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African Journal of Biochemistry Research

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ARTICLES

Full Length Research Paper

Proximate composition of *Rastrineobola argentea* (*Dagaa*) of Lake Victoria-Kenya

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Proximate composition analysis for crude lipid content, crude protein content, moisture content, total ash content and dry weight of *Dagaa* (*Rastrineobola argentea)*: a small pelagic fish which constitutes one of the main fish species in Lake Victoria was determined from selected beaches: Paga, Dunga, Rota, Usari and Nduru around Lake Victoria-Kenya. This was done with a view to provide nutritional data to guide food processing, industrial exploitation, preservation and consumption of the fish. On a wet weight basis (wwb), proximate composition values were established as crude protein content, kjedahl and biuret (19.1 - 21.7% and 1.93 - 5.80 mg/ml respectively), lipid content by Dyer and Bligh and Soxhlet (3.87 - 7.78 and 1.77 - 3.40% respectively), ash content (1.88 - 4.38%), ash content on a dry weight basis was (10 - 14.58%) and moisture content of (72.83 - 76.90%). Analysis of these results showed that there were significant differences (p < 0.01) in crude protein content, crude lipid content, total ash content and moisture content of *Dagaa* from the five landing sites. Hence, the inherent variations in *Dagaa* collected from the different landing sites were attributed to the difference in geographical locations of the sites. *Dagaa* was classified as a fatty fish (fat content >2%) based on Dyer and Bligh method. The high protein, ash and lipid content of *Dagaa* make it a nutritionally dense fish.

Key words: *Dagaa*, Lake Victoria, proximate composition.

INTRODUCTION

In most third world countries, food insecurity is increasingly becoming an issue of national concern (Owaga et al., 2010). Fish is an important food of high nutritive value. It is rich in essential nutrients; high quality protein with high digestibility and made of the ten essential amino acids in desirable quantities for human consumption, essential omega 3 fatty acids, vitamins A, B, D and a variety of minerals such as calcium, potassium, phosphorus, iron, copper and iodine required for supplementing both infant and adult diets (Ackman et al*.,* 1988, Gordon and Ratliff, 1992; Huss, 1988; Owaga et al., 2010; Saito

et al., 1997). As a result, the fish industry has been identified as one of the sectors that if improved would effectively contribute towards alleviation of food insecurity (Owaga et al., 2010). This can be achieved by directly contributing as a food resource and also through income and generation of employment (Baidu, 1997).

To ensure maximized utilization of the fish industry to achieve this goal, several fish species have been investigated for their proximate content (Ghelichpour and Shabanpour, 2011). Lake Sardine, locally known as "*Dagaa*" (*Rastrineobola argentea)* is a small pelagic fish

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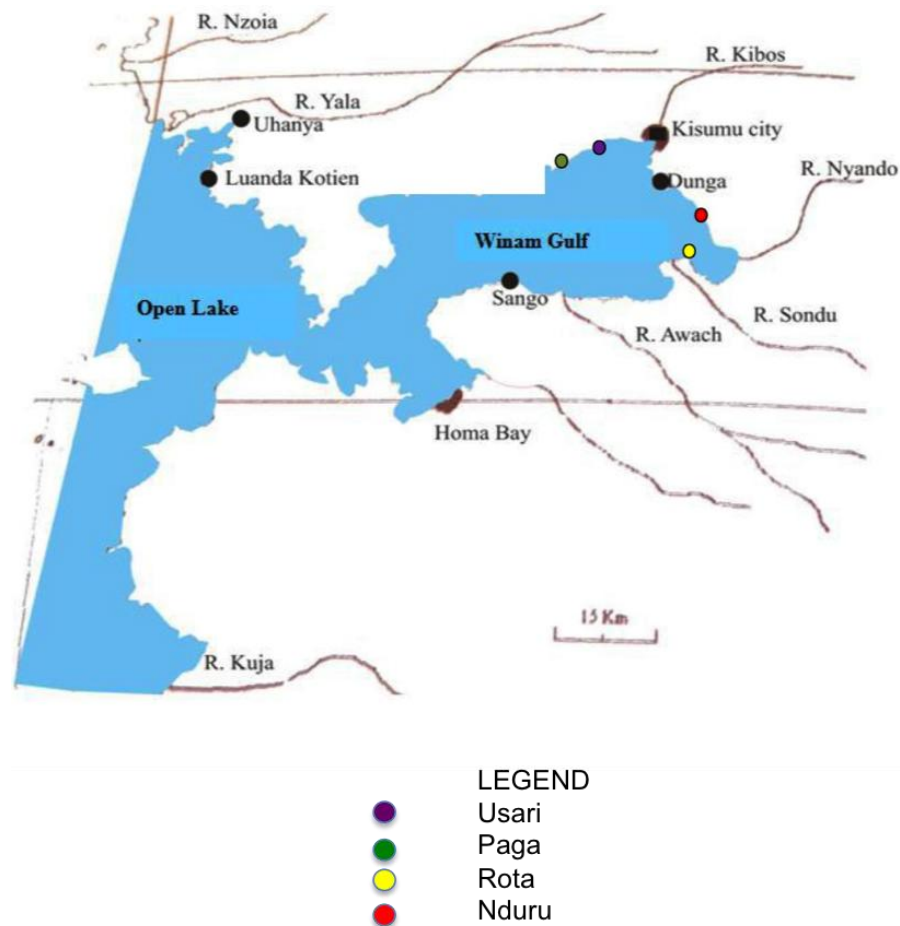
Figure 1. Dunga and other landing sites (Adopted from Onyango et al., 2009 with modifications).

that accounts for the second largest volume (62.9%) of the total fish catch along the Lake Victoria (Nyeko, 2008; Wanink, 1999; Witter et al., 1995).

It is also cheap in comparison with Nile perch (Owaga et al., 2010). Despite the large harvest of *Dagaa*, post harvest losses of 20 - 30% due to endogenous enzymes/-spoilage by microorganism and up to 50% during the rainy season are experienced (Bille and Shemkai, 2006). The rapid nutritive value depreciation due to endogenous enzymes signifies a high nutritive value that could be exploited.

Notwithstanding, its importance as a fish food for low income households, high numbers (30 - 50%) of malnu-trition has been recorded among the population around Lake Victoria (GOK and UNICEF, 1999), an indication that not much attention has been given regarding its nutritional composition. Consequently, its potential, as a rich nutrient source remains largely unexploited Indus-trially and nutritionally.

The objective of this study therefore was to investigate theproximatecompositionof*Dagaa"Omena"*(*Rastrineobola argentea)* from various points along the Lake Victoria in order to provide evidence on nutritional composition of

Dagaa that could guide its use in dietary consumption, food processing, industrial exploitation and preservation.

MATERIALS AND METHODS

Sampling

Fresh *Dagaa* (1 kg each) was sampled in labeled polythene bags from four fishermen from each of the landing sites: Dunga, Nduru, Paga, Rota and Usari around the shores of Lake Victoria (Figure 1) resulting in a sample weight of 4 kg from each landing site and a total sample weight of 20 kg (20 batches). The *Dagaa* was then transported for 2 h via flight to the laboratory (Chiromo campus - University of Nairobi, Kenya) in a cool box at 4°C, and each landing site sample divided into 200 g labeled batches and stored frozen at -20°C until analysis.

Preparation of fish mince

Two types of fish mince were prepared

A homogenate from well-mixed samples from the individual landing sites (Rota, Usari, Paga, Nduru and Dunga) was obtained and apportioned in 200 g batches. Each batch was then blended using a food blender. The individual landing sites' minced samples were

stored in 200 g batches at (-20°C) until analysis.

A second homogenate of the *Dagaa* representative samples (mixture of samples from the five landing sites) was obtained by mixing 200 g of mince from each individual site to constitute a 1 kg representative mince. This was then mixed well and repacked in polythene bags in 200 g batches, labeled numerically and stored frozen at (-20°C) until analysis.

The average *Dagaa* size of the representative sample and *Dagaa* size of the individual landing sites was measured.

Proximate composition

Upon usage, batches of individual sites/*D*agaa representative samples were thawed in a cold room (4°C) for 16 h before analysis

Total moisture/total dry weight

Crucibles were cleaned and dried in an oven (Memmert, USA), cooled to room temperature and then weighed on a weighing balance (Metler, Switzerland). The minced samples for proximate analysis were allowed to attain room temperature to ensure accuracy in weights taken before they were put into the dried crucibles and weighed in triplicates.

Total moisture content/dry weight was determined using air oven method according to AOAC method 950.46 (AOAC, 1995). The dry matter/moisture content was obtained as follows:

$$\text{Dry matter content (\%)} = \frac{\text{Weight of dried sample}}{\text{Original wet weight}} \times 100$$

Total ash

Total ash content was determined from pre-dried *Dagaa* samples according to the AOAC method 920.153 (AOAC, 1995). Ash content was determined as follows:

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Original wet weight}} \times 100$$

Total protein

Crude protein content was determined by the Biuret and micro-Kjedahl methods according to AOAC method 928.08 (AOAC, 1995). A factor of 6.25 was used to convert percent nitrogen to percent protein. Protein content was determined as follows:

$$\text{Total protein} = \text{\% Nitrogen} \times 6.25$$

Total lipid (fat)

Crude fat was determined using the solvent extraction method after soxhlet extraction according to AOAC method 991.36 (AOAC, 1995) and modified method of lipid extraction by Dyer and Bligh (1959). Lipid content was determined using the formula below:

$$\text{Lipid/100 g sample} = \frac{\text{Lipid in the tube} \times (\text{volume chloroform in total})}{\text{Amount of sample weighed in (g)} \times (\text{ml chloroform evaporated})}$$

Comparison of fat analysis methods

The data obtained using modified Dyer and Bligh described above was then compared with those obtained from crude fat analysis using soxhlet extraction method according to AOAC method 991.36 (AOAC, 1995).

Statistical analysis

The proximate values for moisture, lipid, ash and protein were a mean of triplicate laboratory determinations. The mean values for each of the individual landing sites was tested by comparing the respective proximate values for each landing site. The proximate composition determinations were done in a completely randomized block design. The differences in means among the landing sites was measured using ANOVA while Tukey HSD test was used to determine significant differences between means at 5% ($p < 0.05$) level of significance. Statistical analyses were performed using the SPSS statistical package (SPSS, 2007).

RESULTS AND DISCUSSION

This study aimed at determining the proximate composition of *Dagaa* (*Rastrineobola argentea*). The lengths of *Dagaa* edible portion and proximate analysis of *Dagaa* from each of the landing sites are summarized in Tables 1 and 2, respectively. The average *Dagaa* length ranged from 4.0 - 5.0 cm. Moisture content among the landing sites ranged from 72.83 - 76.90%, ash content on a wet weight basis (wwb) and dry weight basis (dwb) was between 1.88 - 4.38% and 10.00 - 14.58%, respectively. Lipid content on a wet weight basis as determined by Dyer and Bligh and soxhlet methods was 3.87 - 7.78% and 1.77 - 3.40%, respectively. Protein content by Kjedahl and Biuret method was recorded at 19.11 - 21.78% and 1.93 - 5.80 mg/ml, respectively.

Protein content (Kjedahl and Biuret), ash content (dwb and wwb), lipid content (Dyer and Bligh/Soxhlet) and moisture content between the landing sites were significantly different ($p < 0.01$) (ANOVA) (SPSS). A further comparison between the landing sites' mean values by Tukey HSD test was conducted. All landing sites showed significant differences between their moisture content at 5% confidence level. On a wet weight basis, ash content of Rota, Nduru and Usari were significantly different ($p < 0.05$) (Tukey HSD), Paga and Dunga had approximately the same ash content. Usari, Paga, Dunga and Nduru had approximately similar lipid content (Dyer and Bligh) whereas Rota had significantly different lipid content (Dyer and Bligh) at 5% confidence level. Dunga, Paga and Rota had a significant difference ($p < 0.05$) in lipid content determined by Soxhlet method. All the landing sites showed significant difference in protein content determined by Biuret method at 5% significant level (Tukey, HSD). Nduru, Dunga and Rota had approximately similar protein content by Kjedahl method. However, Usari and Paga had significantly different protein content as determined by Kjedahl at 5% confidence level level (Tukey, HSD). Co-relational analysis matrix (Table 3) indicates relationship between the proximal parameters.

Table 1. Lengths of *Dagaa* edible portion.

Landing site	Dunga	Nduru	Paga	Rota	Usari	Homogenous	Average
Fish length (cm)	5.0±0.40	4.8±0.05	4.0±0.03	4.5±0.04	4.6±0.02	4.7±0.34	4.6±0.34

The mean lengths; (in cm) of the edible portions of representative *Dagaa* (second homogenate) from the different landing sites along Lake Victoria.

Table 2. Proximate composition of Dagaa on wet weight basis (wet weight basis).

Landing sites	Moisture (%)	Dry weight (%)	Ash content (%) (wwb)	Ash content (%) (dwb)	Lipid content (%) Dyer/Bligh	Lipid content (%) soxhlet	Protein content (%) kjedahl	Protein content Biuret (mg/ml)
Usari	76.35±0.05[as]	23.65±0.05[b]	1.88±0.94[ah]	11.49±0.61[a]	4.35±0.57[a]	1.77±0.00[a]	19.11±1.80[ar]	4.19±0.03[ad]
Nduru	74.05±0.95[as]	25.95±0.95[b]	2.08±0.69[ah]	10.70±0.00[ax]	7.32±0.59[a]	1.82±0.00[a]	21.07±0.19[a]	1.93± 0.15[ad]
Dunga	72.83±1.30[a]	27.17±1.30[b]	4.38±0.38[a]	10.00±0.00[ax]	3.87±0.19[a]	3.40±0.00[aw]	19.53±1.03[a]	5.80± 0.04[ad]
Paga	74.37±0.40[as]	25.63±0.40[b]	2.36±0.14[a]	10.55±0.78[ax]	7.78±0.44[a]	2.18±0.32[aw]	21.78±1.79[ar]	5.22± 0.12[ad]
Rota	76.90±0.30[as]	23.1±0.30[b]	3.08±0.81[ah]	14.58±2.94[a]	5.47±0.10[ay]	2.77±0.00[aw]	20.31±0.88[a]	4.39±0.06[ad]
Homogeneous	78.40±0.89[as]	21.6±0.89[b]	4.26±0.79[ah]	10.83±3.54[ax]	3.11±0.45[ay]	4.90±0.10[aw]	18.77±0.03[a]	4.76± 0.06[ad]

Moisture content, dry weight, ash content (wet weight and dry weight basis), Lipid content (dyer Bligh and soxhlet) and protein content (kjedahl and Biuret) of *Dagaa* from the five landing beaches along Lake Victoria. a- values with significant differences ($p < 0.01$) using ANOVA. Values with same double letters have significant difference ($p < 0.05$) {Tukey (HSD)}. Values are shown as mean ± standard deviation for triplicate analysis of a pooled sample.

There was a true positive correlation ($p < 0.01$) between protein content by kjedahl and lipid content by Dyer and Bligh method. However, there was a negative correlation between lipid content by soxhlet method and protein content by kjedahl. The protein composition of fish affects the post harvest quality and characteristics with respect to oxidative changes in the muscle tissues (Owaga et al., 2010). Previous studies conducted, showed that deep-sea fishes are high in protein and low in fat as compared to pelagic fish (Suseno et al., 2010). However, in this study, Dagaa protein values 19.1 - 21.8% were higher than those reported for deep-sea species (11.9 - 20.6 %). The lipid values (4.4 - 7.8 %) on the other hand were also higher than those reported for deep-sea species whose fat content ranged from 0.01 - 4.84% (Suseno et al., 2010). The high lipid and protein

values for *Dagaa* could be attributed to the fact that the edible portion is 100% (whole fish) despite its average length of only 4.6 cm; that is relatively small as compared to the deep sea fishes whose lengths range from 14 to 49 cm (Suseno et al., 2010).

Fish can be grouped into four categories according to their fat contents: lean fish (<2%), low fat (2 - 4%), medium fat (4 - 8%) and high fat (>8%) according to Dyer and Bligh (Huss, 1988; Ackman, 1989). Also, it could be classified based on the >5% (dwb) fat composition criteria for discriminating lean from fatty fish species according to soxhlet (Owaga et al., 2010). Dyer and Bligh and Soxhlet method classified *Dagaa* as fatty fish (Table 4); fat content >2 and >5% (dwb), respectively.

Previous studies have reported that the percent

moisture content of fresh fish is inversely related to the lipid content (Jahncke and Gooch, 1997). *Dagaa* non-polar lipid content is inversely related to its water content. However, its polar lipid content is directly proportional to water content as was confirmed in this study. *D*agaa from Dunga site, which recorded lowest moisture content (72.83%), reported the highest non-polar lipid content (3.40%) by Soxhlet method. Similarly, Dunga reported the lowest polar lipid content (3.87%) by Dyer and Bligh method.

The difference in the fat yields using the two methods; 3.9 - 7.9% by Dyer and Bligh and 1.8 - 3.40% by soxhlet could be attributed to difference in solvent polarities. Dyer and Bligh is more efficient in extraction of phospholipids (polar lipids) while soxhlet is more efficient for extraction of non-polar lipids e.g. triglycerides. However, the

Table 3. Correlation matrix of the proximate parameters.

Component	Moisture	Dry weight	Ash (wwb)	Ash (dwb)	Lipid (dyer/bligh)	Lipid (soxhlet)	Protein (Kjedahl)
Moisture	1	-.293	-.175	.494[a]	-.395	.402	-.379
Dry weight	-.293	1	-.166	-.182	.408	-.232	.537[a]
Ash (wwb)	-.175	-.166	1	.613[b]	.575[b]	.624[b]	.-.465
Ash (dwb)	.494[a]	-.182	.613[b]	1	-.500[a]	.681[b]	-.455
Lipid (dyer/Bligh)	-.395	.408	-.575[a]	-.500[a]	1	-.700[b]	.815[b]
Lipid (soxhlet)	.402	.232	.624[b]	.681[b]	-.700[b]	1	-.471[a]
Protein (kjedahl)	-.379	.537[a]	-.465	-.455	.815[b]	-.471[a]	1

A correlation study was done using SPSS v. 16.0. 1 shows perfect correlation. a shows significant correlation at the 0.05 level (2-tailed). Whereas, b shows significant correlation at the 0.01 level (2-tailed).

Table 4. Proximate composition of *Dagaa*: Comparison of fat analysis by Dyer and Bligh and soxhlet extraction methods (wet weight basis).

Sample ID	Soxhlet (g lipid /100 g fish)	Dyer and Bligh (g lipid /100 g fish)
Homogenous	4.909 ± 0.10	3.11 ± 0.45
Usari	1.77 ± 0.00	4.35 ± 0.57
Nduru	1.82 ± 0.00	7.32 ± 0.59
Dunga	3.40 ± 0.00	3.87 ± 0.19
Paga	2.18 ± 0.32	7.78 ± 0.44
Rota	2.77 ± 0.00	5.47 ± 0.10

Mean values of lipid content of representative samples from the six landing beaches determined by Soxhlet and Dyer Bligh methods. Values are shown as mean ± standard deviation.

difference in amount of lipids in *D*agaa from the different landing sites could be attributed to, water, temperatures, sex, age, season of the year, food availability and salinity of the different geographical locations of the landing sites (El Tay et al., 1998; Stansby, 1981).

Total ash content of *Dagaa* (1.88 - 4.38%) is relatively higher than those of deep-fish species (Suseno et al., 2010) with lower edible portions. This is because *Dagaa* is consumed whole (edible portion is 100%) unlike deep-sea fish species with edible portions of only 5 - 63% (Suseno et al., 2010). The high total ash content in comparison with other fresh water species is as a direct result of inclusion of skeletal muscles in the edible portion that contains calcium and iron in high amounts; 3600 mg/100 g and 10.2 mg/100 g, respectively (Ghelichpour and Shabanpour, 2011 and Owaga et al., 2010).

Conclusion

Fish industry, through *Dagaa* provides a solution to food insecurity. *D*agaa has been shown to be rich in proteins, minerals and lipids therefore a rich and relatively cheap inclusion for human dietary.

Its high nitrogen (protein) content makes it a rich Industrial bio resource for fish protein hydrolysate production for human/animal consumption. The high protein content also makes it viable for inclusion as an ingredient in bacteriological media. The high nitrogen content coupled with high ash content makes it viable for use as a foliar fertilizer and in aquaculture and poultry as feed. Its high lipid content makes it a viable raw material for bio ethanol production.

This information is important in informing industrial processing parameters for optimum yields, development of more efficient methods of preservation to ensure long shelf life and to preserve nutrient content and quality since the high nutrient content is also adequate for microorganism activity and subsequent spoilage. As well as making informed human dietary consumption choices. The inherent proximate composition variations for all landing sites around the lake should be studied.

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Full Length Research Paper

The protective role of oral consumption of N-acetyl cysteine during a single session of exhaustive exercise in untrained subjects

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The aim of the current study was to verify the effect of N-acetyl cysteine (NAC) supplementation on markers of oxidative stress and inflammatory response during a single session of exhaustive exercise. In a randomized placebo-controlled double-blind clinical trial, thirty healthy, untrained young males and females with a mean age of 21.33±2.39 years, weight of 59.63± 9.24 kg and height of 166.20±10.15 cm were selected and divided into 2 groups. Before starting the supplementation, blood samples were collected from all the participants. The next blood samples were collected just before the exercise started, immediately after the exhaustive exercise and after one hour of rest. Malondialdehyde (MDA), total antioxidative capacity (TAC), CRP, BMI and Vo_2 max were determined. A significant increase was observed in MDA and CRP levels during the experiment in the placebo group. But in the treated group, the concentrations remained the same throughout the experiment. The TAC levels were significantly raised in the samples collected after NAC supplementation as compared to the placebo group. No changes were observed in the time to fatigue. Results of the current study suggest that oral consumption of n-acetyl cysteine for 24 h before a single bout of an exhaustive physical exercise could significantly reduce the harmful effects of oxidative stress.

Key words: N-acetyl cysteine, oxidative stress, exhaustive exercise, reactive oxygen species, time to fatigue.

INTRODUCTION

There have been several investigations to show that, exhaustive exercise especially in untrained individuals can cause oxidative stress and lead to increase in the production of reactive oxygen species in different tissues (Valko et al., 2007). Overproduction of reactive oxygen/-nitrogen species known as pro-oxidants can result from

different stressors including vigorous physical exercise (Poljsak et al., 2011).

As the consumption of oxygen increases during exhaustive physical exercise, it leads to acute state of oxidative stress (Poljsak et al., 2011). It has been shown that the initial stage of inflammatory processes involve an increase in the production of highly reactive oxygen species (Xinyuan et al., 2013). Also it is well known that highly reactive oxygen species can easily react with macromolecules including lipids, proteins and DNA within the cells (Urso and Clarkson, 2003). Polyunsaturated fatty acids in the cell membranes are the most frequent targets; they undergo a chain of oxidative reaction called lipid peroxidation which leads to decrease in the membrane fluidity and making it more difficult for the proteins and nutrients to pass through (Bloomer et al., 2007). Several factors such as duration, intensity, fitness, breed; health, athletic ability and environmental conditions are likely to have an impact on the severity of oxidative stress and damage (Fisher-Wellman et al., 2009; Vollaard et al., 2005).

Following the cessation of a session of exhaustive exercise, especially in untrained subjects, excessive generation of pro- oxidants occurs in the muscles and tissues Sen et al. (2000). Strenuous exercise in an unconditioned individual or someone unaccustomed to exercise will induce oxidative damage and result in oxidative stress and then tissue injury Evans (2000).

Hence, supplementation with varieties of anti oxidants to reduce free radical production and subsequent oxidetive damage during and following an exhaustive physical exercise has been a priority of much research activity (Bloomer et al., 2007; Fisher- Wellman et al., 2009). N-acetyl cysteine (NAC) is a by-product of glutathione which is a tri-peptide in the cytoplasm of the cells and acts as a reducing agent (Childs et al., 2001). Both glutathione and NAC are antioxidants and they have the ability to minimize oxidative stress and its negative effects on different tissues (Kretzschmar et al., 1991). Always intense and unaccustomed exercise could lead to oxidative stress and its detrimental effects (Alessio et al., 2000). N-acetyl cysteine has been used in clinical practice to facilitate glutathione (the major non-enzymatic endogenous antioxidant) biosynthesis, thereby improving the intra-cellular enzymatic antioxidant defense system and possibly decreasing the damaging effects of reactive oxygen species (Zhang et al., 2011). The role of glutathione as a reducing agent in ameliorating the complications of exercise induced oxidative stress has been studied by many investigators (Fisher- Wellman et al., 2009). Furthermore, it has been shown that NAC decreases free radical production and oxidative stress both at rest and during pro-

longed exercise (Jammes et al.,2005). N-acetyl cysteine significantly attenuated the rise in plasma [K+] followed by fatigue during a prolong endurance exercise. This result confirms that the antioxidant NAC attenuates muscle fatigue, in part via improved K^+ regulation, and points to a role for reactive oxygen species in muscle fatigue (McKenna et al., 2006)

To the best of our knowledge there has been no study to show that oral consumption of pure NAC can ameliorate the possible damages caused by the generation of reactive oxygen species during a single session of exhaustive exercise in untrained subjects. The results of an investigation suggest that treatment with NAC represents an important factor in the defense against muscle soreness and has different effects on oxidative damage and pro- and anti-inflammatory cytokines following a session of exhaustive exercise (Silva et al., 2008). The aim of the present study was to evaluate the protective role of oral N-acetyl cysteine during a single bout of exhaustive exercise in untrained young individuals.

MATERIALS AND METHODS

Study design and participants

In a randomized placebo-controlled double-blind clinical trial, thirty healthy untrained young males and females (Age = 21.33±2.39 years) from Azad University of Bushehr, Iran, were divided into two groups; the placebo and treated groups of 15 each. For randomization, the eligible participants received the treatment tablets and the placebo in random order. The order of the treatments was randomly generated by computer and sealed in sequentially numbered opaque envelopes and assigned to subjects in the order of their first study visit. Both the clinical team and participants were blinded from the time of randomization until analysis was complete. A questionnaire containing the information about their foods consumed and the physical activity they had for the past 6 months, was filled by each individual. The main inclusion criteria were participants aged between 21 and 25, either sex, having V_{O_2} max between 35 and 40 ml/kg/min and living in the same dormitory and all having the same type of food. The subjects who were consuming energetic drugs, smokers and those having any type of regular sport activity were excluded from the study. None of the participants were on antioxidants supplementation. All the experimental procedures were performed in accordance with the Helsinki Declaration and the policy statement of the American College of Sports Medicine on research with human subjects. The ethic Committee of Bushehr University of Medical Sciences approved all the experimental protocols. Also, prior to participating in this investigation, informed written consent was obtained from all subjects.

Participants in the intervention group received 600 mg doses (four times with an interval of 6 h) of effervescent tablets of N-acetyl cysteine (Zambon, Srizzera SA, Swissmedics Company), starting from 24 hours before the exhaustive exercise. The last dose was given just one hour before the exercise. The control group consumed dextrose 5% dissolved in water (it has no side effect

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Table 1. The general characteristics and anthropometric parameters of the treated and the control group.

Parameter	Placebo	Treated	P-values
Age (years)	21.33 ± 2.39	21.33 ±2.33	0.823
Height (centimeter)	166.83±11.18	166.2±10.15	0.0923
Weight (kg)	66.2± 12.67	59.63± 9.24	0.543
BMI (kg/ m^2)	23.93±4.45	21.55±2.47	0.323
f.m (kg)	16.89±8.58	14.06±6.12	0.091
f.f.m (kg)	49.37±10.57	45.61±10.46	0.561
f.m (%)	25.20±10.04	23.84±9.98	0.087

Data is presented as mean ±SD, BMI = body mass index, f.f.m = fat free mass, f.m = fat mass and P <0. 05 are considered as significant.

when consumed orally). The clinical trial was registered as Irct ID: IRCT201208238129N2.

Procedures

Anthropometric measurements and the general characteristics of the participants including age, sex, height, weight, fat free mass (f.f.m), fat mass (f.m), Vo$_2$ max and body mass index (BMI), were recorded. Height was measured to the nearest 0.5 cm, and the weight, to the nearest 0.1 kg. Body fat composition was measured by using X-Scan plus II, JAWON Medical, Korea, which provides segmental obesity and segmental edema. Vo$_2$ max was determined according to Cooper Standard Aerobic Test (distance in meters minus 504.9 divided by 74.44). Body mass index was calculated as weight in kilograms divided by the square of height in meters (Grundy et al., 2004). The first blood samples were taken 24 h before starting the experiment (time 1). The second sample was collected at time 2 (just before the experiment started) and immediately after the exhaustive exercise utilizing Bruce examination protocols in the form of flat treadmill running (time 3). The last sample was collected after one hour of rest (time 4). The serum samples were immediately separated and stored at -80°C for further analysis. Malondialdehyde, the byproduct of lipid peroxidation was measured by using an improved thiobarbituric acid reactive substance (TBARS) based method (Manciet and Copeland, 1992). The determination of total antioxidant capacity was performed using commercial Kits from Biovision-Co, USA which uses Trolox equivalents to standardize the antioxidants. C - reactive protein was measured by ELISA technique based on the principle of a solid phase enzyme-linked immonosorbent assay. Inter assay coefficient of variation (CV) for the method was <20%.

Statistical analysis

Normal distribution of the data was controlled with the Kolmogorov-Smirnov test. Probability values <5% were considered statistically significant. The significance of the difference in the results between the two groups was determined with Chi-square analysis using 2 x 2 contingency tables. Independent unpaired t tests was used for continuous normally distributed variables. The Mann-Whitney U Test was used to compare differences between the two independent groups when the dependent variable was continuous but not normally distributed. The Wilcoxon signed-rank test was used as an alternative to the paired Student's t-test for dependent samples when the variables cannot be assumed to be normally distributed. Kruskal-Wallis test was used to compare independent variables across several groups. Spearman correlation coefficient was used to test relationship between different variables. All statistical analy-

ses were performed using the PASW Statistics 16 (SPSS Inc., Chicago, IL).

RESULTS

The general characteristics and anthropometric parameters of the treated and the control group are given in Table 1. There was no difference in age, sex, weight and BMI between the two groups (Table 1). The concentration of CRP in the control group increased during the experimental procedure but, it was significant only at one hour after the exhaustive exercise (Figure 1). However, in the treated group the concentration remained the same during the study (Figure 1). The concentration of malondialdehyde in the serum samples of treated subjects remained the same during the study (Figure 2). However, in the control group the concentrations raised significantly at times 3 and 4 during the experiment (Figure 2). The blood levels of total antioxidant capacity at time 1 (before the consumption of N-acetyl cysteine) was the same in both groups (Figure 3), but at times 2, 3 and 4 the concentrations decreased significantly in the control group (Figure 3). In the treated group, the total antioxidant capacity levels were significantly raised at times 2 and 4 but at time 3 no change was observed (Figure 3). Moreover, oral consumption of n-acetyl cysteine starting from 24 h before the exhaustive exercise had no significant effects on the values of Vo$_2$ max and then, the time to fatigue (Table 2).

DISCUSSION

The novelty of this study was the oral consumption of NAC (600 mg, without any other antioxidant ingredients) by the participants who were doing exhaustive exercise for the first time. The results of the present study showed that the serum concentration of MDA and CRP known as the markers of lipid peroxidation and cell damage respectively, keep on increasing in the subjects undergoing an intense and unaccustomed exercise. This is in accordance with the results from other investigators showing that plasma TBARs and CK levels increased after a 45

Figure 1. The differences in the concentration of CRP between the two groups (treated and the control). The stars show a significant difference.

Figure 2. The differences in the concentration of MDA between the two groups (treated and the control). The stars show a significant difference.

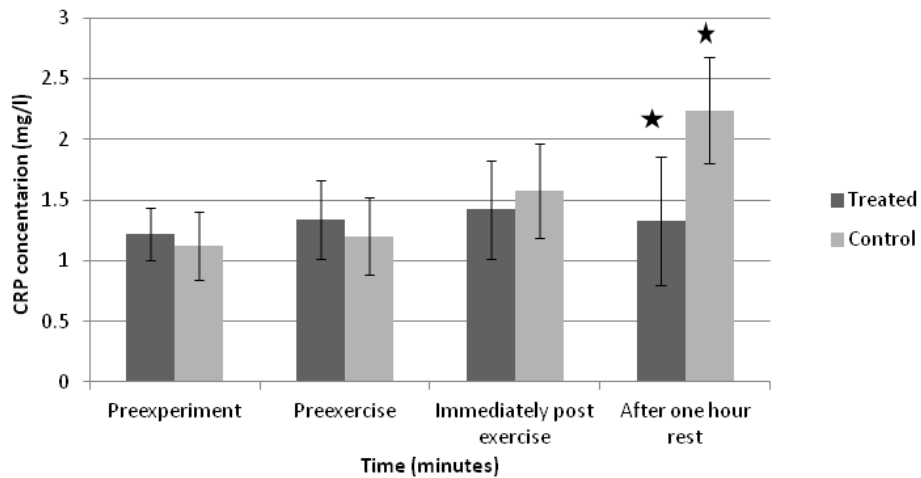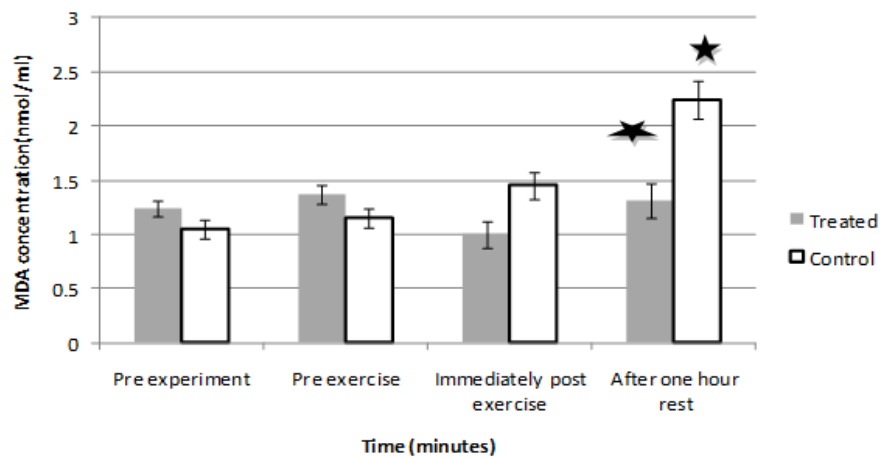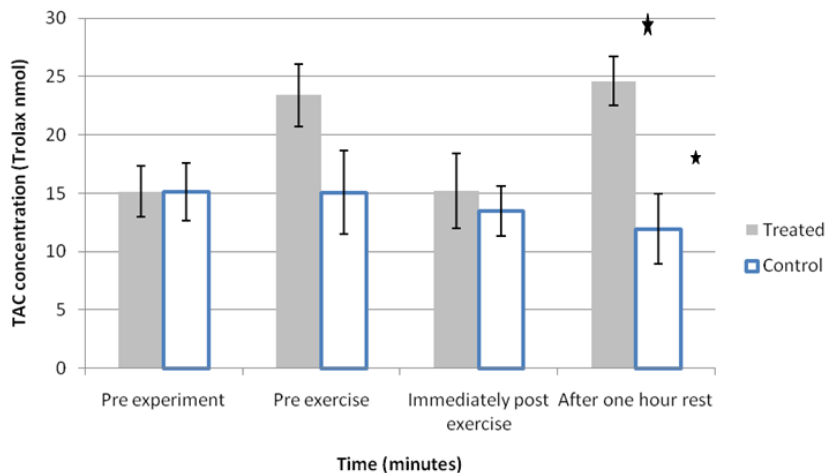**Figure 3.** The differences in the concentration of TAC between the two groups (treated and the control). The stars show a significant difference.

Table 2. The comparison of Vo_2 max between the case and the control group, before and after the exercise.

Treated group		Placebo group	
Before the exercise	After the exercise	Before the exercise	After the exercise
36.07±9.30	20.44±9.35	32.38±6.75	21.95±15.12

Data is presented as mean ±SD.

min session of flat treadmill running (Maughan et al., 1989; Kanter et al., 1988). Different studies by using various forms of exercise have reported significant increase in plasma levels of malondialdehyde (Bryant et al., 2003; Ramel et al., 2004; Rodriguez et al., 2003). Also, generation of reactive oxygen species has been found to be increased after exhaustive aerobic and isometric exercise (Alessio et al., 2000). However, changes in MDA due to the intense exercise are not reported consistently (Kelley et al., 2006). There are several studies suggesting that regular aerobic exercise has the potential to lower the concentrations of inflammatory biomarkers including CRP in individuals with conditions associated with elevated inflammation (Andersson et al., 2010; Lakka et al., 2005; King et al., 2003). Our study has shown elevated CRP level after an intense exhaustive exercise in young untrained individuals with no sign of inflammation. This shows that compared with mild and regular aerobic exercise, intense exhaustive physical training especially in unaccustomed subjects can result in the elevation of inflammatory markers. Several studies show that the more intense the exercise the more production of reactive oxygen species and the oxidative stress (Chevion et al., 2003).

The result of some studies confirmed that a correlation exists between Vo_2 and oxidative stress (Chevion et al., 2003). However, other findings show that intense aerobic exercise does not increase the markers of oxidative stress (Chevion et al., 2003). These contradictory results could be explained by antioxidant nutritional status. It was also found that trained subjects can exhibit oxidative stress as well as unaccustomed subjects (Pincemail et al., 2000; Palazzetti et al., 2003). Potential mechanisms of increased generation of reactive oxygen species include the leakage of electron from electron transport chain during mitochondrial respiration and subsequent production of superoxide anions, activity of xanthine oxidase in the catabolism of nucleoproteins pathway, autooxidation of catecholamines or the activity of NAD(P)H oxidase (Sen et al., 2000).

The results of our study showed that, the serum MDA and CRP levels were not changed in the group taking the antioxidant N-acetyl cysteine, as compared to the control group. This is in line with the result from an investigation which shows that administration of N-Acetyl cysteine (1200 mg), for 8 days and in two doses has reduced malondialdehyde significantly (Zembron-Lacny et al., 2009). The results of previous study indicated that NAC

at 1,200 mg daily can reduce oxidative stress from short exhaustive exercise. However, this still needs more proof because low levels of free radicals that produced in mitochondria are very important for normal muscle function, and they increase more with stronger force (Wilmore et al., 2008). The *in vivo* and *in vitro* studies have shown that the synergic consumption of N-acetyl-cysteine and antioxidants such as vitamin C and vitamin E might act as pro-oxidant by inhibiting the defense mechanism or adaptive response from exercise induced free radicals (Ristow et al., 2009). Also, some studies concluded that production of low concentration of free-radicals during a moderate bout of exercise, in fact induces the expression of intra cellular antioxidant enzymes including super oxide dismutase, catalase and glutathione peroxidase as a defense mechanism (Carmen et al., 2008). However, in many other investigations N-acetyl-cysteine has been used as an effective antioxidant (Wilmore et al., 2008; Waris and Ahsan, 2006). In our study, the blood total antioxidant capacity of the subjects in the control group decreased significantly. However, it was elevated in the treated group during the experiment. This is in agreement with the results from some studies but not all (Peake et al., 2007). The result of an investigation has suggested that even with short bouts of high-intensity exercise, NAC is effective at promoting a positive redox balance within the cell (Peake et al., 2007). This supports our results regarding the benefits of using NAC orally during short sessions of high-intensity exercise. Recently, it was reported that supplementation with vitamin C and NAC together increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise (Childs et al., 2001). In our study, we used pure NAC (without vitamin C) to examine the impact of NAC alone on oxidative stress. It was found that the total antioxidant capacity decreased immediately after the exhaustive time in the treated group; this may be because of the antioxidant components used to quench the over production of harmful radicals at this particular time. This was supported by the investigation which shows that the antioxidant capacity may be temporarily reduced during and immediately post exercise (Teixeira, 2009; Kerksick and Willoughby, 2005; Steinberg et al., 2006). In our study, there was no change observed in the time to fatigue regarding the subjects treated with NAC as compared to the control. This is in agreement with the result reported that N- acetyl cysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans

(Kerksick and Willoughby, 2005). However, the result from an investigation concluded that NAC may be helpful at delaying fatigue as well at the building of oxidative stress; they suggested that more systemic, translational research on humans needs to be conducted (Teixeira et al., 2009). The result from an investigation suggested that even with short sessions of high-intensity exercise, NAC is effective at promoting a positive redox balance within the cell (Massimo et al., 2004; Bloomer and Goldfarb, 2004). However, the result from a recent clinical trial has shown that, thiol-based antioxidant supplementation enhances GSH availability in skeletal muscle, but at the same time it could disrupt the skeletal muscle inflame-matory response and repair capability, potentially because of a blunted activation of redox-sensitive signalling path-way (Michalilidis, 20013). Finally, we conclude that oral administration of N-acetyl cysteine starting from one day before a single session of exhaustive exercise pro-gram can significantly reduce the harmful effects of oxidative stress, but not time to fatigue. We suggest that total level of thiols which are highly altered by NAC and also the activity of antioxidant enzymes such as GPx, GR which are directly related to GSH be determined in future studies.

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Full Length Research Paper

A microassay for quantification of 2,2-diphenyl-1-picrylhydracyl (DPPH) free radical scavenging

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Polyphenols are the second largest abundant organic molecules in nature. One of the principal reported biological activities is the antioxidant capacity. 2,2-Diphenyl-1-picrylhydracyl (DPPH) method has been used to quantify polyphenols antioxidant activity in many different products with reproducible and reliable results. We proposed and validated a methodology to analyze this antioxidant activity with smaller amount of sample and reagents, using DPPH. Technique validation was done through three ways: 1) between reported and proposed methodologies, 2) in the micro-plate assay and 3) between two different laboratories. Results between methods showed no significant differences. Results at different times, in the same day, had differences. Intra-day proposed assay results showed significant differences in days, turn and standard concentration; at the same time, no significant differences were found in sample solutions. Analysis from two different laboratories was validated. This result confirms that microscale DPPH scavenging quantification is a reliable alternative to save reactants and time.

Key words: Antioxidant activity, 2,2-diphenyl-1-picrylhydracyl (DPPH), micro plates, polyphenols.

INTRODUCTION

Antioxidant activity is, in recent times, one of the most important determinations in phytochemistry and cell biology; because several mortal diseases such as cancer are related to free radicals and their interaction with human cells (Daker et al., 2008). To attempt this activity, the trend is to use natural compounds in food formulations or in medicines.

Polyphenols are the second largest abundant organic molecules in nature containing more than one phenolic (C_6H_6OH) group. One of the principal biological activities related to these secondary metabolites is its antioxidant activity (Sreejalekshmi and Krishnan, 2011). On the other

hand, polyphenols extraction, purification and characterization have been done for several plants and addition to food identified as functional foods can be a potential application (Swapna and Sabu, 2011).

There are some techniques to achieve antioxidant activity; they are grouped into two principal categories: assays based on hydrogen atom transfer (HAT) reactions and electron transfer (ET). ET is the most known techniques, because it measures the antioxidant capacity in the reduction of an oxidant, which changes color when is reduced; color change degree is correlated with antioxidant concentration in the tested samples.

Folin-Ciocalteu reagent, Trolox equivalence antioxidant capacity, ferric ion reducing antioxidant power, total antioxidant potential assay using Cu (II) complex as an oxidant, ABTS$^{\cdot+}$ reduction and DPPH methodologies were classified as ET (Huang et al., 2005).

2,2-Diphenyl-1-picrylhydracyl (DPPH) is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH is an excellent monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay. It is a stable free radical because its spare electron delocalization over the whole molecule, gives the violet color to DPPH solutions; when an hydrogen atom is donated to the molecule, a stable non radical form of DPPH was formed changing its color to pale yellow (Szabo et al., 2007). DPPH method has been used as an alternative to quantify polyphenols antioxidant activity in large scale (Bendini et al., 2006) giving reliable results.

DPPH technique is one of the most useful methodologies to determine the antioxidant activity (Bendini et al., 2006); at the same time is one of the most inconsistent methodologies because of its many variables involved. Among the variables that influence DPPH response are ion concentration, sample concentration, concentration and standard type, reaction time and analysis of results. If the researchers keep constant the variables, except one, the methodology become reliable; in this case samples concentrations were identified as dependent variable. Due to the easy method manipulation it has been used to proposed novel analysis in antioxidant activity (Szabo et al., 2007; Locatelli et al., 2009; Ciesla et al., 2012; Amatatongchai et al., 2012).

In this analysis, we proposed a technique to analyze the antioxidant activity of polyphenols samples, using smaller amount of sample and reagents with DPPH method.

MATERIALS AND METHODS

Chemicals

DPPH was purchased from Sigma (St Louis, EUA) and it was dissolved to 60 µM in absolute methanol (Jalmek, Mexico). Tannic acid (Jalmek, Mexico) in concentrations ranging from 10 to 50 ppm was used as standard. As difficult samples, tannic acid solution of 25 ppm and aqueous extracts of fermented oregano wastes were probed. Tannic acid concentrations were adjusted to have similar absorbance profile to aqueous extracts from fermented oregano wastes.

Experimental protocol

Procedure published by Moylenux (2004) for antioxidant assay was used as base for miniaturization of reaction, using tubes as reaction vessels. In the original article, the measurement of antioxidant activity was done with 3 mL of final volume. In our microassay the final volume was adjusted to 200 µL (193 µL of DPPH-methanol and 7 µL of antioxidant sample) and the absorbance of the solutions was determined by a micro-plate reader (Epoch BioTek, USA). Reading of the absorbance of the DPPH-antioxidant

reactions were taken after 30 min; all the assays were done in 3 repetitions of each sample (standard and antioxidant samples) and 3 replicates from one solution.

A DPPH-methanol solution was prepared and its absorption wavelength was determined by a screening in visible range. For this, 2.5 mg DPPH were solved in 5 mL methanol (≈ 1.27 mM). This stock solution was daily prepared, used for the measurements, and kept in the dark at ambient temperature when not used. Using this stock solution enables the measurement of a calibration curve at the wavelength of maximum absorption for calculating the DPPH concentration with a correlation coefficient (R^2) of 0.999. Methanol was used as blank (200 µL) and DPPH-methanol as control. To avoid DPPH light degradation microplates were covered with aluminum foil.

Validation of microassay

To calculate the method´s reproducibility, precision and accuracy, samples absorbance were determined twice a day (intra-day) for 3 consecutive days (inter-days). DPPH reduction percentage was calculated using with the follow equation:

1. $\% \, DPPH \, reduction = \left[(A_{control} - A_{sample})/A_{control} \right] * 100$

Where: $A_{control}$ is methanol-DPPH 60 µM absorbance at 734 nm; A_{sample} is tannic acid solution or aqueous extracts of fermented oregano wastes absorbance at 734 nm.

Method validation was done in three ways: 1) between reported and proposed methodologies, 2) in the micro-plate assay and 3) at two different laboratories.

To obtain the statistic validation of the proposed methodology, results were analyzed according to the guidelines of the international conference on harmonization of technical requirements for registration of pharmaceutical for human use (ICH). Comparison was done with ANOVA, Tukey test and repeatability results. Evaluated parameters were methodology (reported or proposed), standard concentration and reaction time.

Validation of the proposed micro-plate assay was done according to the guidelines of the international conference on harmonization of technical requirements for registration of pharmaceutical for human use (ICH) and recommendations of Wang et al. (2007). Evaluated parameters were days, samples, standard concentration and turns. The results were analyzed by ANOVA, Tukey test, regression coefficient, variability coefficient, repeatability, reproducebility, interaction between the days and turns (I), measurement system repeatability and reproducibility (R&R), measurement system turn variation (V_P) and total measurement system variation (V_T). Equations employed were:

2. $Repeatability = 5.15\sqrt{MSE}$

where MSE is medium square error

3. $Reproducibility = 5.15\sqrt{\dfrac{MSA - MSAB}{bn}}$

where MSA is medium square of days, MSAB is medium square of day*turn interaction, b is turn number and n is repeats number.

4. $I = 5.15\sqrt{\dfrac{MSAB - MSE}{n}}$

5. $R\&R = \sqrt{Repeatability^2 + Reproducibility^2 + I^2}$

Statistic measure	Obtained value
Repeatability	0.092
Reproducibility	0.381
I	0.099
R&R	0.404
V_P	0.077
V_T	0.412
R^2	0.980
Variation coefficient	5.436

6. $V_P = 5.15 \sqrt{\frac{MSB - MSAB}{an}}$

7. $V_T = \sqrt{R\&R^2 + V_P{}^2}$

Data analysis

For inter laboratories validation, the evaluated parameters were days and standard concentration; results were analyzed by ANOVA and Tukey test.

All the ANOVA's, Tukey tests, regression and variation coefficients was calculated using the statistical computer software SAS (Version 9.0), with $\alpha = 0.05$ confidence level; equation solutions was obtained by Excel (Microsoft Office, 2007) and plots were done in Sigma Plot (Version 11.0).

RESULTS AND DISCUSSION

As first step to use DPPH as scavenging H^{+} atoms, the analyst should verify the working wavelength in the selected equipment (Molyneux, 2004). After the screening, in this study selected wavelength was 517 nm. Several literature reports suggest that the wavelength for DPPH quantification must be 515, 517 or 520 nm, but it depends on the lamp equipment (Fernandez-Orozco et al., 2011).

Data analysis for method validation is by using mathematical and statistical approaches (Araujo, 2009). Results between methods (reported in literature and proposed) showed no significant statistical differences. According to the results, there was difference among the concentration of tannic acid used (20 and 25 in one group, and 50 ppm in the other group), as demonstrated by the Tukey test. Regarding time, results show a significant difference among the different data. Repeata-bility value for both methodologies was 0.16.

When a microscale methodological technique is validated, it is necessary that its results are similar to such methodology. Statistical and repeatability results demonstrate that both methodologies gave similar quantitative values; therefore, microscale can be reliable. Significant differences in standards concentrations are attributed to the DPPH molecules content, that react with the poly-phenols molecules scavenging the H^{+}. In higher polyphenols/standard, concentrations H^{+} scavenging is higher because there are more electrons available for transfer (Osman, 2011). Similar effect occurs at different reaction times, since there is more time for molecule interactions and electron/proton exchange (Mishra et al., 2012).

Intra-day proposed assay results are summarized in Table 1. ANOVA results (Table 2), showed significant differences in days, turn and standard concentration; at the same time no significant differences were found among sample solutions.

All the statistics values are below one. The reproducibility was 0.381, which suggest that the results are reliable, because it was similar between days and in the same day. Repeatability value (0.09) was lower than previously reported (0.16); this difference can be attributed to the molecular polyphenols/standard stability, which is modified by reaction times and/or assay temperature. In this study, temperature was not controlled (ambient temperature) and the number of samples was increased for intra-assay analysis, so reaction time, for each block of samples or the analyst sample manipulation, was higher than inter-methodologies assay, previously described.

Interaction day-turn was not significance, which means that the analysis had similar results for the same sample in different periods. R&R value (0.404) confirms that the global results had confidence to use the micro-plate methodology. V_P value (0.077) was lower than V_T value (0.412) because the analysis in one turns was similar than the other made in a different turn. Results show that the methodology is useful in the same assay conditions. Significant differences in days and turns was consequence of the DPPH-methanol solution used for the analysis, because it was made in the first day of analysis and kept under light protection at 4°C, to prevent tempe-rature or light degradation. Molyneux (2004) reported that the stock DPPH solution was stable for 3 days, but results showed that this hypothesis is not consistent; the recommendation for further analysis is to make a fresh DPPH-methanol stock solution, every time before each experiment. Also, Dawidowicz et al. (2012) reported that DPPH scavenging is affected by water content in the solution, higher pH and other factors, so it can influence the differences found in analysis of day three. Significant differences in polyphenol/standard concentration had the same explanation, previously described, for the concentration differences in methodologies.

Data linearity can be obtained with regression coefficient. General result was 0.98; therefore the result of the methodology is accepted (Hibbert, 1999). Tannic acid standard composition is a glucose-phenolics mix and the components can react between them, making the variability perceptible in the regression coefficient, which did not achieve more than 0.98; also the DPPH degradation

Table 2. ANOVA results for statistic validation of a micro scale methodology to quantify antioxidant activity using DPPH.

Variation source	Case 1. Between methodologies			Case 2. Into micro plate assay			Case 3. Between laboratories		
	df	Media square	P	df	Media square	P	df	Media square	P
Repeat (r)	2	0.002	0.108	-	-	-	2	0.000	0.681
Method (m)	1	0.002	0.211	-	-	-	-	-	-
Concentration (c)	2	0.042	<0.0001	5	0.080	<0.0001	1	0.003	0.001
m*c	2	0.004	0.019	-	-	-	-	-	-
Time (t)	2	0.006	0.004	-	-	-	-	-	-
m*t	2	0.001	0.588	2	0.000	0.555	-	-	-
c*t	4	0.001	0.587	5	0.000	0.235	-	-	-
Day (d)	-	-	-	2	2.181	<.0001	1	0.000	0.587
Turn (T)	-	-	-	1	0.079	<.0001	-	-	-
Sample (s)	-	-	-	2	0.001	0.022	-	-	-
d*T	-	-	-	2	0.036	<.0001	-	-	-
d*c	-	-	-	10	0.002	<.0001	1	0.005	0.001
d*T*c	-	-	-	10	0.000	0.525	-	-	-
d*s	-	-	-	4	0.000	0.478	-	-	-
T*s	-	-	-	2	0.000	0.555	-	-	-
d*c*s	-	-	-	20	0.001	0.066	-	-	-
d*T*s	-	-	-	4	0.000	0.517	-	-	-
c*s	-	-	-	10	0.001	<0.0001	-	-	-
T*c*s	-	-	-	10	0.000	0.185	-	-	-
d*T*c*s	-	-	-	18	0.000	0.716	-	-	-

with time cannot allow the same quantification for the analyzed samples.

Another way to achieve statistical validation was described by Wang (2007) who reported that if the experiments were performed with normal operation equipment conditions and the sample treatments are considered in assay normal ranges, obtained data can be validated if they are between confidence intervals (P < 0.05) and three standard deviations (3SD) above and below average. Experimental data was plotted in Figure 1, where all the measurements were located between 3SD limits and the higher values were distributed within confidence intervals. Also, figure shows significant differences were founded between days (specifically day 3), but does not affect method acceptance.

Method accuracy was measured with the error (average - real value); error assay values ranged between 16.90 and 12.64, these results showed wide variability in the assay. This can be attributed to the tannic acid standard; the reagent is a glucose and phenolics mix, which can react and change the solution composition. Tannic acid was selected as standard because most of polyphenols extracts are complex mixes of molecules, such as the standard proposed. The main objective of this assay is to provide a methodology to quantify easy, fast and low
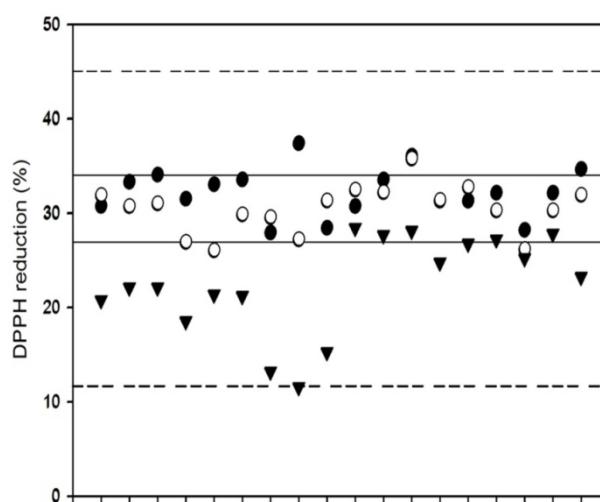

Figure 1. Statistic validation of a micro-plate technique for DPPH scavenging quantification. • Day 1, ○ Day 2, ▼ Day 3, — confidence interval limits, --- 3SD limits.

quantities of the antioxidant activity of different compounds, but specifically polyphenols. If the methodology is done

with pure compounds the results are expected to improve and the variability must diminish. Statistical differences found in all validation tests are attributed to the molecules reaction in DPPH-methanol solution or to the tannic acid standard (Sharma and Bhat, 2009). Even though there are differences, statistical results show a method validation for the microscale scavenging quantification of DPPH, mainly by polyphenols.

Microscale analysis was done in two different laboratories and it was validated, R^2 was 0.99 and 0.92; coefficient of variation was 3.11 and 4.68. There were no statistical differences between them, so the methodology can be applied in different places, with different analysts and different equipments. Hibbert (1999) established that a new methodology is validated if the inter-laboratory precision is usually at least twice that of the intra-laboratory precision, and that after removal of outliers; our results are in agreement with this. Also, Uriarte-Pueyo and Calvo (2009) reported coefficient of variation, from polyphenols micro-plate quantification, similar to those found in this investigation, which validated its methodology.

Conclusion

A rapid assay to evaluate the antioxidant activity of bioactive compounds has been developed. Proposed methodology, using micro plates, for DPPH scavenging quantification, provide confidence and multiples advantages for use as standard technique in polyphenol samples and therefore in other less complex samples. This result confirms that the microscale DPPH scavenging quantifycation is a confidence alternative to save reactants and time.

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Full Length Research Paper

The composition of fatty acids stored in liver, muscle and fat tissues of the African lungfish *Protopterus annectens* (Owen, 1839)

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The composition and profile (%) of fatty acids stored in liver, muscle, and fat tissues of the fresh-water African lungfish *Protopterus annectens* were determined by gas chromatography. High content of total fatty acids (TFA) was found in fat (62.06 ± 3.4 g/100 g) followed by liver (50.68 ± 4.72 g/100 g) and muscle tissues (10.9 ± 0.81 g/100 g). Saturated fatty acids (SFA) formed 72.9% TFA in the liver and muscles and 53.3% in fat. Fat was rich in polyunsaturated fatty acids (PUFA), containing 31.1%, while muscle and liver contributed 12.9 and 10.6 % respectively. Fat and liver also contained, respectively, 9.59 ± 1.1 and 8.38 ± 1.9 g/100 g of monounsaturated acids (MUFA) compared to 1.56 ± 0.3 g/100 g in muscles. The major SFA were C16:0, C18:0 and C14:0. The MUFA were characterized by the Oleic (C18:1n9c) and Palmitoleic (C16:1) acids. The major PUFA in all three types of tissues was the Linoleic acid (C18:2n6c), while C20:3n6 was detected in fat only, and C18:3n3 was detected in muscles only. The n-3/n-6 ratio in the muscles was 0.5. The results of this study show that fatty acid composition in the African lungfish *P. annectens* can considerably vary, depending on the storage organ. According to the present results, *P. annectens* was considered a semi-fatty fish, storing its lipids in muscles, liver and fat tissues. These organs are potential source of essential fatty acids and may play an important role in human health. More focus on lipid extraction from *P. annectens* is recommended.

Key words: Fat, fatty acids, freshwater, liver, lungfish, muscle, *P. annectens*.

INTRODUCTION

Fat is stored in form of fatty globules in various parts of the fish such as the muscle and the liver, and as layers under the skin and in the body cavity (Sheridan, 1988; Ben Smida et al., 2009; Khoddami, et al., 2009). Various fats and oils from both seawater and freshwater fish were found to be rich sources of omega-3 long chain poly-unsaturated fatty acids (PUFAs), particularly eicosapen-tacenoic acid (EPA, 20:5n-3) and docasahexaenoic acid (DHA, 22:6n-3), as well as its precursor, alpha linolenic acid (C18:3n3) (Bays and Lansing, 1994; Shahidi and Wanasundara, 1998; Saba and Muhammad 2000; Ackman, 2002; Su et al., 2004; Bergé and Barnathan, 2005). An increasing interest in the health benefits of fish oil consumption has emerged, because these fatty acids play an important role in the prevention and treatment of cardio-vascular diseases (Nestel, 2000), improving of learning ability and lowering the levels of lipid in blood plasma (Uauy et al., 2001; Lim and Suzuki, 2002).

Fish oils extracted from the livers of fishes are commonly a rich source of beneficial fatty acids, including

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long chain polyunsaturated fatty acids like omega-3s, and fat-soluble vitamins A and D (Covadonga et al., 2004; Mnari et al., 2007; Guil-Guerrero et al., 2010). Fish oils are also extracted from non-consumable parts of the fish, such as head, skin, central bones, viscera (Shahidi et al., 1991; Sathivel et al., 2002; Stocknes et al., 2004; Nuraini et al., 2008; Khoddami et al., 2009). These parts may contribute to the total level of fatty acids, thus increasing the nutritional value of the fish. However, little is known about the fatty acids composition and profiles (%) of the Nile fishes (Elagba and Al-Sabahi, 2011) compared to other freshwater fishes (Ahlgren et al., 1994; Suriah et al., 1995; Zenebe et al., 1998; Bieniarz et al., 2000; Ackman et al., 2002; Fawole et al., 2007; Sharma et al., 2009; Ugoala et al., 2009). The present study is a first approach to determine the fatty acid composition and profiles of the African lungfish *Protopterus annectens*. It is important to analyze the fatty acid composition of the lungfish because is commonly eaten in western states of the Sudan and it is a popular marketed species among consumers in these rural areas. Therefore, the objective of this study was to determine the fatty acid composition and profile (%) in the muscle, liver and fat tissues of the freshwater lungfish *P. annectens*, to encourage the consumer to use these storage organs of the of the fish.

MATERIALS AND METHODS

Collection of samples

Fresh specimens of the African lungfish *P. annectens* (Owen, 1839) collected from the Nile water was obtained from the fish market in Khartoum. Muscle from dorsal and ventral parts of the fish, liver and fat tissues were carefully removed and minced. Three 1 g samples from each of the three tissues of each specimen were taken. The total lipids (TL) were extracted from the tissue samples with chloroform-ethanol mixture (2:1, v/v) according to the extraction method of Folch et al. (1957).

Fatty acids analysis with gas chromatography

Fatty acids were analyzed as their methyl esters with a gas chromatography-mass spectrometry (GC-MS; Hewlett- Packard 5890 GC), according to the procedure of Ahlgren et al. (1994). The different fatty acids in the lungfish were obtained by comparing the retention times of the fatty acids under study and those of a mixture of methyl esters (Supelco, PUFA-3). The concentration of individual fatty acid was calculated using heneicosanoic acid (C21:0) as internal standard. The results (means ± standard deviation, SD) were calculated both as concentration (g/100 g of wet tissue) and weight percentage (fatty acid profile). The results represent the mean values of a series of repetitions (n = 9).

RESULTS AND DISCUSSION

The fatty acid contents in liver, muscle and fat tissues of the African lungfish *P. annectens* are shown in Table 1. Comparatively, the fat tissues express the highest content (62.06 ± 3.4 g/100 g) of TFA followed by the liver

(50.68 ± 4.72 g/100 g) and the muscle tissues (10.9 ± 0.81 g/100 g). The levels of fatty acids stored in different tissues are considered as important criterion of classification for fish species. According to Ackman (1994), fish with a medium lipid storage rate contains 4-8 g/ 100 g of fatty acids in the muscular tissue. Therefore, according to the level of fatty acid found in liver (50.68 ± 4.72 g/100 g) and muscles (10.9 ± 0.81 g/100 g) of the lungfish, *P. annectens* can be considered a semi-fatty fish, storing its lipids in the muscles, the liver and fat tissues. The fat and liver tissues also contain higher levels of MUFA (9.59 ± 1.1 and 8.38 ± 1.9 g/100 g) and PUFA (19.52 ± 4 and 5.37 ± 2.7 g/100 g), respectively, compared to 1.56 ± 0.3 and 1.42 ± 0.3 g/100 g, respectively, for the muscle tissues. The fat tissues contributed 46.7% of TFA as unsaturated *fatty acid* (USFA), of which 31.1% were PUFA, while each of the liver and muscle tissues contributed 27.1% of TFA as USFA. This composition was similarly found in other fish, such as salmon, rainbow trout and some Nile fishes (Ahlgren et al., 1994; Zenebe et al., 1998; Bieniarz et al., 2000; Ackman et al., 2002; Fawole et al., 2007; Mnari et al., 2007; Khoddami et al., 2009; Elagba and Al-Sabahi, 2011).

The total fatty acids (UFA and SFA) in the liver, muscle and fat tissues of the lungfish are expressed in (g/100 g) fresh weight (Figure 1). There was a high level of SFA in the liver and muscle of lungfish (72.9 and 72.9%), respectively, compared to 53.3% in the fat tissues. The fat and liver tissues also contain higher levels of MUFA and PUFA (Figure 2) compared to the muscle tissues. A clear variation between the different organs can be observed. Comparatively, the results which expressed the different proportion of MUFAs and PUFAs with other fish lipids may be due to the environmental effect of tropical fish species (Suriah et al., 1995; Zenebe et al., 1998; Lim and Suzuki, 2002; Kwetegyeka et al., 2008). An analysis of the UFA/SFA ratio (Figure 3) gives a better idea of these differences in distribution and confirms the important variations between the organs. The PUFA are very high in the fat tissues, forming 31.1% of TFA. The PUFA/MUFA ratios (Figure 4) were, respectively, 0.6, 0.9 and 2.0 for the liver, muscle and fat tissues. There was a significant concentration of MUFA (9.59±1.1 and 8.38±1.9 g/100 g) in the fat and liver tissues (Figure 5).

The fatty acid compositions of total lipid (TL) from liver, muscle and fat tissues of the lungfish are presented in Table 1. A total of 17 fatty acids were identified in the analyzed tissues of the lungfish. As shown in the table, the profile of each fatty acid group and the distribution of SFA and unsaturated fatty acid UFA (MUFA + PUFA) vary also in different organs. The predominant SFAs in all samples were tricosanoic, palmitic, stearic and myristic acids. The highest levels of C16:0 (21.7%) were determined in the muscle lipid, and the highest levels of C18:0 (38.2 %) were determined in liver lipid. It was reported that palmitic acid was the predominant in SFA group in freshwater channel catfish, *Ictalurus punctatus*

Table 1. The contents of fatty acids (Mean ± SD, % of TFA) in the muscle, liver and fat tissues of the lungfish *Protopterus annectens*.

Fatty acid	Liver		Muscle		Fat	
	%	g/100 g	%	g/100 g	%	g/100 g
C14:0	13.4	6.79 ± 1.52	11.1	1.22 ± 0.56	8.9	5.44 ±1.39
C15:0	4.4	2.23 ± 0.34	*	*	4.6	2.82 ± 0.1
C16:0	5.5	2.76 ± 15	21.7	2.3 ± 0.05	12.4	7.65 ± 2.13
C17:0	7.8	3.95 ± 0.73	2.8	0.31 ± 0.06	12.4	7.61 ± 0.31
C18:0	38.2	19.36 ± 3.26	14	1.53 ± 0.36	4.4	2.71 ± 0.68
C22:0	*	*	*	*	2.8	1.72 ± 0.03
C23:0	3.6	1.84 ± 2.9	23.3	2.56 ± 0.56	7.8	4.58 ± 0.62
C14:1	*	*	*	*	1.4	0.88 ± 0.1
C15:1	*	*	2.6	0.29 ± 0.05	2.1	1.28 ± 0.1
C16:1	8.3	4.2 ± 1.09	4.1	0.45 ± 0.35	1.5	0.9 ± 0.1
C17:1	*	*	*	*	3	1.84 ± 0.04
C18:1n9c	8.2	4.18 ± 1.8	7.5	0.82 ± 0.29	1.5	0.94 ± 0.07
C20:1	*	*	*	*	6.1	3.75 ± 0.51
C18:2n6c	10.6	5.37 ± 0.99	6.5	0.72 ± 0.13	15	9.6 ± 0.52
C18:3n3	*	*	3.3	0.36 ± 0.1	*	*
C20:2	*	*	3.1	0.34 ± 0.08	*	*
C20:3n6	*	*	*	*	16.1	9.92 ± 3.3
ΣTFA	50.68 ± 4.72		10.9 ± 0.81		62.06 ± 3.4	
ΣSFA	36.93 ± 6.7		7.92 ± 1.1		32.53 ± 2.4	
ΣUFA	13.75 ± 2.24		2.98 ± 0.31		29.53 ± 3.76	
ΣMUFA	8.38 ± 1.9		1.56 ± 0.3		9.59 ± 1.1	
ΣPUFA	5.37 ± 2.7		1.42 ± 0.3		19.52 ± 4	
SFA / TFA	72.9		72.9		53.3	
MUFA / TFA	16.5		14.2		15.6	
PUFA / TFA	10.6		12.9		31.1	
UFA / SFA	0.37		0.38		0.89	
ΣN6	5.37 ± 0.99	10.6	0.72 ± 0.13	6.5	19.52 ± 0.27	31.1
ΣN3	*	*	0.36±0.1	3.3	*	*

SD, Standard deviation; *, Not detected; TFA,total fatty acids; SFA, saturated fatty acids, MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; N, Omega 6; N3, Omega 3.
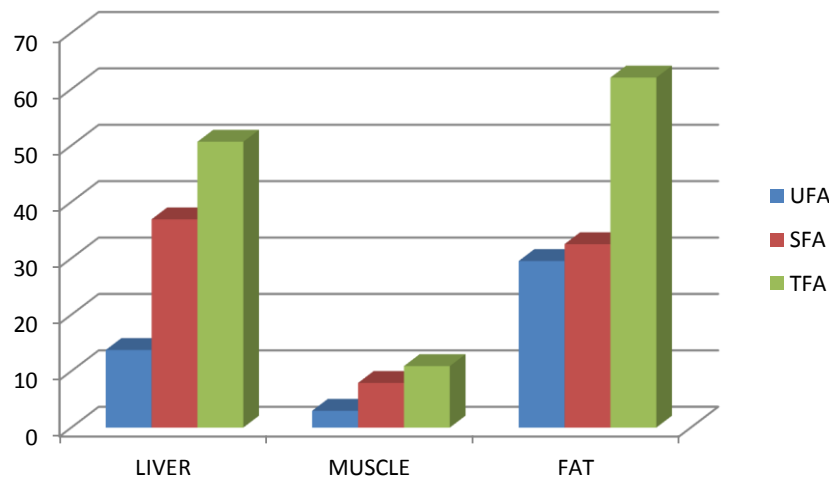

Figure 1. Contents of UFA, SFA and TFA (g/100 g) in different tissues of the lungfish.
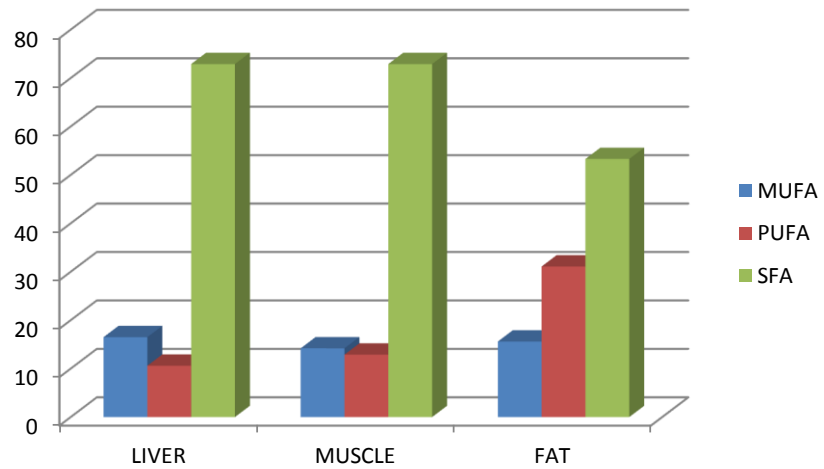
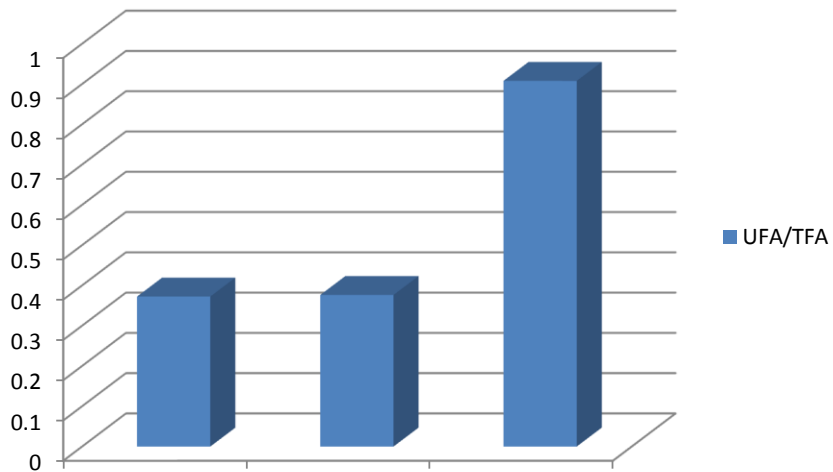**Figure 2.** MUFA, PUFA and SFA in different tissues of the lungfish (as % of the TFA).

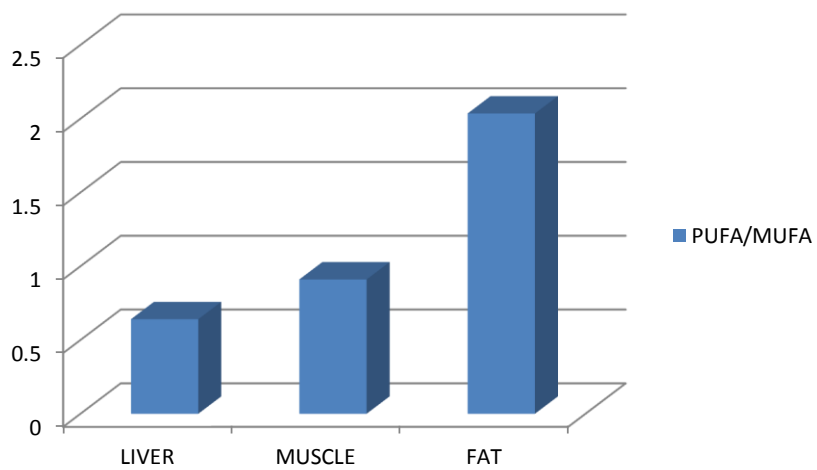**Figure 3.** UFA / SFAin different tissues of the lungfish.

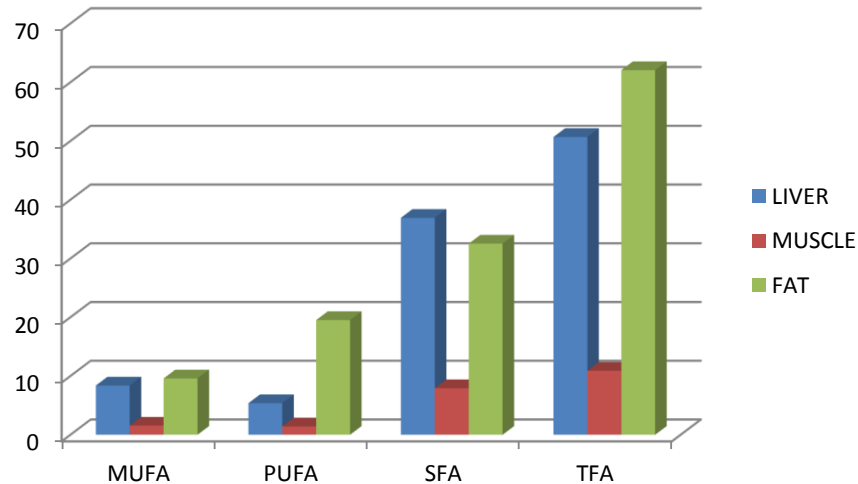**Figure 4.** PUFA/MUFA in different tissues of the lungfish.

Figure 5. Contents of MUFA, PUFA, SFA and TFA (g/100 g) in different tissues of the lungfish
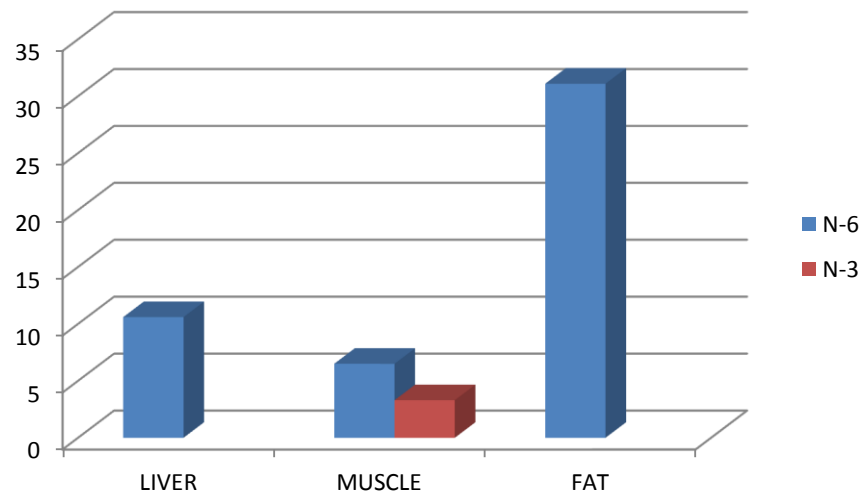

Figure 6. Content of omega-6 (N-6) and omega-3 (N-3) in different tissues of the lungfish (as % of the TFA).

(Sathivel et al., 2002), in freshwater rainbow trout, *Oncorhynchus mykiss* (Haliloglu et al., 2004) and in the catfish *Pangasius hypophthalmus* (Ho and Paul, 2009). The percentage of PUFA in fat, muscle and liver of the lungfish, was 32.1, 12.9 and 10.6%, respectively. The major PUFA was the lenoleic acid (C18:2n6). Eicosatrienoic (C20:3n6) and lenolenic (C18:3n3) acids formed 16.1 and 3.3%, respectively, in the fat. The percentage of monounsaturated fatty acid (MUFA) of liver, fat and muscle lipids was 16.5, 15.6 and 14.2%, respectively. Oleic (C18:1n-9) and palmitoleic (C16:1) acids were the major MUFA. Oleic acid constituted 8.2 and 7.5%, and palmitoleic acid constituted 8.3 and 4.1%, in liver and muscle, respectively. The high level of oleic acid found in the lungfish was consistent with its level in other species of freshwater fish. Steiner-Asiedu et al.

(1991) found that freshwater tilapia (*Tilapia* sp.) had significantly higher level of oleic acid than the flat sardine (*Sardinella* sp.) and sea bream (*Dentex* sp.). The level of oleic acid in the American freshwater channel catfish flesh was also high compared to sardine and sea mullet (Ackman, 1994). Although level of oleic acid was lower than the level found in some freshwater fish (Khoddami et al., 2009), it was almost similar to the level reported in other freshwater fish (Zenebe et al., 1998; Bieniarz et al., 2000; Saba et al., 2000; Ackman et al., 2002; Ben Smida et al., 2009; Sharma et al., 2009; Ugoala et al., 2009; Elagba and Al-Sabahi, 2011). The variation of n-3 PUFA detected in the different studied organs of *P. annectens* (Figure 6) was to some extent within the range found in other fish species (Suriah et al., 1995). The difference may be due simply to differences in the fatty acid content

in the diet or it may be related to environmental conditions, sex and age of the fish (Steffens, 1997).

Conclusions

This study indicates that the lipids of the African lungfish *P. annectens* are rich in linoleic and linolenic acids which are essential for human healthy diet. Although, the level of SFA was high in the liver and fat, PUFA accounted for the highest proportion of FA in the fat. The liver and fat (which are considered as waste) may provide a rich unexploited source of polyunsaturated fatty acids. Therefore, more focus on lipid extraction from the lungfish is needed and more detailed studies are necessary.

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Full Length Research Paper

Collaborative effects of some anti-diabetic plants on the liver marker enzymes of diabetic rats

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The leaves of *Psidium guajava, Anacardium occidentale, Eucalyptus globulus* and fruits of *Xylopia aethiopica* are used in the management of diabetes mellitus. The phytochemical constituents as well as the acute toxicity of the combined chloroform extracts (*A. occidentale + E. globulus* and *P. guajava + X. aethiopica*) and their effects (at graded doses of 100 and 250 mg/kg body weight each) on the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were therefore, assayed in diabetic and normal rats using standard methods. The phytochemical analyses of the four extracts showed the presence of flavonoids, terpenoids and fats and oil in all of them. Each of the combined extract was found to be non-toxic even at a dose as high as 5000 mg/kg body weight. The combined extracts at the tested doses significantly ($p < 0.05$) and dose-dependently decreased the activities of ALT, AST and ALP. The effects of the combined extracts (especially 250 mg/kg body weight of *P. guajava + X. aethiopica*) were better than that of the standard anti-diabetic drug [glibenclamide (5 mg/kg body weight)]. The results generally indicate that the combined chloroform extracts of the leaves of *A. occidentale, E. globulus, P. guajava* and fruits of *X. aethiopica* might be adopted for the management and/or amelioration of diabetes mellitus and its accompanying complications.

Key words: *Psidium guajava, Anacardium occidentale, Eucalyptus globules*, *Xylopia aethiopica,* chloroform extracts

INTRODUCTION

Diabetes mellitus is a heterogenous metabolic disorder characterized by hyperglycemia arising from defective insulin secretion, resistance to insulin action or both. This disease generally affects organs that participate in carbohydrate metabolism such as the liver, a major site of insulin clearance and glucose homeostasis during the concentration of postprandial fasting blood glucose (Gavin et al., 1997). The toxicity of excessively high concentration of plasma glucose arising from prolonged hyperglycemia or postprandial glucose cause the autoxidation of glucose and non-enzymatic glycosylation of proteins leading to the production of advanced glycol-

sylation end products (AGEs) which through their receptors (RAGEs) inactivate enzymes, altering their structures and functions and resulting in free radical formation (McCarthy et al., 2001).

The free radicals through oxidative stress cause lipid peroxidation resulting in cell injury and decreased concentration of cellular anti-oxidants (Hijora et al., 2000). Malondialdehyde (MDA), a product of lipid peroxidation, if elevated in the blood can lead to activation of leucocyte lysosomal membrane rupture releasing the enzymes into general circulation. These results in the increase of cell and parenchymal tissue damage for example, liver cell paren-

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chymal destructions causing liver enzymes to leak into the blood stream and this serves as an indicator for free radial-mediated destruction of liver parenchymal cells (Pratibha et al., 2004). Plants such as *Anacardium occidentale, Eucalyptus globulus, Psidium guajava,* and *Xylopia aethiopica* have been implicated in the treatment of diabetes mellitus because they possess arrays of phytochemicals with multifarious pharmacological effects (Sagheb et al., 2010; Gupta et al., 2011; Ukwenya et al., 2012). Hence, the present study was targeted at evaluating the combined effects of the chloroform extracts of the leaves of *A. occidentale, E. globulus, P. guajava* and fruits of *X. aethiopica* on liver marker enzymes of diabetic rats.

MATERIALS AND METHODS

The plant samples

The leaves of *A. occidentale*, *E. globulus* and *P. guajava* were collected from the premises of University of Nigeria, Nsukka while the fruits of *X. aethiopica* were purchased from a local market in Delta State. The plant samples were identified by Prof. (Mrs.) May Nwosu of the Department of Botany, University of Nigeria, Nsukka where the voucher specimens were deposited in the herbarium.

Preparation of the extracts

The leaves of *A. occidentale, E. globulus, P. guajava* and the fruits of *X. aethiopica* were air dried to constant weight at room temperature and then reduced to powder. 600 g of each plant material was macerated in 2.7 L of analytical grade chloroform. After 48 h, the resulting extracts were filtered and concentrated with rotary evaporator at reduced pressure and the yield of extracts calculated. A standard weight (8 g) of each of the two proportionally combined extracts was dissolved in 16 ml of 10% dimethyl sulphuroxide (DMSO). The doses of each extracts administered was estimated by the method of Tedong et al. (2007) where volumes given were calculated as follows:

$$V \ (ml) = \frac{D \times P}{C}$$

Where D = Dose used (g/kg body weight of test animals); P = Body weight (kg); C = Concentration (g/ml); V = Volume (ml)

Animals

Thirty-five male Wistar albino rats of weight (180-230 g) and 64 male mice of weight (20-30 g) were used for this study. The University Animal Research Ethical Committee approved the experimental protocol. The animals were housed and maintained at a 12 h light and dark cycle and fed with rat diet *ad libitum*. The mice were used for acute oral toxicity study while the rats were made diabetic by a single dose of 180 mg/kg body weight of alloxan monohydrate intraperitonially and 35 rats selected for the study, 72 h after diabetes has been established. Treatments were for 40 h and administrations of the combined extracts were twice daily. After 40 h, rats were sacrificed and their blood collected for further biochemical analyses.

Chemicals and reagents

Dimethyl sulfoxide [DMSO (Serva Heidelberg, New York)], chloroform (Sigma Aldrich Chemicals, Germany), alloxan monohydrate (Sigma Aldrich Chemicals, Germany), sodium chloride, dilute tetraoxosulphate (vi) acid, 2% (v/v) hydrochloric acid, 1% (w/v) picric acid, methyl orange, Dragendorff's reagent, Mayer's reagent, Wagner's reagent, Fehling's solution, 5% (w/v) ferric chloride solution, aluminium chloride solution, lead subacetate solution, ammonium solution and distilled water were used for the study. The commercial kits were purchases from Randox Laboratories Ltd, Crumlin Co Antrim, UK.

Phytochemical analyses

Qualitative phytochemical analyses were carried out on the extracts of the various plant samples according to the procedures outlined by Harborne (1998) and Trease and Evans (1989).

Acute oral toxicity test (LD$_{50}$)

A lethal dose toxicity study of each of the two proportionally combined extracts was carried out by the method described by Lorke (1983).

Experimental procedures

Alanine and aspartate aminotransferase (ALT and AST) activities were assayed using Randox commercial enzyme kit as described by Reitman and Frankel (1957) and Schmidt and Schmidt (1963). Alkaline phosphatase (ALP) activity was estimated using Randox commercial enzyme kit, based on the methods of Rec (1972) and Englehardt (1970).

Statistical analysis

Data generated from this study were represented as mean ± SEM. Variables were analyzed by one-way Analysis of Variance (ANOVA) and comparison done by multiple comparisons using Duncan test.

RESULTS

Qualitative phytochemical constituents of the chloroform extracts of the leaves of *A. occidentale*, *E. globulus* and *P. guajava* and the fruits of *X. aethiopica*

The qualitative phytochemical analyses showed the presence of flavonoids, terpenoids and fats and oil in the four extracts (Table 1). Saponins and tannins were present in the extracts of *A. occidentale*, *E. globulus* and *P.* guajava. Glycosides and alkaloids were not detected in the extracts of *A. occidentale* and *P.* guajava respecttively. Saponins and tannins were not detected in *X. aethiopica* extract.

The acute toxicity and lethality (LD$_{50}$) of the combined plant extracts

There was no lethality or any sign of toxicity in the four groups of four mice each that received 10, 100 and 1000 mg/kg body weight of each of *A. occidentale* + *E. globulus* and *P. guajava* + *X. aethiopica* as well as 5 ml/kg body weight of 10% DMSO respectively at the end

Table 1. Qualitative phytochemical constituents of *Anacardium occidentale*, *Eucalyptus globulus*, *Psidium guajava* and *Xylopia aethiopica.*

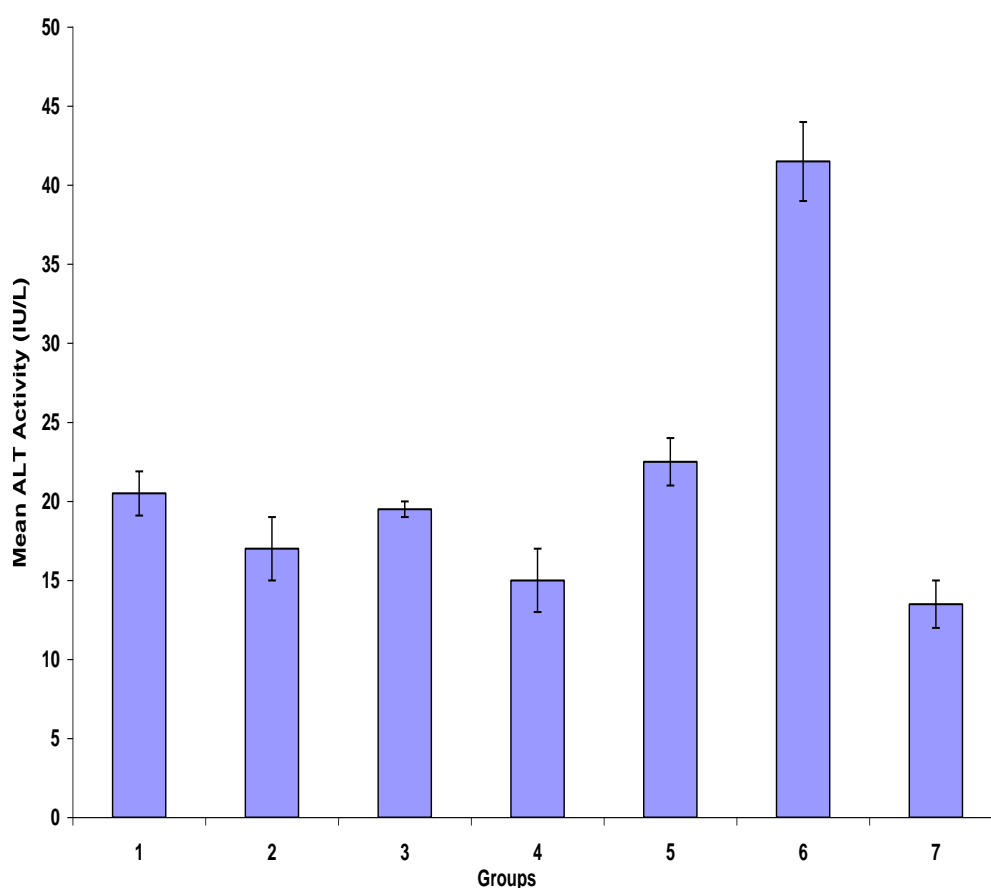| Phytochemical constituent	*Anacardium occidentale*	*Eucalyptus globulus*	*Psidium guajava*	*Xylopia aethiopica*
Alkaloids	+	+	ND	+
Flavonoids	+	+	+	+
Glycosides	ND	+	+	+
Saponins	+	+	+	ND
Tannins	+	+	+	ND
Terpenoids	+	+	+	+
Fats and oil	+	+	+	+

+ = Present; ND = Not detected.

Figure 1. Effects of varying doses of the combined plant extracts on alanine aminotransferase (ALT) activity. Group 1= *A. occidentale* + *E. globulus* (100 mg/kg b.w). Group 2 = *A. occidentale* + *E. globulus* (250 mg/kg b.w). Group 3 = *P. guajava* + *X. aethiopica* (100 mg/kg b.w). Group 4 = *P. guajava* + *X. aethiopica* (250 mg/kg b.w). Group 5 = Glibenclamide (5 mg/kg b.w). Group 6 = Diabetic untreated (5 ml/kg b.w of DMSO). Group 7 = Non diabetic control (5 ml/kg b.w of DMSO).

of the first phase of the study. At the end of the second phase of the study, there was neither death nor obvious sign of toxicity in the groups of mice that received 1900, 2600 and 5000 mg/kg body weight of each of the combined plant extracts.

Effects of varying doses of the combined plant extracts on alanine aminotransferase (ALT) activity

Figure 1 shows that the diabetic untreated group had the highest activity of ALT but administration of the different
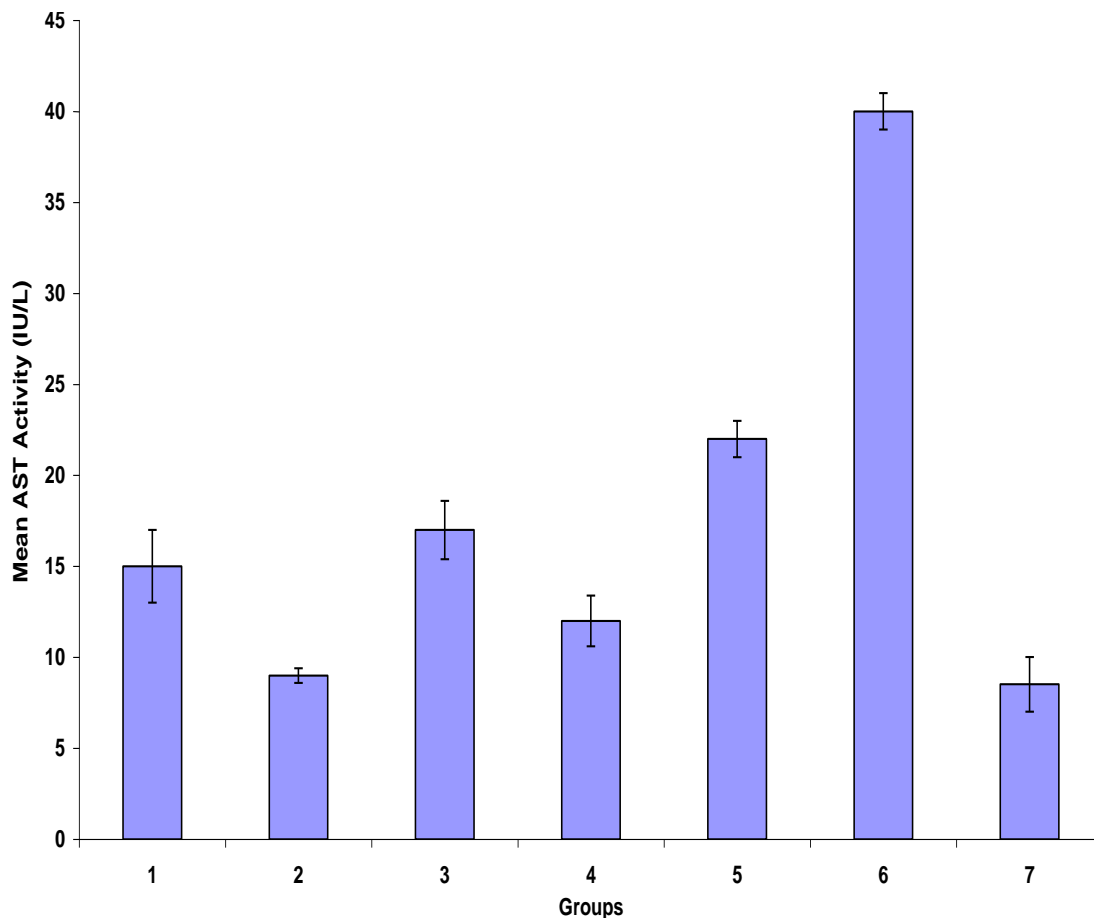
Figure 2. Effects of varying doses of the combined plant extracts on aspartate aminotransferase (AST) activity. Group 1= *A. occidentale* + *E. globulus* (100 mg/kg b.w) Group 2= *A. occidentale* + *E. globulus* (250 mg/kg b.w). Group 3 = *P. guajava* + *X. aethiopica* (100 mg/kg b.w). Group 4 = *P. guajava* + *X. aethiopica* (250 mg/kg b.w). Group 5 = Glibenclamide (5 mg/kg b.w). Group 6 = Diabetic untreated (5 ml/kg b.w of DMSO). Group 7 = Non diabetic control (5 ml/kg b.w of DMSO).

doses of the combined extracts resulted in significant ($p<0.05$) decrease in ALT activity. The 100 and 250 mg/kg body weight of each of *A. occidentale* + *E. globulus* and *P. guajava* + *X. aethiopica* in a similar manner as the standard anti-diabetic drug [glibenclamide (5 mg/kg body weight)] significantly (p<0.05) and dose-dependently reduced the activity of ALT when compared with the value obtained for the diabetic untreated group. However, the 250 mg/kg body weight of *P. guajava* + *X. aethiopica* caused the greatest reduction in the activity of ALT.

Effects of varying doses of the combined plant extracts on aspartate aminotransferase (AST) activity

The AST activities of groups 1, 2, 3, 4, 5 and 7 were significantly ($p<0.05$) and dose-dependently lower than that of the diabetic untreated group (group 6). There were no significant ($p>0.05$) differences between the AST activities of groups 2 and 4 and that of the group 7 [non diabetic control group (5 ml/kg body weight of DMSO)] as shown in Figure 2.

Effects of varying doses of the combined plant extracts on alkaline phosphatase (ALP) activity

As shown in Figure 3, the diabetic untreated group had the highest ALP activity but administration of the different doses of the combined extracts caused significant ($p<0.05$) decrease in ALP activity. The 100 and 250 mg/kg body weight of each of *A. occidentale* + *E. globulus* and *P. guajava* + *X. aethiopica* in a similar man-ner as the standard anti-diabetic drug [glibenclamide (5 mg/kg body weight)] significantly ($p<0.05$) and dose-relatedly led to a drop in the activity of ALP when com-pared with the value obtained for the diabetic untreated group. The 250 mg/kg body weight of *P. guajava* + *X. aethiopica* however, depressed the activity of ALP best.

DISCUSSION

Acute toxicity test on the combined extracts (*A. occidentale* + *E. globulus* and *P. guajava* + *X. aethiopica*) using mice showed an LD_{50} value of greater than 5000
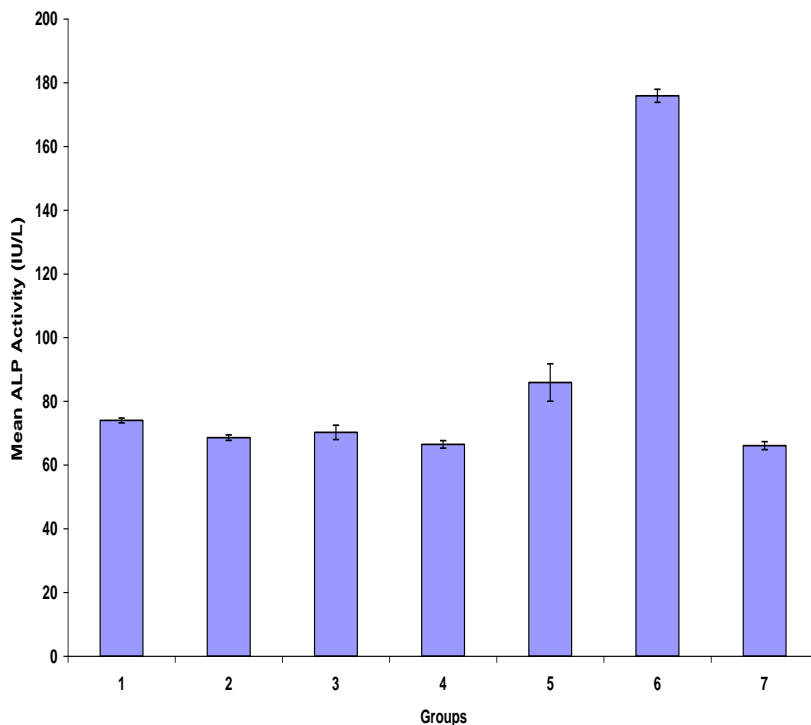
Figure 3. Effects of varying doses of the combined plant extracts on alkaline phosphatase (ALP) activity. Group 1= *A. occidentale* + *E. globulus* (100 mg/kg b.w) Group 2 = *A. occidentale* + *E. globulus* (250 mg/kg b.w). Group 3= *P. guajava* + *X.. aethiopica* (100 mg/kg b.w). Group 4 = *P. guajava* + *X. aethiopica* (250 mg/kg b.w). Group 5 = Glibenclamide (5 mg/kg b.w). Group 6 = Diabetic untreated (5 ml/kg b.w of DMSO). Group 7 = Non diabetic control (5 ml/kg b.w of DMSO).

mg/kg body weight for each combined extract which indicates that the leaves of *A. occidentale, E. globulus, P. guajava* and fruits of *X. aethiopica* have low toxicity.

The 250 mg/kg body weight of the combined extract (*A. occidentale* + *E. globulus*) exerted better effects in the treated rats than all the doses of *P. guajava* + *X. aethiopica* considering all the studied parameters. The remarkable reductions observed in ALT and AST acti-vities could be said to have been caused by the hepato-cellular and cardiac protection offered by the combined extracts. Hepatic and cardiac tissues release aspartate and alanine aminotransferases and therefore, the eleva-tion of plasma concentrations of these enzymes are indicators of hepatic and cardiac damage as in the case of complications in diabetes mellitus (Crook, 2006). The observation is in support of the report of Ogbonnia et al. (2010) who studied the effect of a poly-herbal formulation on liver function enzymes in diabetic rats. It was noted that the administration of the poly-herbal formulation (which has *X. aethiopica* as one of its component) to rats led to a pronounced decrease in ALT and AST activities in the treated rats. The implication of this is that the com-bined extracts did not produce harmful effects on both the cardiac or hepatic tissues of the treated rats while in the diabetic untreated group, there were notable elevations in

the activities of these two enzymes, an indication of hepatic and cardiac tissue damage.

The notable reduction in serum ALP activity recorded is suggestive of cellular membrane/hepatocellular mem-brane protective effects of the combined extracts. ALP functions as a biochemical marker enzyme for maintain-ing membrane integrity. Increase in its plasma activity indicates peroxidation of cell membrane which occurs during diabetes mellitus (Akanji et al., 1993). Uboh et al. (2010) showed that the aqueous extract of *P. guajava* confers hepatocellular protection in rats. The hepato-cellular protection evidenced in the present study might be due to the presence of flavonoids in the plant extracts. Flavonoids have been reported to possess antioxidant activity (Middleton, 1996) and thus, are capable of protec-ting cell membranes from peroxidative actions of free radicals.

In conclusion, this study shows that the combined chlo-roform extracts of the leaves of *A. occidentale, E. globulus, P. guajava* and fruits of *X. aethiopica* as em-ployed in the study had better decreasing effects on the activities of liver marker enzymes than glibenclamide (a standard anti-diabetic drug) and therefore, may be adopted for the management and/or amelioration of diabetes mellitus and its accompanying complications.

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