood glucose by the glucose oxidase method of Marks and Dawson (1965), serum albumin by the method of Doumas *et al.* (1971).

Haematological studies: The haemoglobin concentration was estimated using the cyanome-thaemoglobin photometric method. The packed cell volume was estimated using the micro-haematocrit centrifuge. The red blood cell and differential white blood cell was estimated using the improved Neubauer haemocytometer. Erythrocyte sedimentation rate was determined using the Westergren method (1957).

RESULTS AND DISCUSSION

The result of phytochemical screening shows that a higher content of alkaloids, sterols and resins than cyanogenic glycosides, saponins and tannins were detected in the raw Tigernut tuber. However, in the roasted Tigernut tuber, only alkaloids sterols and resins were detected and no other phytochemical assayed was detected. Alkaloids, saponins and tannins are known to have antimicrobial activity, as well as other physiological activities (Sofowora, 1993; Evans, 2005). Alkaloids are known for their toxicity, but not all alkaloids are toxic. They inhibit certain mammalian enzymic activities such as those of phophodiesterase, prolonging the action of cAMP. They also affect glucagons and thyroid stimulating hormones, while some forms have been reported to be carcinogenic (Okaka *et al.*, 1992). Some have been used either as an analgesic, antispasmodic, bactericidal agents (Frantisek, 1991). Saponins have been reported to be useful in reducing inflammation of upper respiratory passage and also chiefly as foaming and emulsifying agents and detergents (Frantisek, 1991). Tannins have astringent properties that hasten the healing of wounds and prevention of decay. Tannin compounds have antimicrobial activities and are responsible for preventing and treating urinary tract infections and other bacterial infections. The result of the determination of phytochemical test indicated that the tuber possess some biologically active compounds which could serve as potential source of vegetable drugs in herbal medicine. These phytochemicals exhibit diverse pharmacological and biochemical actions when ingested by animals (Amadi *et al.*, 2006). They are usually present at low concentration in edible fruits, nuts, tubers and vegetables. Roasting reduced the amount of these phytochemicals in plant products (Piorrock *et al.*, 1984) as most of these phytochemicals are thermally unstable.

Analysis of the antinutrients composition of the raw tubers of *C. esculentus* showed that it contained 0.60±0.32 g/100 g oxalates, 2.40±0.40 mg/100 g phytates, 0.88±0.02 mg/100 g saponins, 9.62±0.29 g/100 g tannins and 1.08±0.69 mg/100 g cyanogenic glycosides. The roasted *C. esculentus* tuber contained 0.55±0.36 g/100 g oxalates, 1.06±0.24 mg/100 g phytate, 0.67±0.40 mg/100 g saponins, 7.10±0.35 g/100 g tannins and 0.86±0.44 mg/100 g cyanogenic glycosides. The levels of antinutrients analyzed were very low compared to those reported for nuts like the peanuts (Ejigui *et al.*, 2005). The presence of phytates in biological systems may chelate divalent metals like calcium, magnesium, or block the absorption of essential minerals in the intestinal tract (Dan, 2005) thus decreasing their bioavailability (Oberleas, 1973). Phytates chelate with mineral elements thereby having significant effects on the utilization of the minerals. They also react with basic residues of protein. Tannins and to some extent oxalates, binds to proteins thereby making them difficult to digest in the body. Oxalates can remove calcium in the form of calcium oxalate (Savage, 1993) in the blood and thus may result to kidney damage. Saponin reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intra-lumenal physicochemical interaction (Price *et al.*, 1987). They also exhibit structure dependent biological activity (Savage, 1993). The potential toxicity of a food produced from a cyanogenic plant depends on the likelihood that its consumption will produce a concentration of Hydrogen Cyanide (HCN) that is toxic to exposed humans. Cyanide causes an increase in blood glucose and lactic acid levels and a decrease in the ATP/ADP ratio indicating a shift from aerobic to anaerobic metabolism. Cyanide also activates glycogenolysis and shunts glucose to the pentose phosphate pathway decreasing the rate of glycolysis and inhibiting tricarboxylic acid cycle (Akintonwa and Tunwashe, 1992). Odumodu (1992) and Okafor *et al.* (2003) had earlier reported low contents of these antinutrients in tigernut tuber flour compared with other local fruits, nuts, tubers and vegetables. Roasting numerically reduced the antinutrient composition of tigernut tuber flour.

Acute toxicity test are generally the first test conducted in any toxicity study. They provide data on the relative toxicity likely to arise from a single or brief exposure to any substance. Different plant extracts have been known to possess different levels of toxicity which majorly depends on the levels of antinutrients inherent in the plants (Sofowora, 1993). Preliminary investigations on the acute toxicity of the tuber extract of *C. esculentus* in mice showed that the aqueous extract of *C. esculentus* (tigernut) tuber was not toxic to mice at the administered concentrations.

Table 2: The antinutrient composition of the tigernut tuber

	Components				
Sample	Oxalates (g/100 g)	Phytate (mg/100 g)	Saponin (g/100 g)	Tannins (mg/100 g)	Cyanogenic glycosides (mg/100 g)
Raw	0.60±0.32	2.40±0.40	0.88±0.02	9.62±0.29	1.08±0.69
Roasted	0.55±0.36	1.06±0.24	0.67±0.40	7.10±0.35	0.86±0.44

Values are mean±standard deviation of triplicate determination

Table 3: The biochemical parameters of the animals at the end of experimental period

	Groups				
Parameters	Group I NS	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV 1500 mg/kg	Group V 2000 mg/kg
Blood glucose (g/dl)	71.5±4.04	60.25±3.40*	56.75±2.50*	54.00±3.46*	48.50±4.66*
Serum protein (g/dl)	6.92±0.27	7.43±0.63	7.39±0.45	7.16±0.61	7.19±0.35
Serum albumin (g/dl)	3.35±0.48	3.14±0.72	4.08±0.29*	4.18±0.31*	3.93±0.30*
Serum cholesterol (mg/dl)	88.10±15.12	86.49±17.65	91.35±3.24	75.94±18.89	79.91±8.79

Values are mean±standard deviation of quintuplicate determination, *Means significant different ($p<0.05$) compared to the control.
N = 5, NS = Normal Saline

The result of the effect of administration of the various concentrations (500, 1000, 1500 and 2000 mg/kg) of *C. esculentus* tuber extract on biochemical parameters such as blood glucose, serum protein, albumin and cholesterol are presented in Table 3. The result showed that there was significant increase ($p<0.05$) in serum albumin and a significant decrease ($p<0.05$) in blood glucose, but there was no significant effect ($p>0.05$) on serum protein and cholesterol. Since total serum proteins and albumin are generally influenced by total protein intake (Onifade and Tewe, 1993), the results obtained indicate nutritional adequacy of the dietary and the extract proteins. Abnormal serum albumin usually indicates an alteration of normal systemic protein utilization (Apata, 1990). Awosanya *et al.* (1999) have demonstrated the dependence of blood protein on the quality and quantity of protein source. The reported low level of phytate in the tuber could also have led to the increased absorption of protein from the rat diet. Phytate acts as a chelator, forming proteins and mineral bioavailability (Davies and Gathlin, 1991). Since glucose level was significantly ($p<0.05$) lowered and cholesterol levels were not affected abnormally, possibilities of anorexia, diabetes, liver dysfunction and mal-absorption of fat, which are the symptoms of abnormal glucose and cholesterol levels in blood (Bush, 1991) are ruled out. The glucose lowering potentials of the extract may be ascribed to modifications in glucose uptake in the intestine. It is well known that soluble fibres generally increase transit time through the gut, slow emptying of the stomach and slow glucose absorption (Swaminathan, 2002). *Cyperus esculentus* tubers have high dietary fibre content (Umerie and Enebeli, 1997), so they may play a major role in lowering blood glucose level. This observation supports an earlier hypothesis that the tuber may be important for diabetics and those seeking to reduce weight (Kordyias, 1990).

The result of the effect of administration of the various concentrations (500, 1000, 1500 and 2000 mg/kg) of *C. esculentus* (tigernut) aqueous tuber extract on haematological parameters such as red blood cells, total and differential white blood cells, haemoglobin, packed cell volume and erythrocyte sedimentation rate is presented in Table 4. The result show that there was no significant effect ($p>0.05$) on these haematological parameters. The results obtained for all treatment groups indicate nutritional adequacy of the tuber extract and the rat diet since they did not indicate malabsorption or under nutrition (Church *et al.*, 1984). These observations were related to the composition of the tuber extract and health status of the animals since none of the animals died as a result of any diseases. Hackbath *et al.* (1983) had earlier recorded a strong influence of food components on haematological traits, packed cell volume and haemoglobin concentration being very strong indicators of nutritional status of animals. It is well known that various antinutritional substances and xenobiotics can cause haemolysis, nutrients malabsorption and abnormal haemopoesis which could arise from liver damage (Chubb, 1982), antinutrient analysis of the tigernut tuber shows that it has low concentration of these antinutrients. The result of the total and differential white blood cell count indicate that the animals were healthy because decrease in number of white blood cells is an indication of allergic conditions, anaphylactic shock and certain parasitism while elevated value indicate to the existence of a recent infection, usually with bacteria (Ahamefule *et al.*, 2008). The mean body weight change in rats after every six days following administration of 500, 1000, 1500 and 2000 mg/kg body weight extract of *C. esculentus* tuber extract are presented in Table 5. A general increase in physical activities, food and water intake were observed for all the animals during the feeding experiment. There was initial increase in weight which was sustained. The increased weight could be due to increased feed and water intake observed all through the experimental period. The increase in weight of the animals suggests that they increasingly accumulated calories from the normal rat diet and from the nutrient rich extracts.

Table 4: The red blood cell count, total and differential white blood cell count haemoglobin concentration, erythrocyte sedimentation rate and packed cell volume of the animals at the end of experimental period

Haematological indices	Group I NS	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV 1500 mg/kg	Group V 2000 mg/kg
RBC ($x10^6$/µL)	8.50±0.19	8.74±0.58	8.63±0.67	8.54±1.55	8.67±0.15
Hb (g/dl)	17.25±1.28	16.94±1.29	16.99±0.95	17.71±1.00	17.91±0.63
PCV (%)	44.37±2.56	46.00±1.08	45.63±4.23	45.00±0.00	44.13±1.32
ESR (mmHr)	0.76±0.12	0.73±0.07	0.82±0.10	0.70±0.55	0.69±0.07
tWBC ($x10^3$/µL)	13.96±2.64	13.51±1.82	13.57±2.72	16.61±2.72	14.53±1.33
Neutr ($x10^3$/µL)	2.78±0.82	2.52±0.46	1.59±0.44	2.77±1.00	3.18±1.24
Lymph ($x10^3$/µL)	10.69±1.88	10.73±1.50	11.62±2.61	13.38±2.62	11.03±1.42
Eosin ($x10^3$/µL)	0.06±0.07	0.07±0.08	0.11±0.13	0.09±0.10	0.00±0.00
Mono ($x10^3$/µL)	0.39±0.20	0.24±0.15	0.24±0.18	0.33±0.13	0.18±0.67
Baso ($x10^3$/µL)	0.03±0.06	0.03±0.07	0.00±0.00	0.40±0.80	0.12±0.15

Values are mean±standard deviation of quintuplicate determination, *Means significant different ($p<0.05$) compared to the control.
N = 5, NS = Normal Saline. RBC = Red Blood Cell, Hb = Haemoglobin, PCV = Packed Cell Volume, ESR = Erythrocyte Sedimentation Rate, tWBC = total White Blood Cell, Neutr = Neutrophil, Lymph = Lymphocyte, Eosin = Eosinophil, Mono = Monocytes, Baso = Basophils

Table 5: The mean body weight of rat administered aqueous tuber extract of tigernut

Periods	Group I NS	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV 1500 mg/kg	Group V 2000 mg/kg
0 day	113.25±15.09	113.50±6.62	114.74±12.20	111.24±9.62	110.47±5.83
6^{th} day	138.05±8.00	115.20±9.97	139.00±16.02	121.50±17.65	134.40±13.00
12^{th} day	147.30±11.47	130.32±9.35	142.94±15.35	125.38±17.26	139.34±12.42
18^{th} day	157.07±8.60	141.90±8.20	149.40±14.57	133.90±17.92	152.14±14.02
24^{th} day	160.15±9.47	143.80±9.30	158.10±15.06	141.02±18.45	159.14±15.40
30^{th} day	174.95±7.61	149.92±10.45	166.48±15.87	148.36±19.06	171.28±11.53

Values are mean±standard deviation of quintuplicate. N = 5, NS = Normal Saline

Although the animals used in this study were fed with normal rat diet, the tigernut tuber extract might have allowed proper absorption of the nutrients which have allowed proper utilization of the nutrients. Low level of active/toxic principles may have stimulated appetite and increased feed utilization resulting in increased weight gain. The tuber of *C. esculentus* is used in making a refreshing beverage called kuunu in Nigeria which is consumed mostly in the Northern region of Nigeria (Belewa and Abodurin, 2008). There have not been any reported cases of toxicity in humans.

The present study confirms the tigernut tuber contains important nutrients and some essential macro and micro nutrient necessary for good human and animal health. Roasting the tuber as a processing step reduced the antinutrients composition. But unlike several other underutilized crops, it does not produce any undesirable effects even when consumed raw. The findings indicate that the tigernut tuber which is popularly eaten raw is rich in important food properties when compared with other crops has no negative effect, at least in rats and considering the economic situation in Nigeria and the near zero economic value of this tuber, its cultivation and consumption should be encouraged.

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The Relationship Between the Antioxidants Intake and Blood Indices of the Children with Thalassemia in Sabzevar and Mashhad

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Abstract: Most of thalassemic patients have with deficiency of anti-oxidants and increase of oxidative stress and few studies have been performed on deficiency of vitamins A, E, C in these patients. In this research we intend to study the antioxidants intake and its relationship with anemia indices in children with thalassemia. This study is a retrospective study which was performed on the children with thalassemia in Sabzevar and Mashhad cities. The sampling method was target-based. In this study, 85 patients with thalassemia were examined. After taking written consent and filling the demographic questionnaire, their height and weight were measured through the common standard methods. Then, two questionnaires of two day meal reminder and meal frequency were completed for them (in order to determine the rate and pattern of receiving antioxidant in the present and the past) and 5cc of blood was taken from each of them so that the blood indices could be measured. The data gathered using statistical SPSS software and applying the correlation coefficient, linear regression, t test, Analysis of Variance (ANOVA) the relationships were analyzed. In this study 85 patients were studied, 48(56.5%) of whom were females and 37(43.5%) of them were males. The average age of them is 16.97±7.02 years-old and mean BMI of them was 19.03±3.71 kg/m^2. The mean antioxidants received in these patients are vitamin A 883.35±1140.58 mcg, vitamin E 4.08±3.89 mg, vitamin C 204.40±238.71 mg and selenium 160±120 mcg, respectively. Among these antioxidants only vitamin C has a direct and significant correlation with hematocrit in the patients ($p<0.05$). In this study, between receiving vitamin A, MCV and MCH levels in the patients with thalassemia an inverse and significant relationship was observed.

Key words: vitamins A, E, and C, antioxidants, thalassemia

INTRODUCTION

Thalassemia syndromes, heterogenic group is of heredity anemia which is created due to deficiency type I making one or more globin chain. The thalassemia manifestations are widespread and range from asymptotic hypochromic and microcytosis to severe anemias and result in intrauterine death or death during childhood if not treated (Arzanian *et al*., 2005).

Iran is one of the countries in the world which has been located on the thalassemia belt. In the Caspian sea regions and the southern Iran, 10% of people are carriers of thalassemia genes and in the other regions this rate ranges from 4-8% and based on the reports by General Department for Disease Control of the Ministry of Health, Treatment and Medical Education, the number of patients with major beta-thalassemia is estimated to be 12494 people nationwide and considering the cases not yet diagnosed or cases with mean symptoms, it has been estimated to be around 15000 people (Hagh Shenasi *et al*., 1997).

These patients are encountered with various complications such as cardiovascular, pulmonary, neural, psychological, ophtalmological, dermal, bilious, joint, ENT, endocrine disorders and the cardiac complications is one of the most prevalent and important causes of death in these patients which appear at the second decade of life and after manifestation, they cause heart failure within a short time, the workload of the heart increases due to the hypoxia and sclerosis of the systemic vessels and the precipitation of iron in myocardium and subsequently results in the increase of oxidative stress and death (Hagh Shenasi *et al*., 1997; Aessopos *et al*., 2007).

Today, different studies indicate that high levels of oxygen together with hemoglobin containing iron attacking the non-saturated fat acids RBC result in producing free radicals(ROS) in the red blood cells of the thalassemic patients which through changing red-ox status of these patients and intensification of the oxidative stress, the complications of these disease are also intensified (Tesoriere *et al*., 2006; Cheng *et al*., 2005). The antioxidant systems include super-oxide demostasis and catalysis enzymes and the samples containing tioul such as glutathione and peroxyredoxin

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and also vitamin E are able to reduce oxidative stress (Cheng *et al*., 2005). In the recent years, the role of antioxidants on the improvement of oxidative stress and the heart failure has been emphasized. With regard to the fact that most diseases resulting from antioxidant deficiency and the increase of oxidative stress and few studies made in this respect, C, E, A deficiencies in these people have been considered (Tesoriere *et al*., 2006; Dhawan *et al*., 2005; Cheng *et al*., 2005). In this study, we intend to investigate the status of antioxidants received and the relationship between the anemia indexes in the children with thalassemia.

MATERIALS AND METHODS

This study is a retrospective research which has been performed on the thalassemic children in Sabzevar and Mashhad cities. The sampling method is target-based. This study has been performed on 85 subjects selected from among the patients with thalassemia. The sample was obtained concerning vitamin A which the most required sample has been estimated among the variables with type 1 error 0.05 and the test potential of 80%. The thalassemic children referred to the Vasee Hospital and Thalassemia Hospital in Mashhad (Sarver clinic) who have been prescribed blood transfusion and had already done three blood transfusions during a week and received 450 cc pack cell each time, were selected as subjects. After taking written consent and filling demographic questionnaire, their height and weight was measured through standard methods. Then, two day food reminder questionnaire and meal frequency questionnaire were filled for them (in order to determine the level and pattern of receiving antioxidants in the present and the past) and 5cc blood was taken from them for measuring the blood indices.

Data collection instruments in this study include questionnaire and laboratorial checklist. For determination of reliability of the researcher-made questionnaires, content validity and equivalent reliability were used for evaluating the credibility of the research. The questionnaire and lab check lists were assessed by two university professors. The lab methods and food process application are also standard.

The data gathered with the SPSS software and through correlation coefficient, linear regression, t test, ANOVA (for eliminating confounding variables) were analyzed.

RESULTS

In this study, 85 patients were examined [48 (56.5%) were females and 37 (43.5%) were males]. The average age of the patients was 16.97±02 and their average BMI was 19.03±3.71 kg/m^2. Among these patients 47.8% had family background with thalassemia and 52.2% did not have any history of this disease. 86.1% of them were with minor and 5.1% were with major type and both types of thalassemia were seen in the family history of 8.9% patients. 42.9% of the people were affected by splenomegaly after being affected with thalassemia, 30.7% of them were splenectomized. The average antioxidants received and the hematological indices of the patients and their relationship with one another are presented in the Table 1, 2 and 3, respectively.

Table 1: Mean±SD of antioxidants intake in thalassemic patients

Antioxidant	Mean±SD
Vitamin A (mcg)	883.35±1140.58
Vitamin E (mg)	4.08±3.89
Vitamin C (mg)	204.40±238.71
Selenium (mcg)	160±120

Table 2: Mean±SD of blood indices in thalassemic patients

Blood indices	Mean±SD
Hemoglobin	9.72±1.70
Hematocrit	28.61±3.08
Ferritin	3147.94±1855
MCV	80.70±4.83
MCH	26.90±1.95
MCHC	33.54±4.58
RBC	3.51±0.35

DISCUSSION

In this study, the average antioxidants received excluding vitamin C is lower than the standard amounts in the thalassemic patients. Only vitamin C is about three times more than the amount received. Claster *et al*. (2009) indicated that 40-75% of the patients with thalassemia are with antioxidant deficiencies such as vitamins A and C and Selenium (Claster *et al*., 2009).

In the study made by Livrea, in the thalassemic patients as compared with healthy people, the vitamin C rate of serum was 44%, vitamin E of serum was 42%, vitamin A of serum was 44%, Beta carotene of serum was 29% and lycopen of serum was 67% lower. He believes that the low level of vitamins E and A in serum of these patients is due to the disorder in the liver function, increase of oxidative processes and receiving low amount of foods rich in these vitamins (Livrea *et al*., 1996).

Therefore, the results of this study are supported by Ali *et al*. (2003). In the study which was performed on 63 patients who were 2-18 year old with thalassemia and 62 healthy subjects, the vitamin E, selenium and zinc in the serum in the people with thalassemia were lower than the control group (Ali *et al*., 2003).

Livrea *et al*. (1998) studied that 35 patients with thalassemia between 10-60 years of age indicated that the vitamin E level and beta-carotene in the LDL-C of these patients are 45 and 24%, respectively. In the healthy people, it is somewhat correspondent with the results of our study. In addition, in his study, oxidized LDL-C of patients with thalassemia are triple and the MDA level of them is double than the healthy people which represents an increase in the oxidative stress in these patients (Livrea *et al*., 1998).

Table 3: Correlation between antioxidants and blood indices in thalassemic patients

	Hb	Hct	Ferritin	MCV	MCH	MCHC
Vitamin A (mcg)	NS	NS	NS	P = 0/001 R = -0/36	P = 0/01 R = -0/28	NS
Vitamin E (mg)	NS	NS	NS	NS	NS	NS
Vitamin C (mg)	NS	P = 0/007 R = 0/3	NS	NS	P = 0/02 R = 0/25	NS
Selenium (mcg)	NS	NS	NS	NS	NS	NS

Table 4: Mean±SD of energy and nutrients intake in thalassemic patients

Energy and nutrients	Mean±SD
Energy	2397.61±1065.23
Carbohydrate	335.13±136.60
Protein	92.01±57.93
SAFA	23.64±15.60
MUFA	28.56±15.45
PUFA	18.21±9.81
Calcium	1271.64±1271.54
Phosphor	1200.98±743.66
Sodium	1143.33±771.57
Potassium	2681.45±1784.56
Iron	26.10±11.15
Zinc	10.06±7.03

Also, the study made by Dissagabutra (2005) indicated that vitamins C and E levels, glutathione and the Total Antioxidant Content (TAS) in these patients were low. This supports our results about the deficit in vitamin E and contradicts with our results about receiving the vitamin A (Dissagabutra *et al*., 2005). As you can see in the findings of this study, the hematocrit and hemoglobin levels of the serum are lower than the normal and the ferritin of serum is higher than normal. Dissagabutra believes that the low level of hemoglobin in the blood is due to the over-hemolysis of the red blood cells because of synthesis of un-natural hemoglobin and on the other hand due to the increase of producing free radicals such as peroxide hydrogen and destruction of the cell membranes, RBCs (Dissagabutra *et al*., 2005).

Lisboa believes that by inhibiting the destruction of red blood cells, antioxidants are protectors of RBCs and prevent from their hemolysis. In his study performed on 24 thalassemic patients (18 patients with major thalassemia and 18 with thalassemia sickle badge), receiving daily 500-1000 mg vitamin E during one year, then reducing lipid peroxidation and MDA in Red Blood Cells, the half life of the RBCs were increased and the average of annual hemoglobin increased (from 10.52-11.96 gr/deciliter) (Costa, 1986).

Livrea believes that production of free radicals by ferritin of serum has a direct relationship and in his study, he has demonstrated that the concentration of vitamin A of the serum has an inverse relationship with ferritin and the production of free radicals (Livrea *et al*., 1996). In our study, due to the low intake level, no relationship was observed. In Cay and Naziroglu, (1999) also receiving antioxidants such as vitamin A and Selenium for five consecutive weeks by the mice could not change hematological indices: MCV, MCH, MCHC as compared with control group (Cay and Naziroglu, 1999).

In our study, there is a significant and direct relationship only between receiving vitamin C and hematocrit which can be due to the high reception of vitamin C by the patients. The Chen *et al*. (2000) results support our results, these researchers stated that overload of iron in the presence of vitamin C reduces oxidative stress and the production of iso-prostan F2 and vitamin C deficit creates pathologic changes especially as increasing triglycerides of the serum and oxidized products are increased (Chen *et al*., 2000). Gerster indicated that reception of vitamin C has been useful for thalassemic patients and has adversely affected incurrence of heart disease (Gerster, 1999). Dissagabutra states: "Combined consumption of vitamins E and C has promoted the antioxidant status of the thalassemic patients and has improved their liver function in them and bilirubin of the serum has been reduced (Dissagabutra *et al*., 2005).

Furthermore, in this study between receiving vitamin A, MCV and MCH levels, an inverse and significant relationship was observed. Up to the present time, no studies have been made on the effect of the vitamin A on the MCV indexes (average globulin volume) and MCH (mean hemoglobin cells) so that we can compare our findings with them. But Bazuaye has suggested that between the reception of vitamin A and anemia megaloblastic in which MCV increases, there is an inverse relationship (Bazuaye *et al*., 2005). Ozdem *et al*. (2008) showed that patients with acid folic and B12 vitamins deficit are affected with degrees of megaloblastic anemia (Ozdem *et al*., 2008). In our study, reception of vitamin A was at the borderline and such a relationship was observed. Katerels believes that low amount of vitamin A and protein carrying (RBP) in the thalassemic patients can be the cause for the abnormal performance of liver in these patients (Katerelos *et al*., 1979).

Conclusion: The results of this study indicate that there is deficiency in receiving total antioxidants excluding vitamin C in the patients with thalassemia and regarding the benefits of these micronutrients for these patients, the necessity of consuming vegetables and fruits which are replete with antioxidants is recommended.

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Use of Response Surface Methodology in Predicting the Apparent Viscosity of 'Achi' *Brachystegia spp.* Flour

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Abstract: Response Surface Methodology (RSM) was used to obtain the predicted values of the apparent viscosity of 'achi' flours from different toasting time. The processing variables were processing time (pt), salt concentration (sc), palm oil concentration (poc). The data generated from the experiment was analyzed by regression analysis. Linear and quadratic effects of processing time and palm oil concentration were significant ($p<0.05$). Salt concentration had no linear or quadratic effect ($p>0.05$) on apparent viscosity of 'achi' flour. The Coefficient of determinations (R^2) for the fit was 0.816 (82%). This high R^2 value showed that the model developed for the response variables appeared adequate for predictive purposes. The experimental and predictive values were closely related showing that the model correctly predicted the response variables.

Key words: Response surface methodology, predicting, 'achi' flour, apparent viscosity

INTRODUCTION

Food crops including cereals and legumes, roots and tubers are processed for different reasons. For example cocoyam can be processed as soup thickeners or as chips for preservation of the product. Ndjouenkeu *et al.* (1996) reported that many polysaccharides currently available as industrial gums were first used in an empirical way in domestic cookery. Obvious examples include pectin as the setting-agent in jam, carrageenan in milk desserts and starch as thickener in soups and sauces. There are many others, however, that are not yet exploited commercially, but are used extensively in traditional local recipes, particularly in the less industrialized regions of the world. These materials usually come from plants that grow wild or are cultivated only on a very limited scale and their functional properties as food hydrocolloids remain largely unexplored.

In Africa, the main culinary use of indigenous hydrocolloids is in thickening soups and stews and the plants used include okra *(Hibiscus esculentus)*, 'ogbono' dika nut *(Irvingia gabonensis)*, mbol (*Belschmiedia zenkeri*), Kelekelin *(Triumfetta Cordifolia)*, 'akparata' *(Afzelia africana)* and 'achi' *(Brachystegia spp)* (Ndjouenken *et al.*, 1996; Anonymous, 2005). The first two of these are the most common in domestic cooking in the Central and Western parts of Africa.

Okra gum, cocoyam flour, has received some scientific attention, unlike *Brachystegia spp* 'achi' which is popular in the Eastern part of Nigeria. The tree is a woody plant found mostly in the rain forest zone and the seed is seasonal but its use in soup making is not seasonal. The seeds can be processed in large quantity and preserved to eliminate the inconveniences encountered by the home-maker in getting food ready for the table and also to improve their storage potential (Keay *et al.*, 1974; Okaka, 2005).

Food crops may be processed as intermediate products or processed for immediate consumption. When processed as base material for other food manufacture, they should have satisfactory intrinsic properties such as nutritional values and acceptable flavor, color and texture, as well as possessing additional critical functional properties that make them compatible with and if possible, enhance the food to which they are added (Wang and Kinsella, 1976). For example, adding protein prevents fat or water from separating during heating of a meat product and also forms stable emulsions or foams (Iwe, 2003). Herh *et al.* (2000) reported that in food products, small changes in the amount of additives can have a dramatic effect on the final product.

Viscosity is an important functional property of foods such as beverages and batters and design of processing lines (Iwe, 2003). Lewis (1987) and Steffe (1996) reported that viscosity is often very important for quality control, particularly on products that are expected to be of a particular consistency in relation to appearance or mouth feel, for instance cream, yogurt, tomato paste and custards.

The functional properties of food additives, condiments and thickeners are factors to consider before their choice. These properties are often affected by the conditions under which the ingredient is applied, such as medium (fat and water), the presence of any other ingredients, acidity, ion strength and temperature and processing time in particular (Iwe, 2003).

Because of the many processing variables involved in the investigation and their corresponding interactions,

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response surface methodology was employed to obtain regression equation models for predicting the response variables of apparent viscosity.

Response surface methodology (RSM) is a statistical technique for investigating multiple parameters alone or in combination, on response variables. It was developed by Box and Wilson (1951) to study the relationship between a response and several related factors. Its applications in different processing area were reviewed by Hill and Hunter (1966). Myers *et al.* (1989) reviewed the evolution of RSM from 1966 to 1988, including progress in experimental design, data analysis and applications. RSM has been successfully applied for predictions and optimizing of conditions in food research (Sefa-Dedeh and Stanley, 1979; Iwe, 2000).

Prediction of apparent viscosity of 'achi' based on such processing variables as processing time, salt conc. and palm oil conc., has not been widely reported. The objective of this study is to apply response surface methodology to develop a model equation for predicting apparent viscosity of 'achi' flour.

MATERIALS AND METHODS

Sources of samples: Samples of *Brachystegia spp* 'achi' were purchased from Eke-Aba, market in Abakaliki, Ebonyi State, Nigeria.

Preparations of samples: Achi' (*Brachystegia Spp*) seeds were sorted and cleaned. The dry seeds were toasted in a hot sand bath for 6, 9, 12 and 15 minutes respectively using kerosene stove and dehulled while hot with stone. The dehulled seeds were milled four times in a traditionally corn mill (Corona corn mills) to produce 'achi' powder. The milled powders were then sieved with American standard sieve number 40, with aperture of 435µm. The different flours obtained were packaged, labeled and stored in airtight polyethylene before analysis.

Viscosity measurements: The method of Sathe and Salunkhe (1981) was adopted in determining the viscosity of the flour samples. Sample dispersion 2.0 % (w/v) was prepared with distilled water at room temperature (28±2°C) under continuous stirring (British magnetic stirrer). The viscosity of the hydrated dispersion was measured at 28°C±2 using the NDJ-8S digital viscometer. Measurements were made on 2% (w/v) dispersion of each flour sample at constant time intervals of 2 hours with a shear-rate (30/m).

Effect of palm oil concentration on apparent viscosity: Dispersion 2% (w/v) of each flour was prepared with palm oil in concentrations between 0-2.5% (w/w). The dispersions were hydrated for 2 hours with continuous stirring. Apparent viscosity of each dispersion was measured at 25±1°C using the NDJ-8S digital display viscometer.

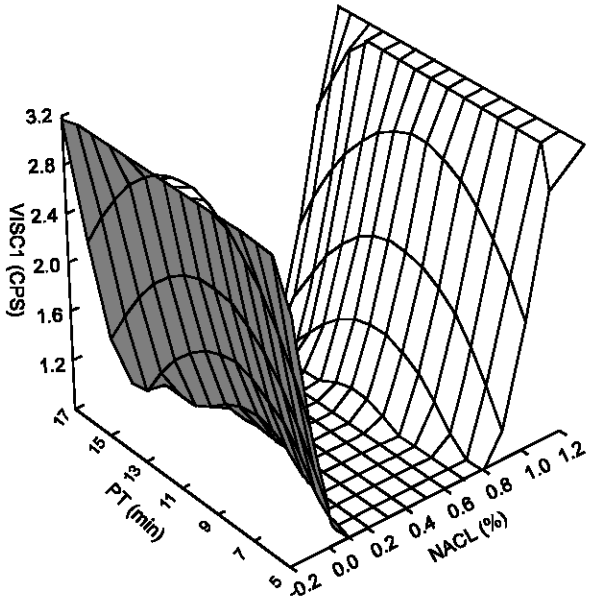

Fig. 1: Effect of processing time and NaCl concentration on the apparent viscosity of 'achi' flour.

Effect of sodium chloride (NaCl) concentration on apparent viscosity: Dispersion 2% (w/v) of each flour was prepared with sodium chloride solution in concentration between 0.0M and 1.0M according to the method of McWalters and Homes (1979). The dispersions were hydrated for 2 hours with continuous stirring. Apparent viscosity of each dispersion was measured at 25±1°C using the NDJ-8S digital display viscometer.

Experimental design: A three factor central composite design CCD (King, 1993; Cochran and Cox, 1957) was used. The independent variables were processing time (pt), palm oil concentration (PoC) and Salt concentration (Sc).

Statistical analysis: Experimental data was analyzed using response surface methodology. The second-order polynomial fitted was

$$\beta_o + \beta_1 pt + \beta_2 PoC + \beta_3 Sc + \beta_{11} pt^2 + \beta_{22} PoC^2 + \beta_{33} Sc^2 + \beta_{12} ptPoC + \beta_{13} ptSc + \beta 23 PoCSc + \beta_{123} ptpoCSc + e$$

Where,

Y is the response variable as previously described to be predicted, β_o is the intercept, β_1, β_2, $\beta_{3\text{---}}$ β123 are the estimated coefficients and pt, poil and Sc are the independent variables.

The regression was fitted using Statistic Program.

RESULTS AND DISCUSSION

The response of dependent variables to the processing conditions is shown in Table 1 and the linear and quadratic effects of the variables, P. oil concentrations

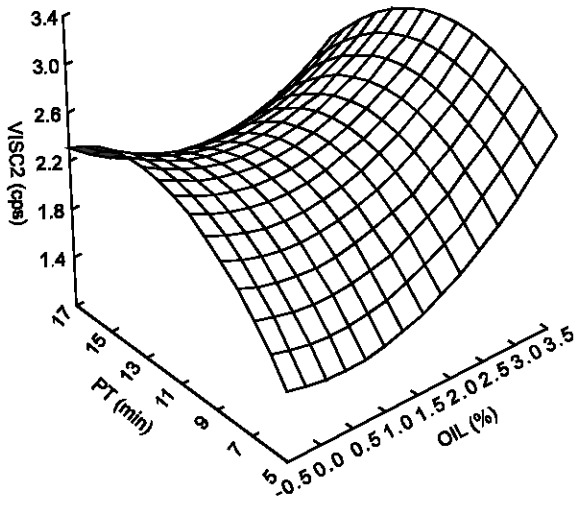

Fig. 2: Effect of processing time and oil concentration on the apparent viscosity of 'achi' flour.

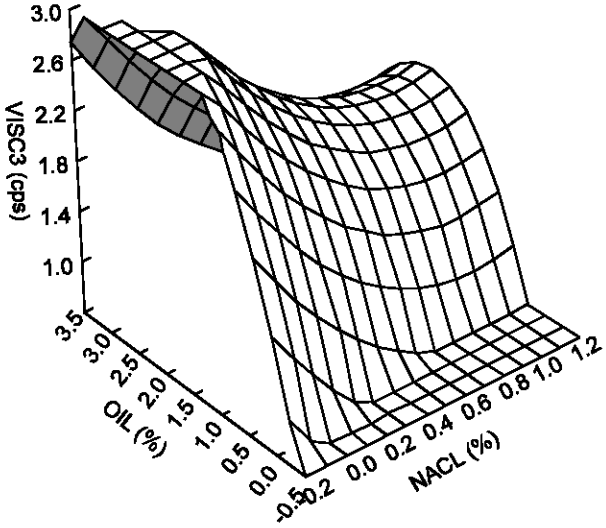

Fig. 3: Effect of oil concentration and salt concentration on the Apparent Viscosity of at different processing level, oil and salt concentrations of 'achi' Flour.

Table 1: Regression coefficient

Parameters	Coefficient	Std Error	p-value
Constant	4.010	1.593	0.025
Pt	0.728	0.257	0.013
PoC.	1.940	0.338	0.000
Sc.	2.693	1.409	0.077
Pt^2	-3.111	0.012	0.020
PoC^2	-0.605	0.095	0.000
Sc^2	-1.195	0.987	0.246
PtPoC.	5.946	0.043	0.193
Pt.Sc.	-9.892	0.093	0.304
PoC Sc.	-1.975	0.022	0.389
R^2	0.806		

Table 2: Analysis of variance (ANOVA) for regression model of apparent viscosity obtained from the surface experiments

Regression	df	Sum of squares	Mean square	F-value	R^2
Regression	1	4.780	0.956	11.620	0.81
Residual	14	1.152	0.082		
Total	19	5.931			

and Pt were significant. Oil concentration had significant ($p<0.05$) linear and quadratic effect on viscosity, while salt concentration showed no significant ($p<0.05$) effect linear and quadratic.

In this study the effect of processing variables on viscosity of "achi" flours presented in Table 1, showed that the coefficient of determination R^2, for the model equation was calculated to be 0.806, which was considered high enough for prediction purposes, this showed that the regression model was very suitable for describing viscosity under varying conditions of processing time, palm oil concentration and salt concentration within the limits of the experimental design. The result demonstrated that the response surface had a maximum point at the code level 10.5 (Pt), 2.475 (PoC) and 2.8708 (Sc).

Table 3: Experimental and predicted values of apparent viscosity of 'achi' flour

S/N. Processing Time (mins).	Palm oil Con (%)	Salt Con (M)	Exptal. value (cps)	Predicted value (cps)
	0.0	0.0	0.917	0.920
	1.0	0.4	2.639	2.640
6.0	1.5	0.6	2.067	2.070
	2.0	0.8	2.017	2.020
	2.5	1.0	1.983	1.980
	0.0	0.0	0.883	0.880
	1.0	0.4	2.783	2.780
9.000	1.5	0.6	2.017	2.020
	2.0	0.8	1.967	1.970
	2.5	1.0	1.550	1.550
	0.0	0.0	0.967	0.970
	1.0	0.4	2.250	2.250
12.000	1.5	0.6	2.050	2.050
	2.0	0.8	1.983	1.980
	2.5	1.0	1.950	1.950
	0.0	0.0	0.900	0.900
	1.0	0.4	1.983	1.980
15.000	1.5	0.6	1.917	1.920
	2.0	0.8	1.617	1.620
	2.5	1.0	1.117	1.120

As can be seen from the response surface plot (Fig. 1, 2 and 3), the apparent viscosity increased when the concentration of salt was decreased and both the processing time and palm oil concentration increased. Fig. 1, shows the interaction between the processing time and salt concentration, with resultant increase in viscosity as salt concentration was decreased, while Figs. 2 and 3 shows the interaction of processing time and palm oil concentration and the interaction of the

three processing variables respectively. There was an increase in apparent viscosity with (Figs. 2 and 3) increase in processing time and palm oil concentration. This increase was probably affected by the conditions under which the ingredients were applied, such as concentration and processing time (Iwe, 2003).

This model was tested for adequacy by the analysis of variance (Table 2). The regression model for apparent viscosity was highly significant ($p<0.001$). Table 2, also suggests that viscosity was primarily determined by the linear term and quadratic terms of the processing time and palm oil concentration. The results also showed that the response surface had a maximum point based on the response surface plot and the response model. The maximum response predicted by the model was 3.10cps.

The experimental and predicted values of the response variable, shown in Table 3 are closely related. This shows that the model correctly predicted the viscosity of "achi" flour. The model equation therefore could be applied in predicting the apparent viscosity of food systems or other legumes with similar processing conditions.

Conclusion: The Response Surface Methodology was effective in predicting the apparent viscosity of 'achi' flours. Results indicated that the variables Pt and PoC were significant on apparent viscosity. The effect of processes variables on apparent viscosity result could be ranked in the following order PoC (x3) > Pt (x1) > Sc (x3). Response variables predicted with model equation under processing conditions were in general agreement with experimental data.

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Tissue and Blood Amino Acids Composition of an Ecotype Cichlid 'Wesafu', *Tilapia zillii* and *Oreochromis niloticus* Using Paper Chromatography

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Abstract: Wesafu is an indigenous ecotype cichlid and a very important part of the fisheries of Epe lagoon in Lagos Nigeria. Investigation of the amino acids composition of tissue and blood samples of Wesafu, *T. zillii* and *O. niloticus* using paper chromatography (Ranjna, 1999) was conducted. Only 14 amino acids (Alanine, Cysteine, Asphatic acid, Phenylalanine, Glycine, Histidine, Isoleucine, Lysine, Leucine, Methionine, Threonine, Valine, Tryptophan, Glutamic acid) were analyzed. In the muscles, 11 amino acids were identified with alanine, guanine and methionine absent in all three fish tissue sampled. Phenylalanine, isoleucine and valine were absent in *O. niloticus* but present in Wesafu and *T. zillii* while Tryptophan and Glutamic acid were present in *O. niloticus* but absent in the tissues of Wesafu and *T. zillii*. However, all 14 AA assayed were present in different proportions in the blood samples of the three species. This report further suggests that the Wesafu is different from either of the two species and warrant species identification at a level of molecular biology.

Key words: Wesafu, *T. zillii*, *O. niloticus*, chromatography, amino acids, tissue, blood

INTRODUCTION

The gradual increase in the population of fish farming over the last sixty years has been linked with advances in aquatic biology and search for alternative food sources. FAO (2003) reported 210 different farmed aquatic animal and plant species to include 131 Finfish species, 42 Mollusca species, 27 Crustacean species, 8 Plant Species and 2 Amphibian and reptile species. Cichlids, commonly called the "Tilapias" of the family Cichlidae are perch-like fish and the family is known to have produced an enormous variety of species in Africa freshwaters. Trewavas (1983) classified cichlid into three genera namely: *Tilapia*, *Oreochromis* and Sarotherodon. These genera are further sub-divided into two groups (Nwadukwe, 1991) based on their breeding habits; they are the guarders and bearers.

New species are genetic resources, within communities and ecosystems and their potentially useful attributes are coded in the genes of these species. Therefore, all concerns with conservation and preservation of new species should be geared toward determination of their identity and potential resource worth for research and science. Domestication for aquaculture is centuries behind crop and livestock breeding. There is a wide array of aquatic species to be accurately identified and their populations characterized for aquaculture potential. Amongst the fin fishes; the tilapias, African fishes of the family Cichlidae, continue to generate high interest for aquaculture around the world.

As tilapia farming progresses and farmed breeds are developed, such characterization will increasingly require descriptors based on molecular genetics. However, given the similarity in appearance of the tilapias (especially in their juvenile stages) and their high propensity to hybridize when transferred for aquaculture or fisheries purposes or when they escape from fish farms. There continues to be the immediate need to characterize new tilapia species and hybrids in natural waters in Nigeria, as a subject for research and aquaculture development studies.

MATERIALS AND METHODS

Experimental fishes: Table sized species of Wesafu, *T. zillii* and *O. niloticus* with average weights and lengths of 1200±52.75 g; 44.55cm±1.26cm, 925±18.12 g; 38±3.45 cm and 765±1.12 g; 36±2.48 cm respectively were used in the study. The fish were obtained from Epe Lagoon and a private fish farm in Badagry, Lagos, Nigeria.

Amino acid profiles: Amino acids profiles in the muscle and blood of Wesafu, *T. zillii* and *O. niloticus* were assayed using paper chromatography to arrive at R_f values of standard amino acids calculated thus:

$$R_f = \frac{\text{Distance traveled by the substance}}{\text{Distance traveled by the solvent front}}$$

(Ranjna, 1999)

Procedure: The following procedure as described by Smith and Feinberg (1973) and Stahl (1969) was followed:

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- A line was drawn along the width of Whatman No. 1 chromatography (20 cm x 20 cm) paper about 8 cm, away from the edge
- Small volumes of each of the amino acids solution were applied with the help of capillary tubes to place a small spot
- The chromatogram was inserted in the chromatography chamber which had earlier been equilibrated with solvent
- The chromatogram was run using solvent N-butanol: Acetic acid: Distilled water in the ratio 12:3:5
- After 3 hours, the chromatogram was removed from the chamber and allowed to dry in air
- The dried paper was sprayed with the Ninhydrin in 1% alcohol solution and dried
- The paper was kept suspended for about 8 hours at room temperature (30°C) after which, each amino acid was noticed to have migrated and gave a purple colour spot
- Each spot was marked and R_f values of standard amino acids calculated, and the identification of the amino acids was done in the given solution

R_f = Distance traveled by the substance/Distance traveled by the solvent front (Ranjna, 1999).

Statistical analysis: Data generated were subjected to Analysis of Variance (ANOVA). The significance of difference between means was determined by Duncan's multiple range test ($p<0.05$) using SPSS for windows (Version 11). Values are expressed as means±SE.

RESULTS

Amino acids composition: The results are presented in Fig. 1, 2; Table 1 and 2 respectively.

Table 1 shows the amino acids compositions in the tissues of Wesafu, *T. zillii* and *O. niloticus*.

Table 2 shows the amino acids compositions in the blood of Wesafu, *T. zillii* and *O. niloticus*, also indicated the 14 AA assayed present in different proportions in the blood samples of the three species.

DISCUSSION

The contribution of Nigeria's cichlid aquaculture to world total output is nil, in spite of the potentials and available resources. Tilapia aquaculture in the country is not attractive for several reasons including stunting, low market value, and the lack of developed commercially viable species. Research into the development of a commercial strain has not been conducted and this, in turn, is responsible for the lull in the Cichlid aquaculture industry in the country. Identification of individual species of fish is therefore, very important in aquaculture production as this help in maintaining the purity of discrete stock and introgression of the cultured fishes into the wild (Betiku and Omiogun, 2006).

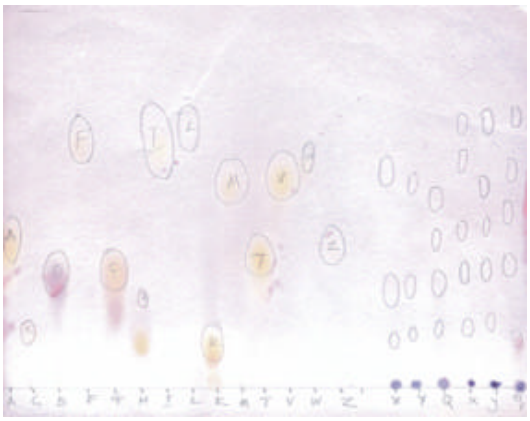

Fig. 1: Amino acids composition in tissue samples of Wesafu, *T. zillii* and *O. niloticus*. A (Alanine), C (Cysteine), D (Asphatic acid), F (Phenylalanine), G (Glycine), H (Histidine), I (Isoleucine), K (Lysine), L (Leucine), M (Methionine), T (Threonine), V (Valine), W (Tryptophan), Z (Glutamic acid), X (Wesafu tissue), Y (*T. zillii* tissue), Q (*O. niloticus* tissue), x (Wesafu blood), y (*T. zillii* blood), q (*O. niloticus* blood)

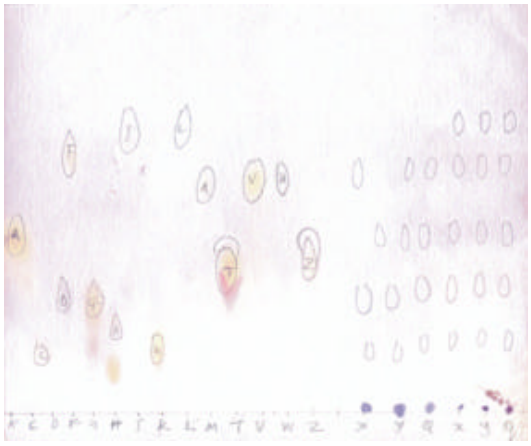

Fig. 2: Amino acids composition in the blood of Wesafu, *T. zillii* and *O. niloticus*. A (Alanine), C (Cysteine), D (Asphatic acid), F (Phenylalanine), G (Glycine), H (Histidine), I (Isoleucine), K (Lysine), L (Leucine), M (Methionine), T (Threonine), V (Valine), W (Tryptophan), Z (Glutamic acid), X (Wesafu tissue), Y (*T. zillii* tissue), Q (*O. niloticus* tissue), x (Wesafu blood), y (*T. zillii* blood), q (*O. niloticus* blood)

Muscle and blood of Wesafu and *O. niloticus* were analyzed for amino acids composition (Table 1 and 2) (10 essential and 4 non essential). The result of assay showed that, 11 amino acids were present in tissue sample of Wesafu, *T. zillii* and *O. niloticus*, with Wesafu

Table 1: Composition of amino acid composition in the tissues of Wesafu, *T. zillii* and *O. niloticus*

		Tissue samples		
Amino acids	Code	Wesafu	*T. zillii*	*O. niloticus*
Alanine	A	-	-	-
Cysteine	C	0.16±0.01[a]	0.18±0.02[a]	0.19±0.01[a]
Asphatic acid	D	0.31±0.02[a]	0.31±0.02[a]	0.33±0.01[a]
Phenylalanine	F	0.61±0.02[a]	0.18±0.01[a]	-
Glycine	G	-	-	-
Histidine	H	0.31±0.00[a]	0.31±0.01[a]	0.33±0.02[a]
Isoleucine	I	0.61±0.02[a]	0.51±0.01[b]	-
Lysine	K	0.16±0.02[a]	0.18±0.01[a]	0.19±0.02[a]
Leucine	L	0.61±0.02[a]	0.51±0.03[b]	-
Methionine	M	-	-	-
Threonine	T	-	-	0.44±0.02[a]
Valine	V	0.61±0.01[a]	0.51±0.02[b]	-
Tryptophan	W	-	-	0.59±0.01[a]
Glutamic acid	Z	-	-	0.44±0.02[a]

Figures in the same horizontal row having the same superscript are not significantly different (p>0.05)

Table 2: Amino acid composition in the blood of Wesafu, *T. zillii* and *O. niloticus*

		Blood samples		
Amino acids	Code	Wesafu	*T. zillii*	*O. niloticus*
Alanine	A	0.45±0.01[a]	0.45±0.01[a]	0.46±0.02[a]
Cysteine	C	0.19±0.01[a]	0.20±0.01[a]	0.18±0.01[a]
Asphatic acid	D	0.33±0.02[a]	0.34±0.01[a]	0.34±0.02[a]
Phenylalanine	F	0.62±0.02[a]	0.74±0.02[b]	0.61±0.03[a]
Glycine	G	0.33±0.01[a]	0.34±0.01[a]	0.34±0.01[a]
Histidine	H	0.33±0.02[a]	0.34±0.01[a]	0.34±0.02[a]
Isoleucine	I	0.72±0.05[a]	0.74±0.04[a]	0.73±0.04[a]
Lysine	K	0.19±0.01[a]	0.20±0.01[a]	0.18±0.02[a]
Leucine	L	0.72±0.02[a]	0.74±0.03[a]	0.73±0.03[a]
Methionine	M	0.45±0.02[a]	0.45±0.03[a]	0.46±0.02[a]
Threonine	T	0.45±0.02[a]	0.45±0.02[a]	0.46±0.03[a]
Valine	V	0.62±0.03[a]	0.74±0.05[b]	0.73±0.04[b]
Tryptophan	W	0.45±0.02[a]	0.45±0.03[a]	0.46±0.03[a]
Glutamic acid	Z	0.45±0.03[a]	0.45±0.03[a]	0.46±0.04[a]

Figures in the same horizontal row having the same superscript are not significantly different (p>0.05)

having the highest value of Phenylalanine, Isoleucine, Leucine and Valine (0.61) followed by *T. zillii* (0.51). These 4 amino acids were completely absent in O. *niloticus* which however had both Tryptophan and Glutamic acid but were absent in Wesafu and *T. zillii* tissues. Alanine, Glycine and Methionine were completely absent in the tissue of all samples analyzed. However, all 14 amino acids analyzed were expressed in the blood samples of the 3 species without significant difference (p>0.05) in individual amino acids. The study suggests that the species are dissimilar and that the ecotype cichlid should not be confused with known species; *T. zillii* and O. *niloticus* buttressing the earlier suggestion of Fashina-Bombata *et al*., 2005a,b, 2006 and 2008 that Wesafu is unidentified cichlid. However, there is the need to establish the identity of Wesafu at a species level by molecular means.

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