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Full Length Research Paper

Various sources of animal protein intake and their association with muscle mass index and insulin resistance in overweight postmenopausal women

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Many epidemiological studies have observed a positive relationship between animal protein intake (API) and the risk of type 2 diabetes (T2D). However, animal proteins are important in the aging population. Forty sedentary and healthy postmenopausal women were recruited in this study. Body composition (dual X-ray absorptiometry method, DXA), 3-d dietary record (API) and insulin resistance (homeostasis model assessment, HOMA) were assessed. Partial correlations were used to examine the relationship between total API (g/day) on muscle mass index (MMI) and HOMA. MMI (r=0.408; p<0.01) was associated to total API. Our results indicate that promoting an increase in animal proteins is important to maintain muscle mass in postmenopausal women.

Key words: Postmenopausal women, animal proteins, processed meats, insulin resistance, muscle mass.

INTRODUCTION

The high intake of animal protein in the Western diet (characterized by a high intake of red meat, processed meat, and high fat dairy products) may be directly or indirectly related to the occurrence of insulin resistance through different mechanisms (Peppa et al., 2002; Schulze et al., 2003; van Dam et al., 2002). To begin, animal proteins are divided into two types of meat, which are clearly differentiated by their color when raw, white or red meat. Red meat includes beef, pork, game (hunted for sport) and some fowl (ducks). Red meat is an important source of saturated fats, which could increase the risk of obesity (French et al., 1994) and cardiovascular disease (Fraser, 1999). On the other hand, white meats have been associated with a more favorable glycemic control and overall health (Montonen et al., 2005a; Montonen et al., 2005b; Villegas et al., 2003). Processed meats (animal proteins with additives for longer storage duration: bacon, sausage, deli) contain nitrites and nitrates, which are toxic for the hepatic beta cells (Wolff, 1993). Whether red meat alone is actually associated with the risk of the development of type 2 diabetes (T2D) is not clearly understood or if this is mostly due to its combination with a unhealthy lifestyle such as high process meat intake (Aune et al., 2009) remains unclear.

Nevertheless, because they contain all essential amino acids and leucine, animal proteins have been suggested to be associated with the maintenance of muscle mass and function in older adults (Bartali et al., 2006; Pannemans et al., 1998). Sarcopenia, which is defined as the loss of muscle mass (Roubenoff and Hughes, 2000) and dynapenia, which is the loss of muscle strength (Clark and Manini, 2010; Roubenoff, 2000) during normal aging are risk factors for functional independence (Janssen, 2006) and frailty in elderly people (Lang et al., 2009). It has been proposed that an adequate total protein intake for elderly individual should sum up to 1.25 g/kg of body weight, instead of the current recommendation of 0.8 g/kg of body weight (Wolfe et al., 2008). Though meaningful, this has been mainly based

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on studies using essential amino acids rather than animal proteins from foods. Intriguingly, our laboratory (Lord et al., 2007) and others (Houston et al., 2008) showed a close relationship between animal protein intake and muscle mass, but did not address the potential impact on glucose metabolism. Based on the aforementioned associations between red meat intake and T2D, it can be speculated that while effective in maintaining muscle mass, animal proteins may be deleterious to glucose metabolism.

The objective of this study was to evaluate if some subclasses of animal proteins can be found beneficial for muscle mass without increasing the risk of T2D in obese postmenopausal women. Our hypothesis is that muscle mass index is related positively with red meat intake and white meat intake but processed meats with homeostasis model assessment (HOMA) value.

MATERIALS AND METHODS

Subjects

Forty healthy overweight postmenopausal women aged between 48 and 69 years were recruited by the use of advertisements in local newspapers to participate in an intervention study. Participants had to meet certain inclusion criteria, as assessed by phone interview: (1) healthy, (2) without major physical disability, (3) non-smoker, (4) non-regular exercisers, (5) moderate drinker (1 alcoholic beverage per day), (6) body mass index (BMI >27 kg/m^2), (7) stable weight (±2 kg) and (8) no medication that could influence metabolism (example: beta-adrenergic blocking agents, hypoglycemiants, etc). Thereafter, the nature and goals of the study were thoroughly explained to the subjects and written informed consent was provided to them. All procedures were approved by the Ethics Committee of the Sherbrooke University Geriatric Institute.

Experimental procedures

After screening, participants were invited for a visit at the metabolic unit of the Research Centre on Aging of the Sherbrooke University Geriatric Institute. Participants were fasted for 12 h upon arrival at the metabolic unit. We measured body composition by dual X-ray absorptiometry method (DXA: GE, Prodigy Lunar, Madison, WI) (BMI, muscle mass index (MMI) kg fat free mass/m^2) and proceeded with a 12 h fasting of glucose and insulin samples (HOMA). Instructions were then given for the completion of the 3 day dietary record and women were asked to return the dietary record one week later. To measure total and animal protein intakes, white meats (e.g. chicken, fish), red meats (e.g. beef, pork), processed meats (e.g. hot dogs) and other, such as eggs, milk and any other meat that did not fit in the aforementioned types. Subjects completed the physical activity scale for the elderly to control for physical activity (PASE).

Body composition

Body weight was measured using an electronic scale (±0.2 kg SECA707, Hamburg, Germany). Standing height was measured using a wall stadiometer (Takei, Tokyo, Japan). BMI was measured as: body weight (kg)/height (m^2). Fat mass (FM) and fat-free mass (FFM) were measured using a DXA (GE Prodigy Lunar Radiation Corp, Madison, WI). In our laboratory, test-retest measures of FM and FFM in ten adults, with a 1-week interval, yielded a mean absolute coefficient of variation of 3.9 and 1.1%, respectively. MMI is generally used as an index of sarcopenia and is calculated as follows: total FFM (kg)/height (m^2).

Dietary intake

Diets were recorded during 3 consecutive days including one weekend day. It has been demonstrated that a 3-day dietary record is valid to estimate dietary intakes in older adults without cognitive impairments (Luhrmann et al., 1999). Each subject was instructed to maintain normal dietary habits throughout the period of data collection. Subjects were provided with a 5 kg food scale and instructed on how to complete a 3-day dietary record. When returned, we analyzed the journal with the participant to ensure validity of the entries. Total proteins were divided as animal and vegetable proteins. Animal proteins intake included all proteins from animal sources (red and white meat, eggs, fish and milk products). Dietary analyses were completed by using CANDAT SYSTEM, version 6.0 software (Candat, London, ON, Canada) to determine daily energy, protein, carbohydrate and lipid intakes.

Insulin resistance

Blood samples were collected after a 12 h overnight fast and a 15 min rest when participants were in a sitting position. Plasma glucose and insulin level were analyzed in the clinical laboratory of the Geriatric Institute. Insulin resistance based on the HOMA index was evaluated according to the following equation (Matthews et al., 1985): insulin (µUI/ml) × glucose (mmol/L)/22.5. HOMA shows a strong correlation with insulin sensitivity measured using the hyperinsulinemic-euglycemic clamp technique (r = 0.88) (Geloneze and Tambascia, 2006). A higher HOMA index indicates a poorer glucose metabolism.

Statistical methods

Values in the text and tables are presented as mean ± standard deviation (SD). Partial correlations were used to examine the relationship between MMI and protein intake. Analysis covariance (ANCOVA) was used to examine differences between tertile groups of total animal protein intake for HOMA. Since age, physical activity, fat mass and total protein intake can affect glucose metabolism and MMI, they were all used as covariates. Significance was accepted at $p \leq 0.05$. All analyses were performed using Statistical Package for Social sciences (SPSS) (version 17.0; Chicago, IL, USA).

RESULTS

Baseline characteristics of the population are presented in Table 1. Partial correlations indicated that total animal protein intake was significantly and positively related to MMI when adjusted for physical activity, age, fat mass and total protein intake (r=0.408, p<0.01) and HOMA (r=0.337, p<0.05) (Table 2), when controlling for physical activity, age, fat mass and total protein intake. Fasting insulin but not glucose was also significantly and positively related to animal protein intake (r=0.352, p=0.035).

Table 1. Baseline characteristics of subjects.

Characteristic	Mean	SD
Age (years)	58.5	5.1
BMI (kg/m^2)	31.1	3.5
Muscle mass (kg)	40.4	4.1
MMI (kgMM/m^2)	16.1	1.7
Fasting glucose (mmol/L)	5.06	0.55
Fasting insulin (pmol/L)	64.2	33.07
Dietary intake (kcal)	2006	506
Total lipid intake (g)	83.4	33.7
Total protein intake (g)	85.2	22.4
Total carbohydrate intake (g)	203	67.9
Animal protein intake (g)	46.8	22.5
Vegetable protein intake (g)	38.4	11.9

Table 2. Partial correlations between protein intakes and MMI or HOMA, controlling for fat mass, age, physical activity, other meats and total protein intake.

Protein intake		MMI (kgFFM/m^2)	HOMA	Fasting insulin (pmol/L)	Fasting glucose (mmol/L)
Animal protein intake (n = 35)	R	0.408	0.337*	0.352*	0.097
	p	0.014	0.044	0.035	0.57
Red meat	R	-0.09	-0.2	-0.16	-0.212
	p	0.623	0.242	0.36	0.221
White meat	R	0.06	0.06	0.03	0.16
	p	0.713	0.738	0.88	0.366
Processed meats	R	0.23	0.34*	0.29	0.23
	p	0.18	0.05	0.08	0.174

*Statistically significant at p<0.05.

Table 3. Relationship between total fiber intake and total animal protein, processed meats and red meat intake.

Parameter		Animal protein intake	Processed meats intake	Red meat intake
Total fiber intake (g)	R	0.076	-0.340*	-0.08*
	p	0.64	0.03	0.623

*Statistically significant at p<0.05.

When dividing total animal protein into different types of meat (red, white, processed or others), only processed meats was positively associated with HOMA (r=0.34, p<0.05), but not white or red meat, no associations were observed for muscle mass (Table 3).

Furthermore, we investigated if different types of meat were related with total fiber intake, which could help us indicate eating habits in our population. As such, processed meat intake was significantly and negatively related with total fiber intake (r=-0.34, p=<0.05). Moreover, red meat intake was not related with total fiber intake (r=-0.08, p=0.62).

DISCUSSION

In this cross-sectional study, we sought to determine if animal protein intake showed significant associations with insulin resistance (HOMA) as well as MMI in postmenopausal women, and which type of meat has an

impact on these variables. The results of our study indicate that a greater intake in total animal proteins is significantly and positively related to fasting insulin levels, HOMA and muscle mass index. However, when dividing the types of meat into three types, processed meats was the only type associated positively with HOMA. Therefore, while total animal protein intake is related to the maintenance of muscle mass, only processed meats increases the risks of insulin resistance.

The results of this study are in agreement with the literature, indicating that high consumers of processed meats are at risk of insulin resistance and T2D (Aune et al., 2009; Montonen et al., 2005b; van Dam et al., 2002). These studies imply that it is not red meat per se, but the association with poor eating habits that is related with the incidence of T2D. Hence, our study seems to reconsider these assumptions by separating the different types of meat and demonstrate that processed meats and not red meats per se has an impact on insulin resistance parameters and that poor eating habits (high intake of processed meats, low total fiber intake) are also related with insulin resistance.

Animal proteins, which include red meat, are a good choice in the context of a healthy diet, because they contain all the essential amino acids, particularly leucine, which regulates muscle protein synthesis (Norton and Layman, 2006). They have been identified as a key nutrient to maintain muscle mass in older adults, but most studies examined essential amino acids supplements rather than proteins from foods (Symons et al., 2007; Volpi et al., 2003).

Interestingly, although counterintuitive, some data indicate that an elevated muscle mass may be associated with some impairments in insulin sensitivity in older adults (Brochu et al., 2008; Goulet et al., 2007). Our results suggest that animal protein intake may be a modulator of the association between muscle mass and glucose metabolism, explaining why a greater muscle mass would be associated with impaired glucose metabolism. Nevertheless, our results indicate that it may be processed meats that contribute to impairments in glucose metabolism. Based on these findings, it seems that protein animals should be recommended to older adults as long as they are educated to select healthy animal proteins such as unprocessed red or white meat and fish. Further studies on additional animal proteins sources such as eggs and milk produce should be conducted to determine if they can also be recommended in that context.

There are some limits to our study. The cross-sectional design of this study and the use of secondary analyses prevent the determination of cause-and-effect relationships. In this sense, we only had access to muscle mass and did not have measures of muscle quality. Also, HOMA is related to central insulin resistance. It would be of interest to measure peripheral glucose homeostasis with the euglycemic-hyperinsulenemic clamp. Furthermore, we did not measure intramuscular lipids in muscle, which could help explain if there is a relationship between low muscle mass and impaired insulin sensitivity. Lastly, the positive relationship between protein intake and hyperinsulinemia is known to be a normal response when there is a high concentration of amino acids in the bloodstream, favoring a better glucose disposal (Manders et al., 2005). However, in our study, fasting glucose was not different between groups.

To conclude, our results suggest that while animal protein intake is favorable for obese postmenopausal women to preserve muscle mass, only processed meats is related to insulin resistance. Although, additional studies need to be conducted with regards to long term high intake of animal proteins on muscle mass and the risk of developing insulin resistance. This study provides interesting data on the intake on animal proteins with regards to the prevention of sarcopenia.

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Full Length Research Paper

In vivo biochemical assessment of aqueous extracts of *Vernonia amygdalina* (Bitter leaf)

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This research was carried out on the aqueous extracts of bitter leaf (*Vernonia amygdalina*) to evaluate its phytochemical, proximate and antioxidant composition and its effects *in vivo* on diabetes and obesity biomarkers, antioxidant and hematological profiles. The phytochemical screening of the bitter leaf extract showed a high concentration of flavonoids as the most abundant phytochemical present. Daily administration of extract to rats led to a slight decrease in the lipid profile of the test rats relative to control and no significant difference in the liver function, kidney function, glucose level and hematological profile of test rats relative to control. Antioxidant assay showed high levels of total antioxidant activity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity that was concentration dependent. *In vivo* antioxidant enzyme assay showed an appreciable increase in the level of the antioxidants, glutathione (GSH), superoxide dismutase (SOD), catalase and malondialdehyde (MDA) of the test rats as compared to control. This suggests improved functionality of the antioxidant system of the test rats probably due to the effect of the phytochemical antioxidants in the extract. It is concluded that aqueous extract of *V. amygdalina* can be consumed as food or as an herbal medicine without plausible toxicity to body organs and tissues.

Key words: Bitter leaf, medicinal plants, lipid profile, hematology, antioxidants.

INTRODUCTION

Vernonia amygdalina is commonly called bitter leaf because of its bitter taste. It is a member of the Asteraceae family and a small ever-green shrub that grows all over Africa. It is reported to be a medicinal plant for diabetes and fever (Crellin et al., 1989). Bitter herbs are reportedly good for the body as they help tone the vital organs of the body like the kidney and liver. Ethnomedically, the leaves are consumed either as a vegetable (macerated leaves in soup) or aqueous extracts as tonics for the treatment of various illnesses (Igile et al., 1995). In the wild, chimpanzees have been observed to ingest the leaves when suffering from parasitic infections (Huffman et al., 1993). The roots of *V. amygdalina* have been used for gingivitis and toothache due to its proven antimicrobial activity (Ademola and Eloff, 2011). In North America, of the 17 species of *Vernonia* all have the same effective properties as a blood purifier, uterus toner and helps also to prevent atherosclerosis (Erasto et al., 2007; Nwanjo, 2005).

Many herbalists and naturopathic doctors recommend aqueous extracts for their patients as treatment for anemia, nausea, diabetes, loss of appetite, dysentery and other gastro intestinal track problems. *V. amygdalina* extracts have also been reported to help suppress, delay, or kill cancerous cells (Kupchan et al., 1969).

This study was designed to investigate the biochemical assessment of bitter leaf extract in the management of conditions like diabetes and obesity. This is because the bitter leaves have been reported to be used ethnomedically to manage these conditions. Various experiments were done to analyze the chemical composition of bitter leaf aqueous extract and carry out *in*

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Table 1. Qualitative phytochemical screening of bitter leaf.

Phytochemical	Result
Tannins	+
Phlobatanin	-
Saponin	+
Flavonoids	+
Cardiac glycosides	+
Alkaloid	+

Phytochemical screening results showed that phytochemicals: tannin, flavonoids, cardiac glycosides, saponins, and alkaloids were present in bitter leaf, while phlobatanin was absent.

vivo studies using the rat model to determine the effects of the extract on hematological, lipid, glucose and antioxidant profiles.

MATERIALS AND METHODS

Chemicals

All chemicals and biochemicals used were of analytical grade, purchased from Sigma Chemical Company, USA and used without further purification.

Collection of plant

Fresh bitter leaves were collected at Yaba Market, Lagos State. The leaves were authenticated by Dr. Kadiri, Department of Botany, University of Lagos and the samples were kept at their herbarium.

Extraction and preparation of extract

The extraction of the bitter leaf sample was done by hot infusion.180 ml of hot water was added to 2 g of plant sample. This was left to stand for 20 min and then filtered. The filtrate was then administered orally to the rats.

Phytochemical screening and quantitative estimation of chemical constituency

Chemical tests were carried out on the bitter leaf extract to identify and quantify its constituents; tannins, flavonoids, alkaloids, saponins, cardiac glycosides and steroids, using standard procedures as described by Rios and Recio (2005), Okwu and Okwu (2004), Sofowora (1993), Evans (1996), and Harbone (1973). A diluted solution was made by mixing 2 ml of sample with 20 ml water.

Antioxidant assay of bitter leaf

0.2 g of the bitter leaf tonic was weighed and diluted with 100 ml of water. From this solution, 20 ml solution with concentrations of 100, 75, 50 and 25 were obtained and labeled appropriately. Tests for ferric reducing power capacity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, antioxidant enzymes, lipid peroxidation and total protein were carried out using previously described methods (Aiyegoro and Okoh, 2009; Edeoga et al., 2005).

Animal study

Forty albino female rats (180 to 200 g) purchased at Animal House, College of Medicine, University of Lagos, Idi Araba, Lagos were used for this experiment. The rats were allowed to acclimatize for a period of fourteen days during which they were fed *ad-libitum* with standard rodents feed (rat chow) and tap water. Then, the rats were equally divided (7 rats/group) into two groups; the test and the control group. The weights of the rats in the test group were measured and used to calculate the dosage of bitter leaf extract, 1 ml/kg/day, to be administered to each rat throughout the three weeks of treatment. Administration was done via the oral route with the aid of oral cannula and syringe. After three weeks of administration, blood was collected from the rats into labelled; lithium-heparin, fluoride and ethylenediaminetetraacetic acid (EDTA) bottles via retro-orbital sinus technique. The rats were then sacrificed using cervical dislocation and their livers were collected into plain bottles and placed in ice. The blood samples and livers were then analyzed for various parameters.

Biochemical assays

The sera of the rats were analyzed using Randox diagnostic kits to assess the liver function, kidney function, lipid profile and glucose level of the rats according to standard protocols as described by Burtis et al. (2011).

Hematological assay

A complete blood count was carried out on the blood of the experimental rats using an Automated Analyzer to measure the levels of white blood cell (WBC), red blood cell (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), neutrophils and lymphocytes, according to standard protocols by Burtis et al. (2011).

Statistical analyses

Data from the various studies are presented as mean ± standard error of mean (SEM). The results were analyzed for statistical significance using Microsoft Excel© software systems (2007). Students'-test and Satterwhaites' method of one way analysis of variance were used to compare mean values between groups. $p<0.05$ was taken to indicate a statistical significance.

RESULTS AND DISCUSSION

The bitter leaf *(V. amygdalina)* extract was analyzed for its phytochemical and nutrient composition and the presence of the alkaloids, tannins, saponins, flavonoids, and cardiac glycosides were detected, with flavonoids as the most preponderant (Table 1 and Figures 1 and 2). Flavonoids have been reported to possess antioxidant, anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities (Edeoga et al., 2005). Their highly antioxidant property present in the extract may act in

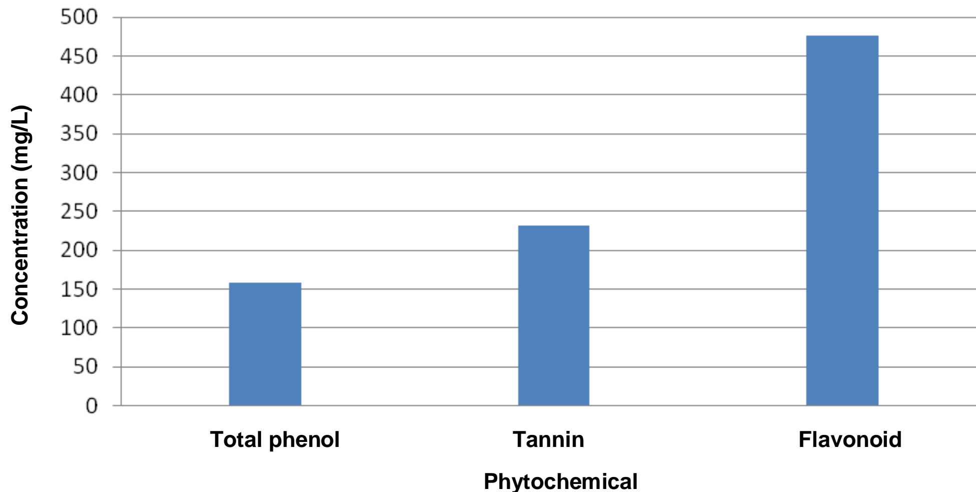

Figure 1. Quantitative estimation of concentration of phytochemicals in bitter leaf. The result shows that flavonoids were present in higher concentration than any other phytochemical.

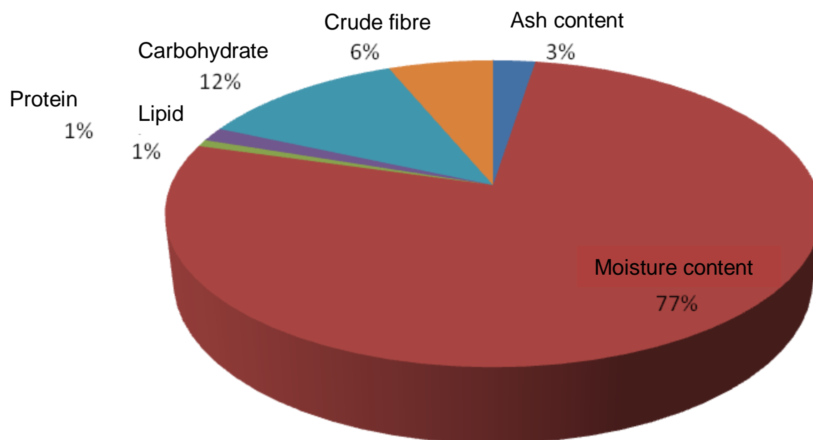

Figure 2. Proximate nutrient composition of bitter leaf.

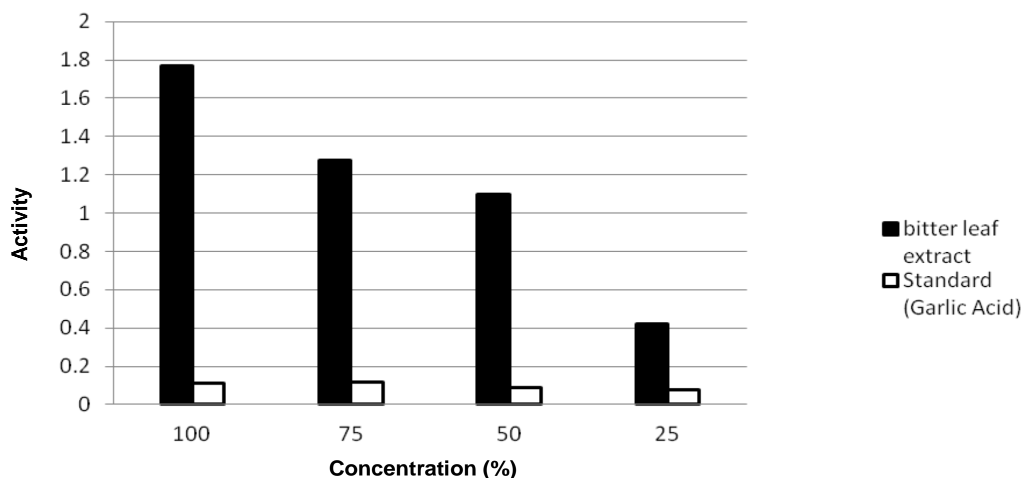

Figure 3. Total reducing power of bitter leaf extract. Determined against that of a known standard at varying concentrations, the reducing power of bitter leaf was very high as compared to the standard at all concentrations.

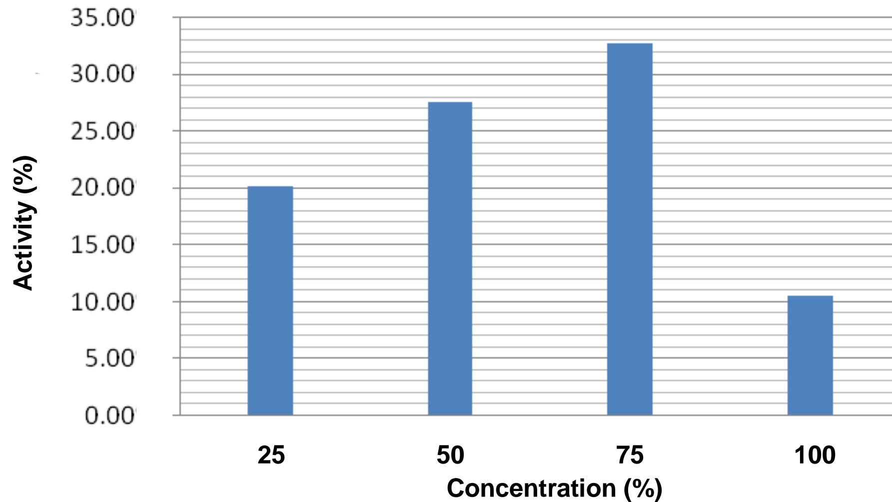

Figure 4. DPPH free radical scavenging activity of bitter leaf extract. From the result, it was noticed that the DPPH free radical scavenging activity of bitter leaf increases with increasing concentration but reduced at the highest concentration.

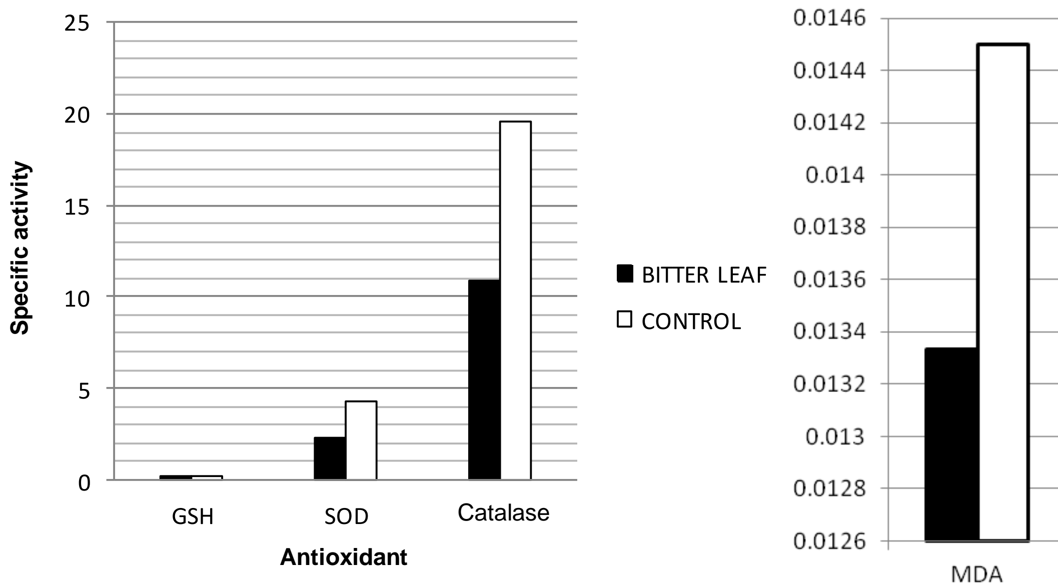

Figure 5. (a) Specific activity of SOD and catalase and GSH in rat liver; (b) Concentration of MDA in rat liver. The results showed that the levels of SOD, CAT, GSH and MDA were lower as compared to the control.

synergy with other phytochemicals present to produce the medicinal benefits inherent in the bitter leaf extract (Akpulu et al., 1994; Boham and Kocipai-Abyazan, 1974).

Antioxidant assay showed high levels of total antioxidant activity and DPPH free radical scavenging activity that was concentration dependent (Figures 3 and 4). *In vivo* antioxidant enzyme assay showed an increase in the level of the antioxidants, glutathione (GSH), superoxide dismutase (SOD), catalase and malondialdehyde (MDA) of the test rats as compared to control (Figure 5) suggesting a possible prevention of lipid peroxidation in tested rats. Unlike a previous report (Opata and Izevbigie, 2006), this suggests improved functionality of the antioxidant system of the test rats probably due to the effect of the phytochemical antioxidants in the extract.

The lipid profile (which involves levels of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and very-low density lipoprotein (VLDL)) serves as diagnostic indices in conditions such as chronic

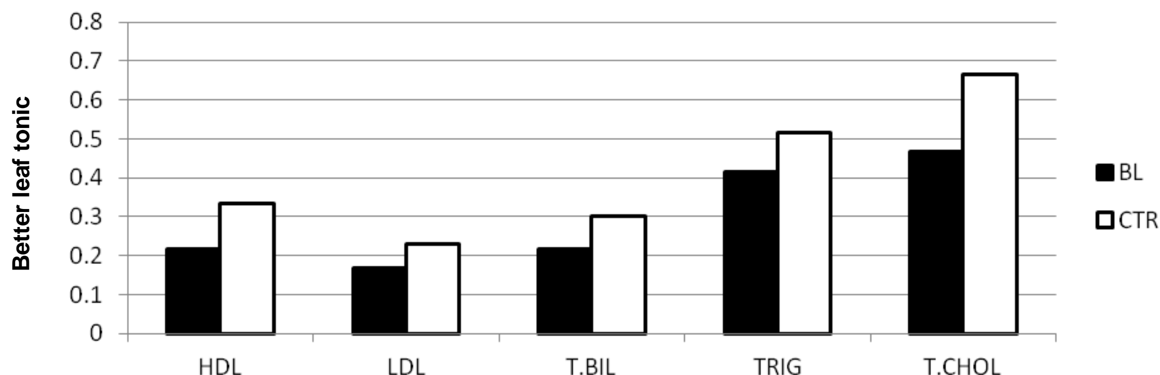

Figure 7. Effect of bitter leaf (BL) tonic on the lipid profile of rats. HDL: High density lipoprotein; LDL: low density lipoprotein; T.BIL: total bilirubin; TRIG: triglycerides; T.CHOL: total cholesterol.

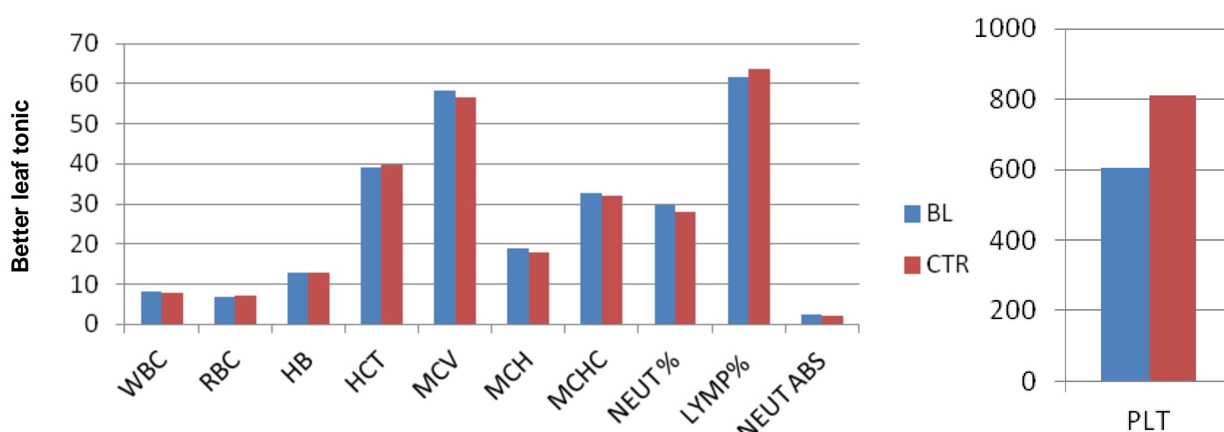

Figure 8. Effect of bitter leaf (BL) tonic on hematological parameters of rats. There was no significant difference at $p<0.05$.WBC: White blood cell; RBC: red blood cell; HB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; NEUT: neutrophils; LYMP: lymphocytes; NEUT ABS: neutrophils absorbance.

obstructive jaundice, hepatitis, coronary heart disease and atherosclerosis. Hyperlipidaemia is one of the risk factors for coronary heart disease while cholesterol is the major lipid constituent of atherosclerotic plague. In support of previous findings (Erasto et al., 2007), extracts of *V. amygdalina* gave a slight decrease in the lipid profile of the test rats relative to control and no significant difference in the liver function, kidney function, glucose level and hematological profile of test rats relative to control (Figures 6 to 8).

The present research shows that there were no significant changes or toxicity potential in most of the investigated parameters following the administration of the aqueous extract of *V. amygdalina*, lending credence to existing reports that *V. amygdalina* is useful in the ethnotherapy of diabetes mellitus (Nwanjo, 2005).

Conclusion

From our study, it can be concluded that aqueous extract of *V. amygdalina* is safe for consumption as food or as herbal medicine without plausible toxicity to body organs and tissues.

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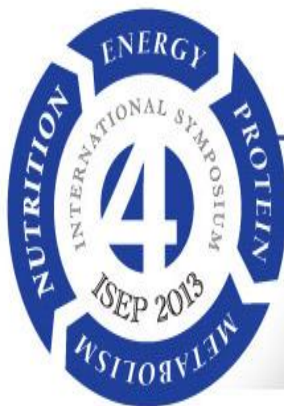

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