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CONTENTS

Original Articles

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Jordan Journal of Biological Sciences

The Effect of *Moringa oleifera* Leaves on Blood Parameters and Body Weights of Albino Rats and Rabbits

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Abstract

This study is aimed at finding out the effect of *Moringa oleifera* on blood parameters and body weights of albino rats (n= 24) and rabbits (n= 10). The rats were divided into four groups; a control group and three experimental groups, while the rabbits were divided into two groups; a control group and an experimental one. The three experimental groups of rats were provided consecutively with100, 200 and 300 mg *M. oleifera* leave extract/kg of body weight daily for 21 days, while the experimental group of rabbits with 2.5 g fresh leaves of *M. oleifera*/Kg of body weight which was added to their feed daily for 21 days, and the control groups were fed on their diets without *M. oleifera*. The results showed significant differences ($P < 0.05$) in mean cell hemoglobin concentration (MCHC) and platelets (PLT) in the third group of rats (AL_3) and red blood cells (RBCs) count, hemoglobin (Hb) and MCHC in the fourth group (AL_4) while no significant differences ($P > 0.05$) were shown in the second group (AL_2). For the rabbits, the mean values of 39.30 ± 1.73, 741.80 ± 65.5 and 5.06 ± 0.54 for PCV, PLT and RBCs in the experimental group were significantly higher ($P < 0.05$) than 33.12 ± 4.32, 344.20 ± 66.6 and 4.68 ± 0.81 for the same parameters in the control group, respectively.

K**eywords:** *Moringa Oleifera,* Medicinal Plant, Blood Parameters, Body Weights, Nutritional Anaemia, Rabbits, Rats.

1. Introduction

Plants as medicinal agents were mentioned in historic documents dating back many thousands of years (Rasonavivo *et al*., 1992). Currently, medicinal herbs as a whole were reported to be used against a wide range of health problems such as cough, cold, stomach, cataract, constipation and many other ailments (Jimenez *et al*., 2003). The plant *M. oleifera* as one of these herbs was reported to prevent effectively, morphological changes and oxidative damage in lens of rats by enhancing the activities of anti-oxidant enzymes, reducing the intensity of lipid peroxidation and inhibiting generation of free radicals (Sreelatha and Padma, 2009). In addition, blood parameters namely: PCV, WBC counts, differentiation of WBC, hemoglobin (Hb) and platelets (PLT) were also found to be positively affected by using this plant (Chinwe and Isitua, 2010). Moreover, *M. oleifera* was found to be of a nutritional value as it contains a number of important vitamins, including: vitamins A, B complex (B1, B3, B6 and B7), C, D, E and K (Dorga and Tandon, 1975; Booth and Wickens, 1988). However, for treatment it was used against high blood pressure, diarrhea, inflammation of colon, intestinal worms, skin antiseptic, as a diuretic agent (Lowell, 2002) and to maintain the levels of blood glucose in diabetic patients (Jaiswal *et al*., 2009, Chinwe and Isitua, 2010). Moreover, *M. oleifera* was used as antimicrobial agent (Caceres *et al.,* 1990), to treat ulcers (Pal and Sahib, 1995) and to promote the immune system against various infections (Jaiswal *et al.,* 2009). So far, most of the work about the effect of *M. oleifera* was carried out on the seeds of this plant. In this paper, we aim to find out the effect of leaves of this plant on various blood parameters as well as the body weights in albino rats and rabbits.

2. Materials and Methods

2.1. Experimental Animals

Albino rats (n= 24, average body weight= 275 g) and rabbits (n= 10, local breed, average body weight = 685g) were used in this study. Body weights of animals before and after experiments were measured using Mettler sensitive balance (number 202845). Albino rats were divided into four groups of six animals; one to act as a control group and denoted AL_1 and the other three to act as experimental groups and denoted AL_2, AL_3 and AL_4. Similarly, rabbits were divided into two groups of five animals; one control (RG_1) and one experimental (RG_2). The control group of rats was provided with normal diet concentrate (dried meat, milk powder, oil and flour in some water) without *M. oleifera* while the experimental groups were provided, in addition to the concentrate, with doses of 100 mg/kg, 200 mg/kg and 300 mg/kg of *M. oleifera* leave extraction, respectively, for 21 days. However, for the rabbits, the control group was provided with fresh clover leaves only, whereas the experimental

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group was fed with both fresh leaves of clover and *M. oleifera* (2.5 g/kg of body weight) for 21 days.

2.2. Preparation of M. oleifera *Leaves Extract*

Leaves of *M. oleifera* were first dried in the shade, left in ethanol (70%) for more than two days in Soxhlet apparatus. Then the 70% ethanol extract was dried in Rotary Evaporator apparatus, weighed and dissolved in distilled water to give the final concentration of 100 mg extract/kg, 200 mg extract /kg and 300 mg extract /kg and were administrated orally by Gavage for the three groups of rats; AL_2, AL_3, and AL_4, for 21 days.

2.3. Hematological Measurements

Blood samples were collected from retro-orbital of the experimental rats in capillary tubes coated with ethylene diamine tetra-acetic acid (EDTA). The tubes were immediately capped, kept at – 4 °C and were immediately analyzed for blood parameters using automated coagulating Sysmex apparatus of the type 8999. The parameters included: hemoglobin (Hb), mean cell volume (MCV), red blood cells count (RBCs), white blood cells count (WBCs), mean cell hemoglobin concentration (MCHC), platelets (PLT), lymphocytes (LYM) and packed cell volume (PCV). However, MCV and MCHC values were calculated from RBCs count, Hb and PCV (Androw, 1972; Merghani, 2010).

2.4. Statistical Analysis

Mean values of blood parameters and body weights were analyzed by student *t*- test using computer package program (PASW statistics 18).

3. Results

3.1. Blood parameters

The results of blood parameters in rats are shown in Table 1. The results show that MCHC and platelets numbers increased significantly ($P < 0.05$), in group 3 (AL_3) and RBCs count Hb concentration and MCHC increased similarly in group 4 (AL_4). However, the remaining blood parameters changed slightly, but insignificantly (Table 1).

Blood parameters in rabbits are shown in Table 2. Only RBCs, platelets and PCV numbers were increased significantly ($P < 0.05$), whereas the other blood parameters remained more or less unchanged (Table 2).

Table 1. Mean values of blood parameters in rats provided daily with *M. oleifera* leave extracts for 21 days in captivity (mean ± SD)

Parameters	AL_1	AL_2	AL_3	AL_4	Normal range[Ⅱ]
WBCsx10^3/mm^3	7.4 ± 0.42	7.6 ± 0.72	12.9 ± 3.63	10.3 ± 4.4	6.9 -11.2
RBCsx10^6/mm^3	6.9 ± 0.43	6.8± 0.42	7.4 ± 0.54	7.08±0.56**	6.9 -11.2
Hb g/dl	12.6 ± 0.64	13.2± 1.25	12.4 ± 3.63	13.7±0.67**	10 – 14
MCV mm^3	51 ± 1.71	52.3± 2.82	55.1 ± 4.9	53.3 ± 2	41 – 48
PCV %	36.2 ±0.87	36.6± 0.23	36.5 ± 0.3	36.8 ± 0.45	30 – 48
MCHC%	32 ± 0.84	33.0± 0.76	34.1 ± 0.92**	35.8±1.46**	28.2 – 32.4
PLTx10^3/mm^3	1075±259.8	1093±108.9	1121.8±262.8**	901 ± 81.7	500–1300
LYM%	53 ± 29.8	74.1 ± 7.9	44.4 ± 11	69.3 ± 15.8	65 – 85

** Highly significant. [Ⅱ](David *et al.*, 2002).

Table 2. Mean values of blood parameters in rabbits provided daily with fresh leaves of *M. oleifera* being mixed with their clover feed for 21 days in captivity (mean ± SD)

Parameters	RG_1	RG2	Normal range[Ⅱ]
WBCs x10^3/mm^3	6.0±3.2	6.5±1.2	5.6 – 16.5
RBCs x 10^6/mm^3	4.68 ± 0.81	5.06± 0.54**	3.7 – 7.5
Hb g/dl	9.46 ± 1.29	9.52 ± 1.75	8.9 – 15.5
MCHC %	31.5 ± 0.91	32.7 ± 1.43	31.1 – 37
PLT x 10^3/mm^3	344.20 ± 66.6	741.80± 65.5**	112 – 795
MCV mm^3	65.54 ± 2.31	68.08 ± 2.57	58 –79.6
PCV %	33.12 ±4.32	39.30 ± 1.73**	26.7 – 47.2
LYM %	60.0 ± 3.39	49.3 ± 18.91	43 – 80

** Highly significant. [Ⅱ] (Hewltt *et al.*, 1989).

3.2. Body weights

Mean values of body weights of rats are shown in Table 3. With the exception of group 2 (AL_2), rats in groups 3 and 4 (AL_3 and AL_4) showed significant ($P < 0.05$) increase in their body weights compared to the control group (AL_1).

For rabbits, the mean values of their body weights are shown in Table 4. In these animals, the results revealed that they didn't change significantly in their body weights ($P > 0.05$).

Table 3. Mean body weight (g) of rats provided daily with *M. oleifera* leave extracts for 21 days in captivity

Parameters	AL_1	AL_2	AL_3	AL_4
Initial weight	292.2 ± 25.4	288.3± 32.8	263.2 ± 37.1	292.5 ± 48
Final weight	296.3 ± 22.7	292.5± 25.4	300.3± 23.6	312.5 ± 42.1
Difference (g)	3.9 ± 2.7	4.2 ± 7.4	37.1± 13.5**	20.3± 5.9**
Difference (%)	1.3%	1.4%	14%**	6.9%**
Weight gained (g/day)	0.19	0.2	1.8	0.96

**Highly significant.

Table 4. Mean body weight (g) of rabbits fed with fresh leaves of *M. oleifera* daily for 21 days in captivity

Parameters	RG_1	RG_2
Initial weight	718 ± 108.1	653.8±125.5
Final weight	720 ± 108.4	674 ± 124.4
Difference (%)	0.28%	3.2%
Weight gained (g/day)	0.1	1

4. Discussion

The tree, *M. oleifera* (*Moringaceae*), is cultivated widely around the world (Odee, 1998; Jed and Fahey, 2008) and used for various purposes one of which is as a feed supplement to livestock (Martin, 2007; Fadiyimu *et al.*, 2010). In this study, albino rats and rabbits were used to test the nutritional values of *M. oleifera* via its effect on blood parameters as well as on changes in the animals' body weights. Dietary components of *M. oleifera* were reported to have measurable effect on blood constituents (Church *et al.*, 1984). With the exception of MCHC, Hb, RBCs and platelets in rats and PCV, RBCs and platelets in rabbits, the other blood parameters did not change significantly with inclusion of *M. oleifera* leaf extract in rats and fresh leaves in rabbis. However, mean values of each parameter were within the normal range (Hewltt *et al.*, 1989; David *et al.*, 2002). In contrast, the body weights of rats increased significantly with increased *M. oleifera* concentration, while no significant change occurred in rabbits. The highest body weight gain of rats in AL_3 could support earlier reports that *M. oleifera* is of a high nutritional value (Ram, 1994; Makkar and Becker,1996; Anwar *et al.*, 2007), but this was not reflected in increased lymphocytes as reported by Fox (2006).

The increase in the body weight of rats might be due to the fact that *M. oleifera* is rich in amino acids, vitamins and minerals particularly iron (Subadra *et al.*, 1997; Faye, 2011). The significant increase in body weights of rats might also be attributed to captivity, where energy expenditure is minimal (Fadi *et al.*, 2010).

5. Conclusion

In conclusion, the results of this study supported the reports about *M. oleifera* in having medicinal effect in curing some health problems associated with nutritional status (Mahajan *et al.*, 2007) and this was indicated in this study by its positive effect on some blood parameters and body weights of the experimental animals.

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Enzymatic Modification of Sea Buckthorn Dietary Fiber by Xylanase from *Streptomyces rameus* L2001: Characterization of its Physicochemical Properties and Physiological Effects on *Bifidobacterium*

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Abstract

Xylanase from *Streptomyces rameus* L2001 was used to modify sea buckthorn dietary fiber to enhance the amount of soluble fiber in this study. We then investigated the physicochemical and physiological properties of the resulting dietary fiber. Conventional single factor methods and response surface methods were used to optimize the conditions and the outcomes were as follows: hydrolysis time, 5 h; enzyme content, 46 U/g; temperature, 55.7°C. Under these conditions, the yield of modified soluble dietary fiber was 42.84 mg/g, as compared with 14.04 mg/g for unmodified fiber. We also conducted studies to determine the properties of modified sea buckthorn dietary fiber, including water holding capacity, oil binding capacity and swelling capacity, which were 110.7%, 106.9% and 116.5%, respectively, relative to unmodified fiber. We next cultivated *Bifidobacterium* with the soluble dietary fiber (derived from modified sea buckthorn) and compared growth with that of bacteria maintained on glucose. Supplying *Bifidobacterium longum* and *B. infantis* with the soluble component aided their proliferation, particularly of *B. longum.* This is the first report on sea buckthorn dietary fiber and its modification with xylanase.

Keywords: Sea Buckthorn Dietary Fiber, Xylanase, Modification, Physicochemical and Physiological Properties

1. Introduction

Sea buckthorn is a new multifunctional food with significant agricultural, ecological, nutritional, medical and ornamental value. (Ruan and Li, 2005) Because of its commercial and nutritional value, sea buckthorn has been the focus of much research to determine its basic physiochemical characteristics, as well as the physiological properties of different components and compounds, including seed oil, flavones, tocopherol and phenolic acid, and the putative associations between these compounds and human health. Larmo *et al*. (2009) researched the effects of sea buckthorn berries on circulating concentrations of cholesterol, triacylglycerols and flavones in healthy adults. They found that sea buckthorn berries have benefits on human health in terms of cardiovascular disease risk by reducing C-reactive protein concentration. A similar conclusion was reached by Xu *et al*. (2011). Other studies have shown that sea buckthorn berries are a source of phenols and proanthocyanidins that may help suppress the growth of human colon and liver cancer cells (Grey *et al*., 2010). Moreover, because of the different phytonutrients and bioactive substances present in sea buckthorn, it has beneficial effects on wound healing (Gupta *et al*., 2006), immune function, oxidative stress by stabilizing membrane structure in animals and increasing the activities of essential enzymes (Yang and Kallio, 2002).

Research into sea buckhorn has also focused on the identification and extraction of its components. High-performance liquid chromatographic fingerprinting has been used to identify the different origins of sea buckthorn berries (Chen *et al*., 2007). However, no systemic research studies of sea buckthorn dietary fiber have been performed to date. Several observational studies have shown that dietary fiber intake is associated with a number of health benefits, and that these effects are dependent on the composition (i.e., soluble and insoluble components) and physicochemical properties of the dietary fiber (Cornfine

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et al., 2010). It is now well established that certain sources of dietary fiber, such as psillium, pectin and oats, can lower serum cholesterol and blood sugar concentrations, independently of the fat or carbohydrate content of the diet (Hu *et al*., 2008), as well as decrease the prevalence of cancer, particularly colon and breast cancer, improve body weight management and may indirectly reduce the risk of heart disease (Kendall *et al*., 2010). Some studies have indicated that the quantity and activity of the microflora in the gut are related to the relative utilization of fermentable dietary fiber (Guillon and Champ, 2000). As described above, research into sea buckthorn fiber is important, not only in terms of empirical evidence, but also considering its potential effects on human health. Further research on sea buckthorn fibers is important to determine the relationship between the components and properties of dietary fiber with health. Such research will also increase the availability of fiber-rich food to provide more nutritional materials for use in daily life.

The purpose of this study was to modify sea buckthorn dietary fiber with xylanase obtained from *Streptomyces rameus* L2001, and evaluate the physicochemical properties of modified dietary fiber (MDF), total dietary fiber (TDF), soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). Outcomes of interest included the water holding capacity (WHC), oil binding capacity (OBC) and swelling capacity (SC). The optimized conditions were determined using the single factor method in addition to response surface methodology with a three-level, three-variable central composite rotational design.

Several small-scale clinical studies with various fermentable dietary fibers have shown significant, but small, clinical benefits of these products either alone or in combination with probiotics on a number of intestinal diseases and disorders (Bittner *et al*., 2007). Based on these properties of dietary fiber, bacteria capable of fermenting carbohydrates (*B. longum* and *B. infants*) were cultured with soluble dietary fiber (derived from modified sea buckthorn) to investigate its effects on bacterial growth.

2. Materials and Methods

2.1. Xylanase Purification and Assay

Streptomyces rameus L2001, isolated from soil samples (Tianshan, Xinjiang), was used in this study, and the purified process was according to Xiuting Li et al. (2010). Briefly, the strain was fermented in culture broth for 5 days at 40℃, and the production of the xylanase was nearly 2000U/mL. After centrifuging (8000×g for 10 min at 4ºC) and ammonium sulfate precipitation (40-60%) , the dialyzed enzyme solution was applied to a DEAE-52 column (1.0cm×10cm) and CM Sepharose Fast Flow column (1.0cm×10cm), which were pre-equilibrated with 20mM Tris-HCl buffer (pH=7.0) and 20mM acetate buffer (pH=5.3) , respectively. The purpose protien were eluted with 0-0.05M NaCl gradient at a flow rate of 1.0 mL/min. All purification procedures above were performed at 4 ºC unless stated otherwise. Ultimately, the purified xylanase was checked by SDS-PAGE to determine the purity and protein molecular.

The reducing sugar produced in this experiment was assayed by the dinitrosalicylic (DNS) acid method with xylose as the standard, and the one unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1μmol of xylose equivalent in one minute under the assay conditions.

2.2. Preparation of Sea Buckthorn Dietary Fiber

Sea buckthorn was provided by Qinghai kangpu biological technology Co., Ltd (Qinghai, PR China), milled through a 300-μm mesh, and heated at 65°C in a air-drying oven (Modle BA0-35A, Shidokai Shanghai Equipment Co., Ltd., Shanghai, China). A sample of sea buckthorn was then immersed in a 10-fold volume of acetate buffer (pH 5.8) and heated to 70°C for 30 min with continuous stirring. In order to gain more soluble fiber, 2 mL of neutral protease (Novozymes Biological Technology Co., Ltd., Beijing, China) was added to the mixture to remove the protein. A conical flask (500 mL) containing the sea buckthorn suspension was heated to 45°C in a water bath (Model SHY-2, Jinchengguosheng Instrument Plant, Jiangsu, China) for 2 h with agitation at 180 rpm. After enzyme hydrolysis, 4 volumes of 95% ethanol were added to precipitate the polysaccharides, and incubated for 4 h at 4°C. The precipitate was collected by centrifugation at 5000 ×*g* for 15 min, followed by vacuum drying to obtain the dietary fiber (DF) used in this study.

2.3. Composition and Physical Properties of Dietary Fiber

The TDF, IDF and SDF in dietary fiber and XMF were determined using the AOAC method. WHC, OBC and SC were determined as previously described (Sangnark and Noomhorm, 2003; Escalada-Pla, 2007).

2.4. Processing of Sea Buckthorn Dietary Fiber by Xylanase from Streptomyces rameus L2001

Hydrolysis of sea buckthorn dietary fiber (5g) was performed in a 50-mL conical flask containing acetate buffer (pH 5.8). The initial temperature and fluid material ratio were 50°C and 10. Based on the pilot studies performed to determine the characteristics of xylanase hydrolysis, the following experimental conditions were used: hydrolysis time, 1–6 h; enzyme dose, 15–65 U/g, temperature, 45–65°C.

2.5. Experimental design and statistical analysis

A three-level, three-variable central composite rotatable design was applied using Design-Expert software version 8.0.1 (State-Ease, Inc., Minneapolis, MN, USA) to determine the optimal enzymatic conditions. Based on our single-factor experiment, the three variables were hydrolysis time, 2–4 h; enzyme dose, 35–55 U/g, and hydrolysis temperature, 50–60°C. The response variable was SDF content (mg/g). Each variable was coded at three levels (–1, 0 and 1), as shown in Table 1.

Table 1.Variables and their levels used in a central composite rotatable design for optimization of xylanase hydrolysis conditions

Variable	Coded level		
	–1	0	1
Hydrolysis time (h)	2	3	4
Enzyme dosage (U/g)	35	45	55
Hydrolysis temperature (°C)	50	55	60

Taking into account the main, quadratic and interaction effects, the quadratic response surface analysis was based on the multiple linear regressions presented in Eq. (1). As the three parameters were varied, 10 α-coefficients were to be estimated, corresponding to the three main effects, three quadratic effects, three interactions and one constant.

$$Y=\alpha_0+\sum_{i=1}^{3}\alpha_i x_i+\sum_{i=1}^{3}\alpha_{ii}x_i^{\,2}+\sum_{i=1}^{2}\sum_{j=i+1}^{3}\alpha_{ij}x_i x_j+e \quad (1)$$

The response function was Y=mg/g of modified soluble sea buckthorn dietary fiber.

2.6. Bifidobacterium *Species and Proliferation Assay*

B. longum and *B. infantis* were provided by the Chinese Academy of Agricultural Sciences (Beijing, China). Both strains were activated using basal medium (g/L) containing 10 g tryptone, 10 g yeast extract, 10 g beef extract, 2 g cysteine hydrochloride, 20 g glucose, 0.25 g K_2HPO_4, 0.58 g $MgSO_4{\cdot}7H_2O$, 0.25 g $MnSO_4$, 5 g sodium acetate anhydrous (pH 6.8). The bacterial strains all grew statically in this medium for 24 h in an anaerobic chamber (Model 1029, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C.

To assess the effect on bacterial proliferation, sea buckthorn SDF (derived from MDF) was added to the culture medium instead of glucose (20 g/L, enriched medium), while the other nutritional components were retained. The growth of *B. longum* and *B. infantis* were assayed by measuring absorbance at 620 nm using a spectrophotometer (Shanghai Lingguang Technology Co., Ltd., Shanghai, China). Briefly, two culture flasks were prepared; one contained 50 mL of basal medium while the other contained 50 mL of enriched medium. Then, 4% activated bacterial suspension was added to each flask, followed by culture for 48 h at 37°C in an anaerobic chamber. Bacterial growth was assayed every 6 h and growth curves were plotted. Proliferation studies were performed at least in duplicate and the mean was calculated.

3. Results and Discussion

3.1. TDF, IDF and SDF Content of Sea Buckthorn

The composition of sea buckthorn dietary fiber was determined before modification. As shown in Table 2, the amounts of TDF, SDF and IDF were 68.03±0.12%, 2.88±0.21% and 65.15±0.33%, respectively. These data indicate that sea buckthorn has abundant fibers. Compare with other fruits, the TDF and IDF contents were 0.3% and 0.6% in watermelon, and 9.1% and 10.9% in sapota, respectively. Meanwhile, the SDF content was 0.3% in watermelon and 2.4% in fig (Ramulu and Rao, 2003). Almost 25 different fruits were assayed, and the values of TDF, IDF and SDF were consistently lower than those in sea buckthorn dietary fiber in this study. These results indicate that sea buckthorn contains much higher dietary fiber than other fruits and it may become a new and good resource of fibers for daily life, even for food by-prodcut or industry as other plants and fruits.

Table 2. Composition of sea buckthorn

Composition	Content
SDF	2.88±0.21%
IDF	65.15±0.33%
TDF	68.03±0.12%

The insoluble and soluble properties of dietary fiber are related to differences in their physiological functions and effects (Jimenez-Escrig and Sanchez-Muniz, 2000). Insoluble fibers are characterized by their porosity, low density, and their abilities to increase fecal bulk and decrease intestinal transit. In contrast, soluble fibers are characterized by their capacity to increase viscosity, and to reduce the glycemic response and plasma cholesterol levels (Abdul-Hamid and Luan, 2000). Moreover, foods high in SDF, such as fenugreek (Al-Habori and Raman, 1998), guar gum, oat and psyllium have hypoglycemic and hypocholesterolemic effects in experimental animals and in humans (Bittner *et al*., 2007). In this paper, we used xylanase to modify sea buckthorn dietary fiber to enhance the SDF content and investigated the physicochemical properties of modified sea buckthorn fiber to assess its function and biological value.

3.2. Effects of xylanase hydrolysis time on SDF content

Figure 1 shows the effect of hydrolysis time on the SDF content of sea buckthorn dietary fiber. Between 1 and 3 h, the SDF increased, reaching a peak of 36.16 mg/g. Meanwhile, extending the reaction time over 3 h decreased SDF content.

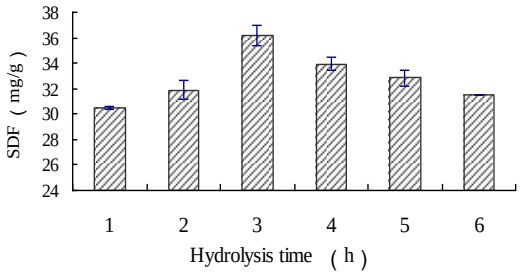

Figure 1. Effects of hydrolysis time on the SDF content of sea buckthorn (mg/g). Reaction conditions: 50 mL acetic acid (pH 5.3), 40 U/g enzyme, temperature 50°C, and incubation for 1–6 h.

The substrate and enzyme concentrations have marked effects on the kinetics of enzymatic hydrolysis (Yoon *et*

al., 2005). During the early phase of the reaction, a high substrate concentration and low levels of polysaccharide had a weak inhibitory effect on xylanase activity, allowing the hydrolysis reaction to occur rapidly. However, as the reaction proceeding, the polysaccharide concentration increased and competitively inhibited the enzymatic reaction, reducing its speed.

3.3. Effects of Enzyme Dose on SDF Content

Figure 2 shows the effects of various doses of xylanase on the yield of SDF. And the greatest yield (37.25 mg/g) was at an enzyme dose of 45 U/g. This experiment showed that increasing the doses of xylanase in a suitable range, increasing the yield of SDF by converting insoluble dietary fiber into polysaccharides, oligosaccharides or monosaccharides.

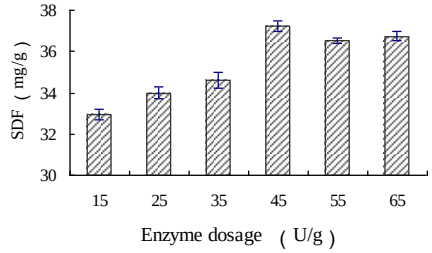

Figure 2. Effects of enzyme dose on the SDF content of sea buckthorn (mg/g). Reaction conditions: 50 mL acetic acid (pH 5.3), hydrolysis time 3 h, temperature 50°C, enzyme dose 15–65 U/g.

3.4. Effects of Hydrolysis Temperature on SDF Content

Figure 3 shows the effects of hydrolysis temperature on SDF content. SDF content increased with increasing temperature until 55°C, and then decreased at higher temperature.

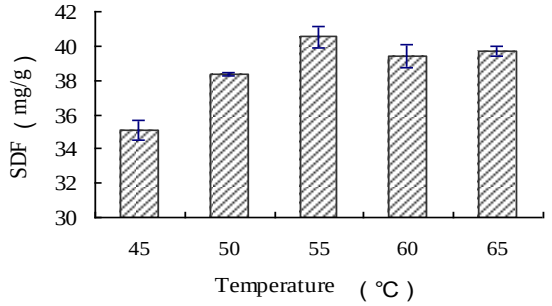

Figure 3. Effects of different hydrolysis temperatures on SDF content of sea buckthorn (mg/g). Reaction conditions: 50 mL acetic acid (pH 5.3), hydrolysis time 3 h, enzyme dose 45 U/g, and hydrolysis temperature 45–65°C**.**

3.5. Optimization of the Xylanase Modification Conditions

According to the different hydrolysis conditions that affected the content of SDF, as shown in Figs. 1–3, we selected a hydrolysis time of 3 h, enzyme dose of 40 U/g and hydrolysis temperature of 55°C as the central conditions of the response surface analysis to optimize the production of SDF.

The coefficients derived from analysis of variance are shown in Table 3.

Table 3. Analysis of variance for the response surface quadratic model

Source	df	Sum of Squares	Mean Square	F	Pr>F	
X_1	1	4.82	4.82	3.78	0.0928	
X_2	1	7.30	7.30	5.73	0.0479	*
X_3	1	0.86	0.86	0.68	0.4372	
X_1X_2	1	0.099	0.099	0.078	0.7882	
X_1X_3	1	0.16	0.16	0.13	0.7335	
X_2X_3	1	0.027	0.027	0.021	0.8879	
X_1^2	1	0.049	0.049	0.039	0.8499	
X_2^2	1	54.10	54.10	42.47	0.0003	*
X_3^2	1	29.28	29.28	22.99	0.0020	*
Lack of fit	3	8.43	2.81	23.20	0.0054	*
Model	9	101.32	11.26	8.84	0.0045	*
Pure error	4	0.48	0.12			

X_1: Hydrolysis time (h); X_2: enzyme dose (U/g); X_3: Hydrolysis temperature (°C); *: significant; [a] Coefficient of determination (R^2) = 0.9191; [b]CV% = 3.00%.

The linear coefficients of enzyme dose and the two quadratic terms for enzyme dose and hydrolysis temperature were statistically significant (P<0.05). While, interactions between the three independent variables was not statistically significance (P>0.05), suggesting little interaction between these parameters. The total coefficient of determination (R^2) and the Model index was 0.9191 and 0.0045 (P<0.005), respectively, indicaed a reasonable fit of the model to the experimental data. And the predicted yield of modified SDF was calculated using the regression model and were compared with the experimental values in Figure 4.

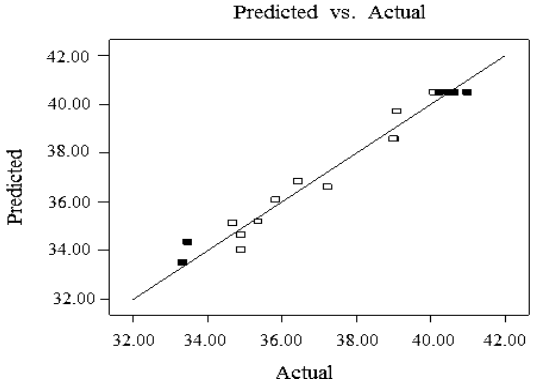

Figure 4. Comparison between the predicted and actual yields of modified SDF.

$$Y=40.48+0.78X_1+0.96X_2+0.33X_3-0.16X_1X_2+0.\mathbf{2X_1X_3}+0.082X_2X_3+0.11X_1^2-3.58X_2^2-2.64X_3^2 \quad (2)$$

To determine the optimum conditions for greatest SDF yield, three-dimensional response surface plots were constructed based on Eq. (2).

Figure 5 shows the linear effects of hydrolysis time and quadratic effects of enzyme dose on SDF content. SDF yield reached at a maximum value near the central condition, and with the hydrolysis proceeding, the production was increased at any enzyme dosage.

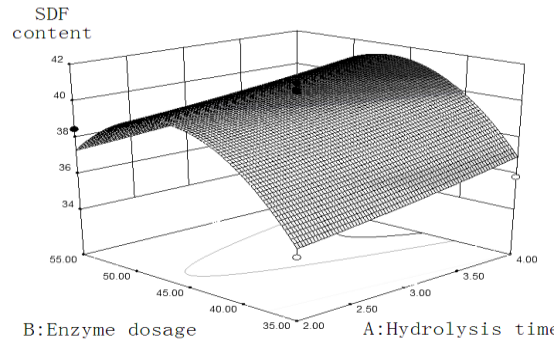

Figure 5. Response surface plot showing the effects of hydrolysis time (h) and enzyme dose (U/g) on SDF content. Hydrolysis temperature (55°C), 50 mL acetic acid and pH (5.3) were kept constant

Figure 6 shows the quadratic effects of hydrolysis temperature and linear effects of hydrolysis time on SDF content; it was similar to Fig. 5.

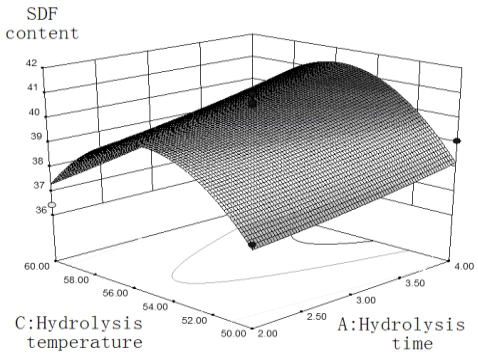

Figure 6. Response surface plot showing the effects of hydrolysis time (h) and hydrolysis temperature (°C) on SDF content. Enzyme dose (45 U/g), 50 mL acetic acid and pH (5.3) were kept constant.

The contour plot shows both quadratic effects of hydrolysis temperature and enzyme dose on SDF content in Figure 7.

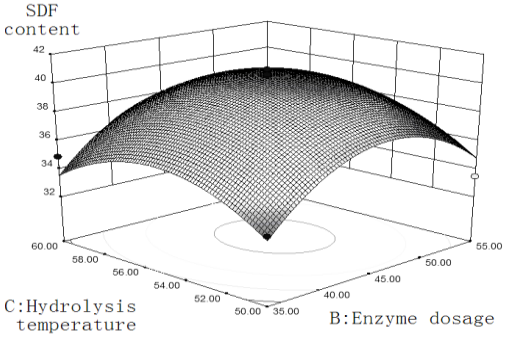

Figure 7. Response surface plot showing the effects of enzyme dose (U/g) and hydrolysis temperature (°C) on SDF content. Hydrolysis time (3h), 50 mL acetic acid and pH (5.3) were kept constant.

This figure shows that the response surface had a maximum point, when the optimum hydrolysis temperature and enzyme dose were 55°C and 45 U/g, the maximum SDF content was 40.53 mg/g.

The optimal conditions derived from the model were as follows: hydrolysis time, 5 h; enzyme dose, 46 U/g; hydrolysis temperature, 55.7°C. After xylanase modification, the amount of soluble dietary fiber increased from 2.88% to 4.28%. Although the enzymatic method to modify the sea buckthorn dietary fiber is not desirable than we expectation, it is also meaningful to reveal the crude xylanase has ability to modify the cereal fiber to gain solube fibers. Meyera *et al*. (2009) modified potato pulp with an enzyme derived from fungi, and found that this did not alter the SDF yield or the relative amount of soluble fiber in the total solubilized dry matter. Meanwhile, other studies focus on digestibility and short chain fatty acid production, rather than SDF concentration (Carneiro *et al*., 2008). In recent years, more researches have been conducted on the physical modification of dietary fibers. For example, Repo-Carrasco-Valencia *et al*. (2009) modified *Amaranthus caudatus* fiber by extrusion, which increased the SDF content from 2.45% to 3.06% in one variety, whereas a slight decrease from 1.65% to 1.46% was noted in another variety. Because the food sensorial properties of food may be affected by chemical treatments and mechanical procedures are associated with low yield, the use of enzymes for modifying cereal-based foods may offer an approach to overcome such limitations (Napolitano *et al*., 2009).

3.6. Physical Properties of Sea Buckthorn IDF, SDF, TDF and MDF

Figure 8 shows the value of WHC of the SDF and IDF. Interestingly, the OBC and SC tended to be greater for SDF than for IDF. The SC and WHC are indices of fiber hydration and provide useful information for fiber-supplemented foods. These properties will also help us to predict the behavior of fiber in foods or during gut transit (Xu *et al*., 2011).

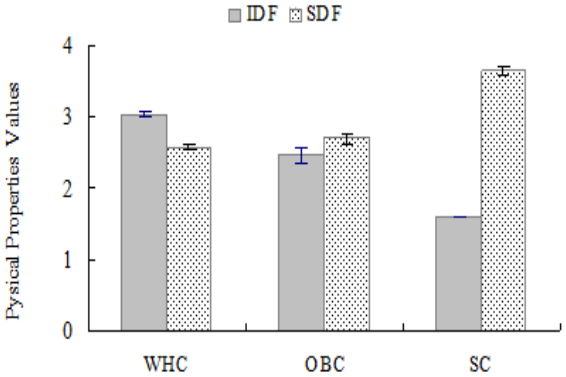

Figure 8. Water holding capacity (WHC), oil binding capacity (OBC) and swelling capacity (SC) of insoluble (IDF) and soluble (SDF) dietary fiber.

As shown in Figure 9 the WHC, OBC and SC of TDF were 3.31±0.034, 2.86±0.084 and 1.70±0.1, respectively. Compared with other cereal and fruits, the SC was much lower in TDF than in apple fiber (3.8) and citrus fiber (8.6). While the WHC was similar to that of wheat bran (3.0), maize bran (2.4) and resistant starch (3.1), as reported by Guillon and Champ (2000). Finally, the OBC

was favorable compared with that of dry okra (3.18) (Schneeman, 1999).

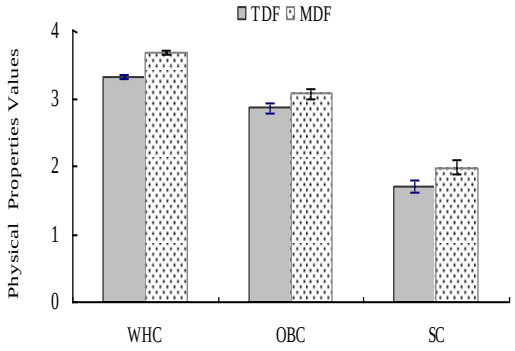

Figure 9. Water holding capacity (WHC), oil binding capacity (OBC) and swelling capacity (SC) of total (TDF) and modified (MDF) dietary fiber.

In a comparison of these parameters between TDF and MDF, the values tended to be higher for MDF than for TDF. This indicates that xylanase modification improved the physical characteristics of sea buckthorn, and these improvements were likely due to the modification of the physical structure of the dietary fiber (McCleary *et al*., 2001). Enzymatic modification is useful to convert the insoluble fiber into soluble oligosaccharides, and alter the structure of dietary fiber, including particle size and surface area, and thus influence the hydration properties. Although the enzymatic degradation role of xylanase is limited and the mechanisms involved in the changes about the physical properties of MDF are still not fully understood, it seems that partial degradation of the dietary fiber by xylanase is likely to lose the structure of dietary fiber and enable swelling. Consequently, oil and water molecules can bind more easily with the individual sea buckthorn fibers particle.

Processes, such as grinding, drying, heating or extrusion cooking, if they modify the physical properties of the fiber matrix, also affect the hydration properties (i.e., WHC and SC) (Renard *et al*., 1994). Raghavendra *et al*. (2006) reported that decreasing particle size from 1127 to 550 μm using a screw press increased the hydration properties. Physical processes have some disadvantages, including wastage of material and the cost due to use more resources and power to obtain the desired products. Accordingly, finding an appropriate method to modify the dietary fiber is becoming increasingly important and many researchers are now focusing on enzyme to modify dietary fiber because its process and approach is more moderate than physical methods, and greater yields can be obtained.

3.7. Effect Of Dietary Fiber On Proliferation Of Bifidobacteria

Bifidobacteria are gram-positive, saccharolytic anaerobic bacteria, and comprise up to 25% of the cultivable gut microflora (Duncan *et al*., 2007), they obtain carbon and energy by fermenting the host's dietary carbohydrates. Furthermore, the different species of *Bifidobacteria* differ in their fermentation profiles when cultured with different fermentable carbohydrates (Yuan *et al*., 2005), and IDF is generally more resistant to colonic fermentation than SDF (Jenkins and Kendall, 2000). Among the *Bifidobacteria*, *B. longum* and *B. infantis* have been studied for their effects on human health and incorporated into dairy products and therapeutic preparations (Biavati and Mattarelli, 2001). Therefore, we used SDF derived from MDF as the carbon source and compared fermentation profiles and growth with both of *Bifidobacteria* cultured with glucose.

Figure10 shows the effects of SDF and glucose as the sole carbon source on the growth of *B. longum*. By 6 h, bacteria in both groups had started to proliferate, with a more pronounced effect in bacteria grown in SDF-enriched medium. By 18 h, the optical density (OD) values started to reach a plateau in the SDF group, indicating that the growth of *B. longum* had started to stabilize. In contrast, the growth of *B. longum* cultured in glucose was much slower over 0–24 h, and the plateau was evident at 24 h. Based on these data, the use of SDF as the fermentation carbohydrate source achieves more rapid growth of *B. longum* than glucose.

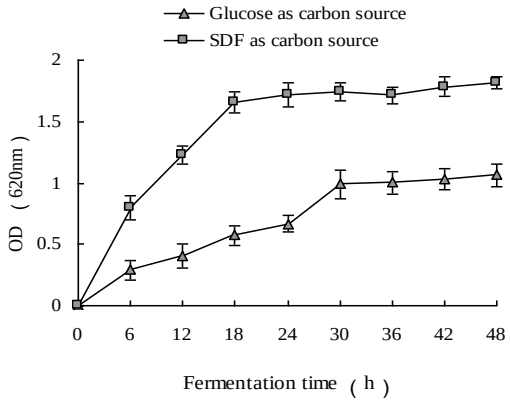

Figure 10. Comparison of the effects of SDF and glucose on the growth of Bifidobacterium longum. Experimental conditions: medium 50 ml (2% modified SDF and glucose in enriched and basal medium, respectively), fermentation time 48 h, temperature 37°C, anaerobic fermentation.

A similar study was performed by Arrigoni *et al*. (2002), who used a wheat germ preparation to cultivate *Bifidobacteria* in vitro. They found that the wheat germ preparation increased the proportion of *Bifidobacteria* from 15% to 24% of all bacteria detected in the faecal matter. Although their results and methodology differ somewhat from our own, the results of both studies indicate that dietary fiber is beneficial for microbial growth and symbiosis in the human intestine.

We next tested the effects of SDF on the growth of *B. infantis*. As shown in Figure 11, the growth curves of *B. infantis* was similar to those of *B. longum*. As before, the OD value was consistently higher for bacteria grown in the SDF-enriched medium than those grown in the basal glucose medium, which was apparent by 6 h. Bacterial growth in the SDF-enriched medium continued to increase over time, reaching the plateau phase at 36 h. In comparison, bacteria grown in the basal glucose medium reached the plateau phase at 42 h. This indicates that *B. infantis* and could catabolize SDF as the carbohydrate source more efficiently than glucose.

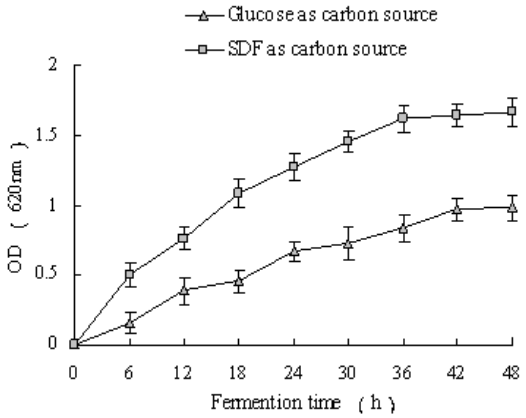

Figure 11. Comparison of the effects of SDF and glucose on the growth of *Bifidobacterium infantis*. Experimental conditions: medium 50 ml (2% modified SDF and glucose in enriched and basal medium, respectively), fermentation time 48 h, temperature 37°C, anaerobic fermentation.

In this study, both *B. longum* and *B. infantis* showed enhanced growth in SDF-enriched medium than in basal glucose medium. These results demonstrate that the dietary fiber has marked effects on proliferation of bacterial communities. Another study has shown that the type of fiber is a major factor that affects the bacterial community, and that administration of dietary fiber may promote animal or human health aiding bacterial growth and fermentation to short chain fatty acids (Amado and Arrigoni, 1992). In addition, it seems that the chemical composition of dietary fiber, particularly SDF, may affect bacterial fermentation. This concept is supported by a study performed by Lebet *et al*. (1998) who found that apple pomace and celery cell wall pectic substances were easily metabolized by the microflora and that oat bran fermentation was characterized by rapid degradation of mixed-linked β-glucans and starch. It was therefore concluded that soluble polysaccharides are more beneficial for bacterial fermentation and the amount of SDF seems to be the most important determinant for the fermentability of fiber-rich substrates because this fraction is easily and completely fermentable (Cummings and Macfarlane, 1991) and its monomer composition dictates the short-chain fatty acid profiles. Accordingly, we conducted enzymatic modification increased the content of SDF and altered the structure and physicochemical properties of sea buckthorn dietary fiber.

The growth of a microorganism on a particular oligosaccharide may be strain specific because of differences in the transport systems of oligosaccharides (Holt *et al*., 2005). Figure 12 compares the effects of sea buckthorn SDF on the growth of *B. infantis* and *B. longum*. By comparing the OD values, *B. longum* seems to grow more efficiently on sea buckthorn SDF than *B. infantis*. Wang *et al*. (2010) compared the utilization of xylooligosaccharides (XOS) with that of wheat bran dietary fiber by *B. adolescentis*, *B. longum*, *B. bifidum* and *B. breve*. They found that *B. adolescentis* displayed the highest growth rate on XOS, followed by *B. longum*. Based on these observations, it is apparent that different species of bacteria metabolize dietary fiber and carbohydrates differently.

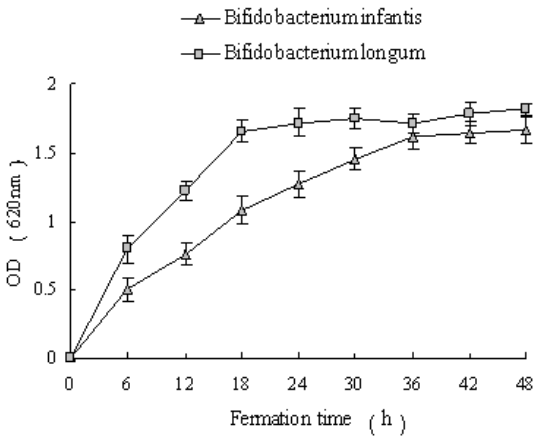

Figure 12. Comparison of growth of *Bifidobacterium infantis* and *Bifidobacterium longum* in enriched medium (SDF 0.2%).

The physiological effects of dietary fiber are highly dependent on the physicochemical properties of the ingested material, such as the WHC, the molecular weight distribution and the viscosity. Meanwhile, the chemical and physical properties of dietary fibers can dictate the degree of fermentability and possibly the composition of intestinal microbiota (Hughes *et al*., 2008). A conclusion was reached by Guillona *et al*. (1998), who described the relevance of the processing of dietary fiber in terms of modifying its physicochemical properties and bacterial metabolism. Decreasing particle size by enzymatic modification increases the surface area of the dietary fiber and hence increases the area exposed to bacteria. Our observations on the physical properties of unmodified and modified sea buckthorn dietary fiber and the *Bifidobacterium* proliferation experiment appear to support this statement.

4. Conclusions

The results of this study indicate that a crude enzyme could be used to increase the SDF content of cereal crops, such as sea buckthorn, and other agricultural plants. Compared with physical methods, the enzymatic process used here increases the yield of SDF and requires less power and fewer resources. In this study, we characterized the physicochemical properties of sea buckthorn fiber and determined the *in vitro* physiological effects on *Bifidobacterium*. Overall, we found that modification by xylanase improved the physicochemical and physiological properties of sea buckthorn dietary fiber.

Enzymatic modification is a mild technique to process dietary fibers and generates the desired products in an efficient manner. A number of studies have reported enzymatic modification of dietary fiber and it is well known that the structure and composition of dietary fiber determine its properties and physiological function. In this study, we determined some of the physicochemical and physiological properties of MDF, but further research of these properties is still necessary. In particular, its potential impact on human health remains to be determined.

Acknowledgments

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Evaluation of Antihyperglycemic and other Complication Effects of Extracts of *Thespesia lampas* Dalz and Gibs on Streptozotocin Induced Diabetic Rats

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Abstract

The methanol and aqueous extracts of *Thespesia lampas* Dalz and Gibs were tested for anti-hyperglycemic, anti-hyperlipidaemic and spermatogenetic effects of streptozotocin (STZ) induced diabetic rats. Diabetes was induced in adult male albino Wister rats by intra peritoneal (i.p) injection of streptozotocin at a dose of 150 mg/kg. Methanol and aqueous extracts of *T. lampas* (METL and AETL) at doses of 300 and 600 mg/kg b. wt were administered as a single dose per day to diabetes rats for the period of 15 days, respectively. The blood glucose levels and serum lipid profiles like total cholesterol, triglycerides, phospholipids, low density, very low density and high density lipoprotein and serum enzymes like ALT, ASP, ALP were measured in the diabetic and non diabetic rats. An oral glucose tolerance test (OGTT) was also performed, in which there was a significant improvement in glucose tolerance in rats treated with extracts. A comparison was made between the extracts and standard anti-diabetic drug glibenclamide. The extracts exhibited significant anti-hyperglycemic and anti-hyperlipidaemic effects on STZ induced diabetic rats when compared to that of standard drug (Glibenclamide 500µg/kg). The present investigation of this plant established pharmacological evidence to support the folklore claim that it is an anti-diabetic agent.

Key Words*:* Anti-Hyperglycemic, Anti-Hyperlipidaemic, Streptozotocin, Spermatogenesis, *Thespesia lampas.*

1. Introduction

Diabetes is a syndrome characterized by deranged carbohydrate metabolism resulting in abnormally high blood sugar level (hyperglycemia). It is caused by hereditary, increasing age, poor diet, imperfect digestion, obesity, sedentary lifestyle, stress, drug-induced, infection in pancreas, hypertension, high serum lipid and lipoproteins, less glucose utilization and other factors. It is estimated that the diabetic patients in India will increase by 195% in the near future**.** The treatment of diabetes with synthetic drugs is costly and chances of side effects are high. For example, long-term use of *Exenetide* (Byetta) has lead to side effects such as nausea, vomiting, diarrhea, dizziness, headache, jittery feeling and acidity (Sarita singh, 2009).

Thespesia lampas Dalz and Gibs (Nadkarni, 1954; Gamble, 1984) belongs to the family Malvaceae and its roots and fruits are used for treating gonorrhea, jaundices, syphilis (The wealth of India, 1976) anti-microbial (Vasaraj, 1997) and earlier report like hepatoprotective activity, antihyperlipidaemic activity and *In-vitro* antioxidant and antihyperglycemic studies were carried out (Sangameswaran, 2008).

2. Materials and Methods

2.1. Plant material

The plant part was collected from the foot hill of Yercaud, Salem, in the month of September 2005. The plant was identified and authenticated by a Botanist and a voucher specimen (TL-12) has been kept in our museum for future reference. The plant material was collected and shade dried at room temperature for 10 d and coarsely powdered and the powder was passed through sieve No.60.

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2.2. Preparation of the Extract

The powdered material of roots of *T. lampas* was extracted with methanol by soxhlet apparatus and water by cold maceration, separately. After extraction, the extracts were concentrated under reduced pressure. The dried extracts were subjected to various chemical tests to detect the presence of different phytoconstituents.

2.3. Preliminary Phytochemical Screening

The methanol and aqueous extracts were subjected to preliminary screening for various active phytochemical constituents (Kokate, 2005).

2.4. Preparation of extracts and standard drug

The extracts were administered orally to rats at various doses, as a suspension with Tween 80. Glibenclamide (500 µg/kg) was made suspension with Tween 80 and administered orally.

2.5. Animals

Male albino rats, 8-12 weeks old with an average weight of 200-250 g were purchased from M/S Venkateshwara enterprises (P) Ltd, Bangalore and used for the study. They were housed in polypropylene cages and fed with standard chow diet and water *ad libitum*. The animals were exposed to alternate cycle of 12 h of darkness and light each. Before each test, the animals were fasted for at least 12h. The experimental protocols were approved by the Institutional Animal Ethics Committee and were cleared by the same (IAEC NO: P.Cog-1/06).

2.6. Toxicity Evaluation in Mice

To determine acute toxicity of a single oral administration of the methanol and aqueous extracts (Separately) of *T. lampas* different doses (200, 300, 400, 500 and 600 mg/kg) were administrated to different groups of mice. The animals were observed continuously for the initial 4 h and intermittently for the next 6 h and then again at 24 h and 48 h following drug administration. Mortality and general behavior of the animals were observed periodically for 48 h (Ghosh, 2007).

2.7. Drugs and Chemicals Used

Glibenclamide was procured from Aventis Pharma, Mumbai. India. Streptozotocin (STZ), hematoxylin, eosin and assay kits for Serum alanine aminotransferase (ALAT), aspartate aminotranferase (ASAT) and alkaline phosphates (ALP) were purchased from sigma chemicals Co Bangalore.

2.8. Oral Glucose Tolerance Test

After overnight fasting, a 0 min blood sample was taken from the rats in normal control (Group I), diabetic control (Group II), diabetic + glibenclamide (Group III), diabetic + methanol extract (300 and 600 mg/kg) (Groups IV and V) and diabetic + aqueous extract (300 and 600 mg/kg) (Groups VI and VII). Glucose solution (2 g/kg) was administered orally immediately (Sweety Lanjhiyana, *etal.*, 2011). Four more samples were taken at 30, 60, 90 and 120 min after glucose administration. All blood samples were collected in potassium oxalate and sodium fluoride containing tubes and used for the estimation of blood glucose.

2.9. Streptozotocin-Induced Diabetic Rats

Streptozotocin (STZ) was dissolved in 0.9% ice-cold saline immediately before use. Diabetes was induced in rats by intra peritoneal (i.p) injection of streptozotocin at a dose of 150 mg/kg, dissolved in saline (Pulok K Mukarjee, 2012). Forty eight hours after streptozotocin administration, blood samples were drawn from tail and glucose levels determined to confirm diabetes. The diabetic rats exhibiting blood glucose levels higher than 200 mg/dl were selected for the studies.

2.10. Experimental Procedure

In experiment, a total of 42 rats were used (36 diabetic surviving rats, 6 control rats). The rats were divided into seven groups

Group I Control rats (Vehicle treated).

Group II Diabetic control (Received 0.5 ml of 5% Tween 80).

Group III Diabetic rats given Glibenclamide 500 µg/kg (Received 0.5 ml of 5% Tween 80).

Group IV and V Diabetic rats given METL 300 and 600 mg/kg b. wt,.

Group VI and VII Diabetic rats given AETL 300 and 600 mg/kg b. wt, respectively.

Blood samples were collected from the tail for glucose estimation just before drug administration on 1st day and 1 h after drug administration on days 4, 7, 10 and 15. Blood samples were collected and centrifuged to separate serum for estimation of lipid profile and other biochemical parameters.

2.11. Determination of Serum Insulin

To determine the effect of extracts of *T. lampas* on serum insulin levels, same group of animals were used (Procedure described earlier). Blood samples were collected from experimental animals on first day and end of experiment. Serum was separated from the samples and insulin levels were determined. Insulin levels were measured by radio-immuno-assay method using kits obtained from Board of Radiation and Isotope Technology, Mumbai, India.

2.12. Anti-Hyperlipidaemic activity

Total cholesterol, HDL- C, LDL-C, VLDL-C, phospholipids, triglycerides and total cholesterol were analyzed from serum. (Vinoth kumar, 2010)

2.13. Determination of Biological Assay

Serum was separated by centrifugation at 3000 rpm at 25^{0}C for 15 min and analyzed for assorted biochemical parameters. The serum ALT, AST and ALP levels were measured using the respective spectrophotometer diagnostic kit obtained from Biosino Biotechnology (ompany Ltd (Beijing, PR China). (Sayed M. Rawi, 2011)

2.14. Statistical Evaluation

All the data are presented as mean ± SEM. The differences between group were evaluated by one-way analysis of variance (ANOVA) followed by the Dunnette multiple comparisons test. $P<0.01$ were considered to be significant.

3. Results and Discussion

3.1. Phytochemical Screening

Preliminary phytochemical screening revealed that the presence of triterpenoids, carbohydrates, vitamins, amino acids, proteins, tannins, saponin glycosides, phyotsterol and steroids.

3.2. Oral Glucose Tolerance Test

Table 1 shows the changes in the levels of blood glucose in normal, diabetic control and experimental groups after oral administration of glucose (3 g/kg). The diabetic rats showed that significant increase in the blood glucose at 30 and 120 min. In extracts and glibenclamide treated animals blood glucose concentration was significantly decreased after 60 min.

The hyperglycemic animals showed significant decrease in the glucose level on long term treatment for 15 days model at the doses of 300 and 600mg/kg of methanol and aqueous extracts of T. lampas (Group-IV-VII).

Oral administration of methanol and aqueous extracts of T. lampas to STZ induced diabetic rats significantly reduced blood glucose levels. Diabetic rats treated with methanol extract (300 and 600 mg/kg) showed significant reduction in blood glucose from 348.32 ± 7.81 to 190.77 ± 6.31 and 352.33 ± 3.41 to 188.6 ± 2.2 mg/dl, and aqueous extract (300 and 600 mg/kg) from 358.26 ± 5.2 to 187.6 ± 2.2 and 346.2 ± 4.8 to 174.33 ± 2.97 mg/dl, in comparison to untreated diabetic control, whereas standard drug (Glibenclamide 500µg/kg) treated rats also showed significant reduction in blood glucose concentration from 346.8 ± 8.2 to 123.3 ± 4.8 mg/dl, respectively. The results were recorded in table 2.

Table 1. Effect of oral glucose tolerance test

Treatment/	Blood glucose levels (mg/dL) after different times			
mg/kg	0 min	30 min	60 min	120 min
Normal control	95.16 ± 2.90	169.17 ± 3.06	134.00 ± 2.59	108.5 ± 1.99
Diabetic control	412.33 ± 4.83	418.00 ± 4.53	427.50 ± 4.68	420.50 ± 4.63
Glibenclamide 500 µg/kg	200.00 ± 2.53	213.33 ± 2.29**	202.0 ± 2.04**	213.50 ± 2.36**
METL 300	225.83±2.54	230.75±2.44*	238.4±6.09*	235.2±1.94*
METL 600	236.41±1.62	242.2±5.01*	239.83±2.25**	234.75±0.93**
AETL 300	227.83 ± 4.82	236.50 ± 4.53*	233.16±3.18**	228.33 ± 4.31**
AETL 600	236.16 ± 4.13*	240.16 ± 4.67*	232.16± 3.18**	234.50 ± 3.82**

METL- Methanolic extract of T. lampas, AETL- Aqueous extract T. lampasValues are mean ±SEM, n= 6. *,**, statistically significance of $P<0.05$, $P<0.001$, when compared with respective diabetic control. (One way ANOVA Followed by Dunnette multiple comparison test)*Anti*-Hyperglycemic Activity..\

Table 2. Anti-hyperglycemic effects of methanol and aqueous extracts of T. lampas on STZ induced rats

Treatment/	Blood glucose level mg/dl					Serum insulin level IU	
mg/kg	1st Day	4th Day	7th Day	10th Day	15th Day	1st Day	15th Day
Norma control	94.7 ± 2.3	95.2 ± 2.5	94.8 ± 2.3	94.3 ± 1.9	94.5 ± 2.3	17.2 ± 3.0	18.0 ± 2.9
Diabetic control	352.0 ± 7.4	363.5 ± 6.1	373.3 ± 7.0	400.3 ± 8.5	434.3 ± 10.5	17.4 ± 2.8	14.3 ± 2.8
Glibenclamide 500 µg/kg	346.8 ± 8.2	333.7 ± 9.6*	248.3 ± 8.10*	193.5 ± 6.3**	123.3 ± 4.8**	14.3 ± 2.8	16.4 ± 2.9**
METL 300	348.32 ± 7.81	330.83 ± 3.98*	264.50 ± 3.60*	218.33 ± 2.98*	190.77± 6.31*	14.8 ± 4.8	18.8 ± 0.2*
METL 600	352.33 ± 3.41	334.2±3.2**	250.0±6.2**	202.8±4.8**	188.6±2.2**	15.2 ± 2.6	19.3 ± 3.0**
AETL 300	358.26 ±5.2	332.2±5.2*	268.2±4.2*	200.2±4.6*	187.6±2.2*	15.4± 4.8	19.8± 2.2**
AETL 600	328.00 ±4.63	328.00 ±4.63**	246.50 ± 6.29**	193.00 ± 8.04**	174.33 ± 2.97**	14.6± 3.2	21.2± 2.6**

METL- Methanolic extract of *T. lampas,* AETL- Aqueous extract *T. lampas*Values are mean ±SEM, n= 6. *, **, statistically significance of $P<0.05$, $P<0.001$, when compared with respective diabetic control. (One way ANOVA Followed by Dunnette multiple comparison test).

3.3. Determination of Serum Insulin Level

The serum insulin levels in all groups of animals were estimated at first and 15th day after extract administration and the values were recorded.

The methanol and aqueous extracts of *T. lampas* (300 and 600 mg/kg) treated rats were significantly influence serum insulin levels from 14.8 ± 4.8 to 18.8 ± 0.2 and 15.2 ± 2.6 to 19.3 ± 3.0, and 15.4± 4.8 to 19.8± 2.2 and 14.6± 3.2 to 21.2± 2.6 IU in STZ induced diabetic animals, respectively. In diabetic control (untreated group) group of rats, decreases in the serum insulin level (Table 2).

3.4. Anti-Hyperlipidaemic Activity

The lipid profiles in control and experimental rats are depicted in Table 3 in STZ induced diabetic rats, there was a significant ($P<0.001$) increase of total cholesterol, triglycerides, phospholipids, and low density lipoproteins

(LDL) and very low density lipoprotein (VLDL) cholesterol and significant (p<0.001) decreases in high density lipoprotein (HDL) cholesterol in serum compared with normal control. The extracts treated rats were significantly (p<0.001) decreased the total cholesterol, triglycerides, phospholipids and LDL and VLDL cholesterol and significantly (p<0.001) increased HDL cholesterol.

Table 3. Anti-hyperlipidaemic effects of extracts of T. lampas on STZ induced diabetic rats.

Treatment/ mg/kg	Changes in mg/dl level					
	Serum total Cholestrol	Triglycerides	Serum HDL	Serum LDL	Serum VLDL	Serum Phospholipids
Normal control	81.00±7.21	87.50± 7.99	23.16 ± 2.00	95.33 ± 5.92	15.50 ± 1.23	48.50 ± 7.85
Diabetic control	196.83±9.58*	168.00± 5.72*	13.50 ± 1.50*	186.83 ± 5.05*	30.66 ± 1.83*	252.50 ± 9.91*
Glibenclamide 500 µg/kg	128.67±8.53**	116.50± 2.89**	21.33 ± 1.43*	123.00 ± 3.23**	17.83± 2.0**	177.83 ± 7.6*
METL 300	152.42±4.8*	132.02±4.2**	20.22 ± 0.82**	139.52 ±8.22**	22.62 ±2.20**	192.42 ±6.6**
METL 600	145.26±2.2**	126.24±2.4**	22.24 ± 0.84**	136.24 ±4.2**	18.20 ±1.2**	192.26 ±2.8**
AETL 300	153.50±10.25*	128.00±4.38**	18.33 ± 0.98**	137.50 ± 5.32**	21.66 ± 1.92**	190.62 ± 9.52**
AETL 600	142.67±6.26**	121.33±3.65**	19.16 ± 0.94**	134.17 ± 9.38**	17.66 ±1.30**	186.23 ± 8.46**

METL- Methanolic extract of *T. lampas,* AETL- Aqueous extract *T. lampas*Values are mean ±SEM, n= 6. *, **, statistically significance of P<0.05, P<0.001, when compared with respective diabetic control. (One way ANOVA Followed by Dunnette multiple comparison test) .

3.5. Determination Biochemical Parameters

Serum enzymes (ALP, AST and ASP) total protein levels are shown in Table 4. ALP, ASP levels were increased significantly (p<0.001) in STZ treated diabetic rats in comparison with normal animals.

The extracts significantly (p<0.001) decreased the elevated ALP, AST and ASP levels in treated rats. A significant (p<0.05) decrease in total protein level was observed with control rats

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Table 4. Biochemical parameters of methanol and aqueous extracts of *T. lampas* treated in STZ induced diabetic rats on 15th Day

Treatment /mg/kg	Changes in biochemical parameters			
	ALT U/L	AST U/L	ALP U/L	T. Bilirubin
Normal control	54.2 ± 1.02	34.4 ± 3.26	89.2 ± 3.16	0.20± 0.02
Diabetic control	82.59 ± 1.90	48.2± 6.4	92.4 ± 0.02	0.26± 0.06
Glibenclamide 500 µg/kg	58.26± 4.6**	39.0± 4.6**	98.20± 0.04**	0.32±0.06**
METL 300	53.2 ± 1.22*	36.4 ± 2.20*	91.2 ± 3.12*	0.18 ± 0.06*
METL 600	52.6 ± 2.26**	33.4 ± 2.2**	92.4 ± 2.4**	0.19 ± 0.04**
AETL 300	52.8 ±2.2*	38.2 ±3.22*	90.6 ±2.2*	0.20 ± 0.02*
AETL 600	53.2± 0.22**	34.6± 2.2**	89.4± 0.4**	0.19± 0.04**

METL- Methanolic extract of *T. lampas,* AETL- Aqueous extract *T. lampas*Values are mean ±SEM, n= 6. *, **, statistically significance of P<0.05, P<0.001, when compared with respective diabetic control. (One way ANOVA Followed by Dunnette multiple comparison test) .

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Effect of Tomato and Guava juices on Oxidative Stress in Rats after Strenuous Exercise

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Abstract

Oxidative stress is thought to play an important role in the pathogenesis of numerous degenerative and chronic diseases. Some antioxidants in tomato and guava juices were found to have a powerful antioxidant effect. Sixty adult male albino rats were used to compare the effect of tomato and guava juices supplementation on oxidative stress. Xanthine oxidase (XO), Glutathion reductase (GR), Vitamin C, Malondialdehyde (MDA), Myeloperoxidase (MPO) and some parameter of liver and kidney functions were measured in groups of rats subjected to strenuous exercises fed on basal diet supplemented with different doses of tomato or guava juices compared with non supplemented rats as a control group. This study found that guava juice is more effective than tomato juice in impairment of oxidative damage caused by strenuous exercise leading to significant decrease in plasma and muscle XO, MDA as well as muscle MPO after exercise comparing with tomato juice. The difference in plasma vitamin C level between the group that given guava juice was significantly higher compared with group that given tomato juice (P<0.05).

Key Words : Tomato juice, guava juice, oxidative stress, antioxidant**.**

1. Introduction

Oxygen is an element obligatory for life, living systems have evolved to survive in the presence of molecular oxygen and for most biological systems. Oxygen has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events (Shinde *et al.,* 2006). Physical exercise is characterized by an increase in oxygen consumption particularly by muscles. The increase in oxygen uptake is associated with a rise in the production of reactive oxygen species which lead to an increase in lipid peroxidation and impairment of antioxidant defense systems of target tissues and blood (Clarkson and Thompson, 2000). Oxidative stress is thought to play an important role in the pathogenesis of numerous degenerative and chronic diseases. Oxidative stress is characterized by an imbalance between antioxidant capacity and reactive oxygen species (ROS) generation. Over- production of (ROS) increased during aging and contributed to many pathological events such as cancer and cardiovascular disease (Moroni *et al.*, 2004).

Xanthine oxidase is an important source of oxygen free radicals and can catalyses the reduction of oxygen, leading to formation of superoxide (O_2) and H_2O_2 which is proposed as a central mechanism of oxidative injury in some tissues (Mc Cord, 1985). Myeloperoxidase is a marker for neutrophil infiltration which is associated with strenuous exercise-induced tissue damage. A part from its host defense, involvement of (MPO) has been described in numerous non infectious diseases such as atherosclerosis, lung cancer, Alzheimer and multiple sclerosis (Tidball, 2005). The reaction of polyunsaturated fatty acids with activated oxygen species result in primary lipid peroxidation production (lipid hydroperoxide). Lipid peroxidation production degraded to secondary lipid peroxidation product like malondialdehyde (MPO) which is used as marker of lipid peroxidation. (MPO) can react with DNA leading to DNA aberration with altered gene product and peptide bonds are broken through the impact of (MPO) in proteins (Sarkar *et al*., 1997).

Lycopene, a carotenoid found in tomato juice which is a powerful antioxidant with a single oxygen quenching capacity 100, times greater than vitamin E and has been hypothesized to be responsible for health benefits of tomatoes (Dimasicio *et al.,* 1989).

Recent epidemiologic studies focusing on tomato and tomato products associated their intake with a reduced risk of degenerative diseases (Giovannacci, 2002). Guava (*Psidium guajava*) is an important tropical fruit and widely

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cultivated in Egypt, mostly consumed fresh. The fruit contains saponin combined with oleanolic acid and flavonoids, guaijavarin (Arima and Danno, 2002). Guava fruit is considered a highly nutritious fruit because it contains a high level of ascorbic acid (50–300 mg/100 g fresh weight), which is three to six times higher than oranges. Guava contain both carotenoids and polyphenols the major classes of antioxidant and pigments giving them relatively high potential antioxidant value among plant foods (Jordan *et al*., 2003). Phenolic compounds found in guava juice were gallic acid, catechin, vanillic acid, trans-cinnamic acid and ferulic acid (Zabidah *et al.*, 2011). Guava contains antioxidant (quercetin) which block enzymes that are responsible for building of sorbitol and quercetin also combats free radical produced during metabolism and aids in preventing age related chronic diseases such as al-zheimers, cataract, heart disease and rheumatoid arthritis (Thaipong *et al.*, 2006).

Accordingly, the purpose of the present study was to compare the effect of tomato and guava juices supplementation on oxidative stress, lipid peroxidation and anti –oxidant substance in plasma and tissues of rats after strenuous exercises.

The present work is the first study aimed to compare the effect of tomato and guava juices on oxidative stress by strenuous exercise, as protective for oxidative damage.

2. Subject and Methods

2.1. Preparation of Tomato and Guava

Tomato (*Lycopersicon esculentum)* and Guava (*Psidium guajava*) were Egyptian species and collected from local market. After sorting and grading operation only red tomato and yellow guava were chosen. They were then sliced with a slicer. The seeded portion of tomato and guava was removed and the fresh flesh was collected. The flesh was cut into small pieces and blended in electric blender to get fine juice. Then the juices were diluted by 25% of distilled water.

2.2. Animals

Sixty adult male albino rats of local species weighing 230-250 grams were used in the present study. All rats were given normal diet and water *ad libitum* and housed in room maintained at 25±5 ^{o}C and a 12 h light-dark cycle. Tomato (*Lycopersicon esculentum)* and Guava (*Psidium guajava*) added to rat's basal diet with different doses were obtained from Egyptian species.

Rats were divided into four equal groups:

Group I: (10 rats) sedentary control group (control negative).

Group II: (10 rats) subjected to strenuous exercises (control strenuous exercises).

Group III: (20 rats) subjected to strenuous exercises plus low doses (25 g/day) of tomato juice (10 rats) or guava juice (10 rats) for 30 days.

Group IV: (20 rats) subjected to strenuous exercises plus high doses (75 g/day) of tomato juice (10 rats) or guava juice (10 rats) for 30 days.

2.3. Exercises Protocol

On the morning day of samples collection, the exercise groups were introduced to motorized treadmill running device .According to Brook and White 1978, rats warmed up for 15 minute running at speed of 15 m/min, then rats progressed to running for 15 minute at 20 m/Min., and 30 minute at 25 m/min., finally, rats ran to exhaustion at a final speed 30m/Min. The point of exhaustion was determined as when the rats were unable to right it when placed on its back.

2.4. Samples Preparation and Measurement

The rats in the sedentary group were anaesthetized with fluothane and sacrificed after 12 h fasting. Then, the rats in the exercise groups were sacrificed immediately after strenuous exercise. Blood samples were collected from abdominal aorta, then heparinized and centrifuged at 3000 rpm for 15 minute. Plasma was used for determination of plasma parameters but for erythrocyte content (GR), erythrocyte washed four times with ice-cold saline for lysis of RBCs, centrifuged at 10000 xg for 10 minutes. Then, supernatant was collected (erythrocyte lysate) and stored with plasma at – 70^{o}C till the assay time. For vitamin C, Blood samples were drawn into vacutainers without anticoagulants, stored for 30 min before centrifugation and acidification, then centrifuged at 2000 *xg* for 10 min for generation of serum ,all samples were treated immediately with an equal volume of 10% metaphosphoric acid (MPA), centrifuged at 3000 x*g* for 15 min and the supernatant frozen at – 70^{o}C till analysis of vitamin C.

*Skeletal muscle tissues***:** The muscle tissues were perfused prior to dissection with a PBs (phosphate buffer saline) solution, pH 7.4 to remove any blood cells and clots, 200mg of different muscle tissues were homogenized in 5-10 ml of cold buffer (i.e 50 mM potassium phosphates, pH 7.5, 1 mM EDTA). Then the mixture was centrifuged at 10000 xg for 10 minutes, the supernatant fluid was removed and stored at – 70 ^{o}C till the assay time.

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), Lactate dehydrogenase (LDH), creatin kinase (CK), and uric acid (UA) were measured using a kit from BioMerieux (France). The concentrations were determined spectrophotometrically (Hitachi, Japan).

Xanthine oxidase (XO): Skeletal muscle and plasma (XO) were determined by ELISA kit (Cayman's xanthine oxidase, USA), The assay is multi step enzymatic reaction in which xanthine first produce H_2O_2 during oxidation of hypoxanthine, in the presence of horseradish peroxidase , H_2O_2 react with ADHP (10-acetyl-3-7-Dihydroxyphenoxazine) to produce highly fluorescent compound which can be measured at wave length from 520-550 nm.

Glutathion reductase (GR): Erythrocyte and skeletal muscle tissues glutathione reductase were measured by ELISA kit (Cayman's Glutathion reductase assay kits, USA), which measure the rate of NADPH oxidation.

$$GSSG + NADPH + H^{+} \rightarrow 2\ GSH + NADP^{+}$$

The oxidation of NADPH to $NADP^{+}$ is accompanied by a decrease in the absorbance at 340 nm and is directly proportional to (GR) activity in erythrocytes and tissues.

*Vitamin C***:** Plasma vitamin C concentrations estimated with a fluorometric assay by Bio-Assay system, USA kit.

*Malondialdehyde (MDA)***:** MDA in plasma and tissue was measured by HPLC with fluorescence detection using commercial kit (Immunodiagnostic kit, Germany).

Myeloperoxidase (MPO): Myeloperoxidase in muscle tissue was measured by ELISA using commercial kit (Immunodiagnostic kit, Germany). The assay utilizes the two-site sandwich technique with two selected polyclonal antibodies that bind to MPO.

2.5. Statistical Analysis

The SPSS (10.00) soft ware was used to data management and analysis, while Microsoft Excel was used for charts .The results were expressed as mean ± standard deviation of the mean. Statistical analysis was carried out using student *t*- test. $P < 0.05$ was accepted as significant.

3. Results

As shown in (Table 1 and 2), plasma levels of AST, ALT, LDH, CK and UA were significantly increased ($P<0.05$) in strenuous exercise group (control exercise (group II)) as compared with sedentary control group (group I). However, there were no significant difference for these parameters in group III and group IV that subjected to strenuous exercises and fed on low and high doses of tomato and guava juices, as compared with group II that subjected to strenuous exercises (control exercise).

From data present in (Table 1 and 2), it was observed that, the liver functions, LDH, CK and uric acid were significantly increased after the exercise. Moreover, both concentrations of tomato or guava juices had no significant effect on decreasing their concentrations near to control negative level for rats subjected to strenuous exercises.

As shown in (Table 3), XO and MDA of plasma and muscle in group II (control strenuous exercise) were significantly increased ($P<0.05$) as compared with group I (sedentary control). However, the muscular XO, MDA and plasma MDA were significantly lower with strenuous exercise fed on low dose (group III) and strenuous exercise fed on high dose (group IV) of tomato juice, as compared with group II.

Also, MPO activity in muscle showed significant increase ($P<0.05$) in control strenuous exercise group (group II) as compared to sedentary control group (group I). However, muscular MPO activity was significantly decrease ($P<0.05$) with strenuous exercise that fed on low dose (group III) and strenuous exercise that fed on high dose (group IV) of tomato juice, as compared with group II (control strenuous exercise).

There was no significant difference in the GR concentration in strenuous exercise group (group II), as compared to sedentary control group (group I). Also, no significant changes in GR concentration in group III and group IV as compared to group II (control strenuous exercise).

There was no significant difference in plasma and muscle parameters in strenuous exercise group with high dose of tomato juice (group IV) as compared to strenuous exercise group with low dose (group III).

The same finding for tomato juice was reported in guava juice (Table 4), except the significant effect of low and high doses of guava juice (group III and IV) in decreasing plasma xathine oxidase, as compared to group II (control strenuous exercise) ($P<0.05$).

As shown in (Table 3 and 4) , the decrease in plasma level of vitamin C in group (II) (control strenuous exercise) was not significant as compared with group (I) (control sedentary group). However, plasma level of vitamin C was significantly increased with strenuous exercise that fed on low dose of tomato or guava juices(group III) and strenuous exercise that fed on high dose of tomato or guava juices (group IV), as compared with group II (control strenuous exercise) ($P<0.05$).

Table 1. Effect of different doses of tomato juice supplementation on plasma ALT, AST, LDH, CK and UA levels after strenuous exercise

Parameters / Groups (N=10)	ALT U/L	AST U/L	LDH U/L	CK U/L	UA mg/dL
Group I	20±2.1	40±3.3	108±4.5	52±2.9	4.5 ±2.9
Group II	33±8.2 *P<0.05*	70±4.3 *P<0.05*	200±6.3 *P<0.05*	240±6.3 *P<0.05*	12.4±2.6 *P<0.05*
Group III	32±2.8 *P>0.05*	67±4.1 *P>0.05*	191±4.6 *P>0.05*	231±10.1 *P>0.05*	11.5±2.1 *P>0.05*
Group IV	31±2.9 *P>0.05*	64±4.7 *P>0.05*	185±6.2 *P>0.05*	225±9.3 *P>0.05*	10.8±5.1 *P>0.05*

N = number of animals in each group values given are mean ± standards deviation Group II compared to group I (control group) group III and IV are compared to group II P<0.05 is significant

Table 2. Effect of different doses of guava juice supplementation on plasma ALT, AST, LDH, CK, and UA levels after strenuous exercise

Parameters / Groups (N=10)	**ALT** U/L	**AST** U/L	**LDH** U/L	**CK** U/L	**UA** mg/dL
Group I	20±2.1	40±3.3	108±4.5	52±2.9	4.5 ±2.9
Group II	33±8.2 *P<0.05*	70±4.3 *P<0.05*	200±6.3 *P<0.05*	240±6.3 *P<0.05*	12.4±2.6 *P<0.05*
Group III	31±3.2 *P>0.05*	65±5.2 *P>0.05*	187±6.7 *P>0.05*	229±8.4 *P>0.05*	11.0±2.1 *P>0.05*
Group IV	30±3.9 *P>0.05*	63±3.9 *P>0.05*	185±7.9 *P>0.05*	226±7.5 *P>0.05*	9.9±2.1 *P>0.05*

N = number of animals in each group values given are mean ± standards deviation Group II compared to group I (control group) group III and IV are compared to group II P<0.05 is significant.

Table 3. Effect of tomato juice on lipid peroxidation product and antioxidant

Parameters / Group N=10	Plasma				Muscle			
	XO uU/ml	**MDA** Umol/l	**GR (Erythrocyte)** Umol/g	**Vitamin C** Umol/l	**XO** uU/g	**MPO** ng/ml	**MDA** Umol/g	**GR** Umol/g
Group I	5.2±0.9	9.3±1.8	26.1 ±3.7	35.3 ±8.4	0.5±0.3	1.6±0.1	21.4 ±2.9	58.7 ±4.6
Group II	8.3±1.2 *P<0.05*	18.4±3.4 *P<0.05*	25.3±4.5 *P>0.05*	33.4±5.3 *P>0.05*	1.4±0.1 *P<0.05*	2.8±0.3 *P<0.05*	40.3±4.7 *P<0.05*	55.5±6.4 *P>0.05*
Group III	8.0±1.4 *P>0.05*	12.1±1.7 *P<0.05*	23.7±2.9 *P>0.05*	42.3±6.2 *P<0.05*	0.9±0.2 *P<0.05*	2.1±0.4 *P<0.05*	30.6±3.5 *P<0.05*	53.6±4.3 *P>0.05*
Group IV	7.9±1.6 *P>0.05*	10.4±2.3 *P<0.05*	24.2±2.3 *P>0.05*	45.4±5.3 *P< 0.05*	0.87±0.7 *P<0.05*	1.9±0.2 *P<0.05*	28.6±2.8 *P<0.05*	55.9±3.9 *P>0.05*

Table 4. Effect of guava juice on lipid peroxidation product and antioxidant

Parameters / Group N=10	Plasma				Muscle			
	XO uU/ml	MDA umol/l	GR (Erythrocyte) Umol/g	Vitamin C Umol/l	XO uU/g	MPO ng/ml	MDA umol/g	GR Umol/g
Group I	5.2±0.9	9.3±1.8	26.1 ±3.7	35.3 ±8.4	0.5±0.3	1.6±0.1	21.4 ±2.9	58.7 ±4.6
Group II	8.3±1.2 *P<0.05*	18.4±3.4 *P<0.05*	25.3±4.5 *P>0.05*	33.4±5.3 *P>0.05*	1.4±0.1 *P<0.05*	2.8±0.3 *P<0.05*	40.3±4.7 *P<0.05*	55.5±6.4 *P>0.05*
Group III	5.9±0.7 *P<0.05*	9.8±2.2 *P<0.05*	26.4±3.7 *P>0.05*	50.8±5.6 *P<0.05*	0.62±0.4 *P<0.05*	1.7±0.6 *P<0.05*	23.8±2.5 *P<0.05*	56.2±4.6 *P>0.05*
Group IV	5.5±2.3 *P<0.05*	9.5±1.3 *P<0.05*	27.5±2.4 *P>0.05*	52.4±3.9 *P<0.05*	0.58±0.3 *P<0.05*	1.5±0.4 *P<0.05*	22.7±3.6 *P<0.05*	56.5±3.7 *P>0.05*

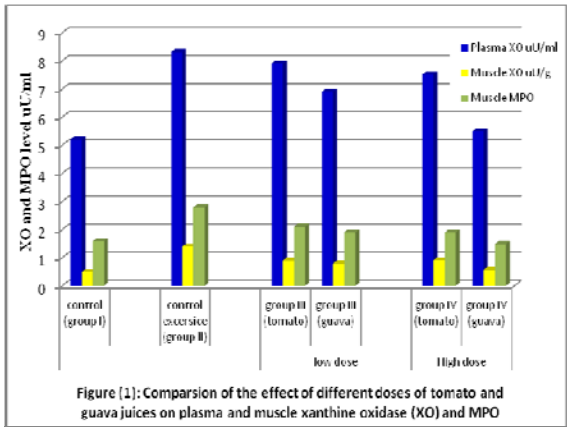

Figure (1): Comparsion of the effect of different doses of tomato and guava juices on plasma and muscle xanthine oxidase (XO) and MPO

As shown in figure 1 ,the plasma levels of XO, muscle XO and MPO were significantly decreased in rats with strenuous exercise and fed on high dose of guava juice (group IV), as compared with rats that strenuous exercised and fed on high dose of tomato juice (group IV) (P<0.05).

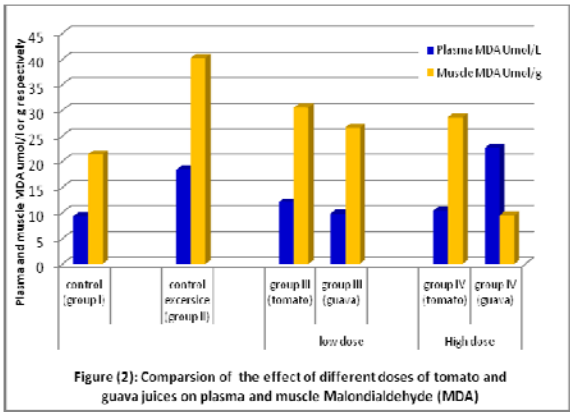

Figure (2): Comparsion of the effect of different doses of tomato and guava juices on plasma and muscle Malondialdehyde (MDA)

As shown in figure 2 ,the MDA for plasma and muscle was significantly decreased in rats with strenuous exercise and fed on high dose of guava juice (group IV), as compared with rat that strenuous exercised and fed on high dose of tomato juice (group IV) (P<0.05).

As shown in figure 3, the plasma levels of vitamin C in group III and IV of rats fed on guava juice were

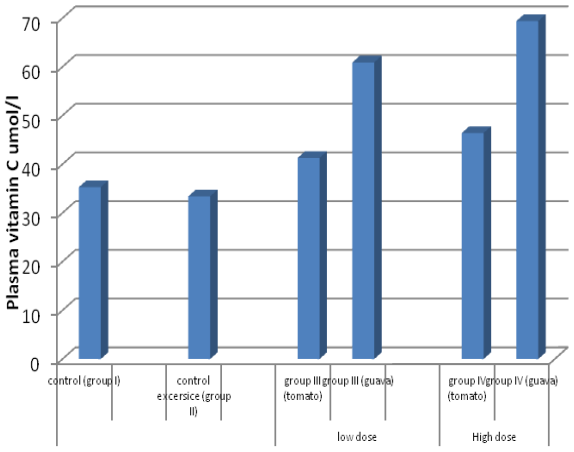

Figure (3): Comparsion of the effect of different doses of tomato and guava juices on plasma vitamin C

significantly increased when compared with the same groups of rats fed on tomato juice (P<0.05).

No significant difference was detected for GR between rats fed on tomato juice group III and IV, as compared with the same groups of rats fed on guava juice (P >0.05).

4. Discussion

A single bout of physical exercise has been shown to induce formation of reactive oxygen species, nitrogen species and the related oxidative damage. On the other hand, regular training is known to increase the resistance against reactive oxygen species induced lipid peroxidation, and to decrease oxidative protein and DNA damage (Quindry *et al*., 2003).

In the present study, the strenuous exercise showed significant increase in liver ALT and AST produced by liver and other tissues as compared with sedentary control group; this result is in agreement with finding by (Bowers *et al*., 1978) who showed that strenuous exercises increase the severity of liver damage. In additional, human study showed that, the liver function parameters, AST and ALT, were significantly increased for at least 7 days after the exercise (Pettersson *et al.,* 2007). Also, in the present study, the plasma LDH activity of strenuous exercise group was significantly increased as compared with sedentary control group. This result is similar to the one found by (Chieh-chung *et a.l*, 2005) which showed that the plasma level of LDH increases three fold with strenuous exercise. Researchers speculated that free radicals resulting from strenuous exercise cause cardiac injury which lead to the release of LDH from cardiocytes into the blood (Vina *et al.*, 2000). However, tomato juices supplementation did not display any significant changes in plasma level of LDH as compared with strenuous exercise group.

In the present study, the plasma CK of strenuous exercise group compared with sedentary control group, was significantly increased. This result corresponds with many researchers (Marquez *et al.*, 2001 and Zajac *et al.*, 2001) who reported that strenuous exercise elevates CK activity in skeletal muscle. Also, the plasma level of uric acid was significantly increased with strenuous exercise group compared with sedentary control group. Goto *et al.*, (1989) reported that strenuous exercise affects the renal function resulting in reduction of glomerular filtration rate and uric acid excretion.

In the present study, the MDA concentration in plasma and muscle was significantly increased in strenuous exercise group as compared with sedentary control group. This result agrees with (Chieh-chung *et al.*, 2005) who reported that MDA in plasma and muscle significantly increased by 0.8 and l.l fold respectively with strenuous exercises caused oxidation damage, increasing lipid peroxidation and decreasing antioxidant. Huang *et al.*, (2008) reported that strenuous exercise elevate MDA, XO and MPO levels of myocardial, muscular, hepatic, pulmonary and renal tissues. In the current research, tomato and guava juices supplementation significantly decreases MDA level in plasma and muscular tissues. In addition, guava juice has more effect for reduction of MDA than tomato juice. The current results were consistent with another study which confirmed that, guava juice had the highest inhibitory effect on MDA formation among the almost fruit juices (Zabidah *et al*., 2011). However, the lycopene supplement through guava or tomato juices did not affect GR levels, both in the erythrocytes and muscle. Moreover, in another study, guava juice caused significant increase for both glutathione peroxidase and glutathione reductase and may reduce the risk of disease caused by free radical activities (Asmah *et al*., 2006).

Also, this study found that, the muscular MPO activity was significantly increased in strenuous exercise group comparing with sedentary control group. Morozov *et al.*, 2003 found that the strenuous exercise lead to a raise of MPO from neutrophils and then induced severe oxidative damage. Wan-teng *et al.* (2006) showed that strenuous exercise increase XO, MPO and lipid peroxidation end product (MDA) levels in myocardial tissues compared with control group. Sean and Kelvin (2007) reported that strenuous exercise generated free radical from mitochondria, neutrophils and XO.

This study demonstrated that tomato and guava juices supplementation with low and high doses significantly decreased muscular MPO activity as compared with strenuous exercise group. This result is similar to (Reifen *et al*., 2004) who reported that MPO activity in muscle was decreased by lycopene administration provided by tomato juice.

In other studies study, Guava juice showed anti-hypertensive (Ayub *et al*., 2010) and lipid-lowering properties (Norazmir and Ayub, 2010).

The recent study demonstrated that the level of plasma vitamin C is significantly increased in guava juice fed rats in low and high doses compared with rats fed on the same doses of tomato juice. The results obtained in this study clarified that, guava juice is more effective than tomato juice in impairment of oxidative damage caused by strenuous exercise by lowering plasma and muscle XO, MDA as well as muscle MPO after exercise more than tomato juice. It may be due to higher content of antioxidant in guava juice more than tomato juice which concede with the previous study published by Thaipong *et al*. (2006) which reported that guava juice is a good source of phenolic compounds much better than other fruits juice .

5. Conclusion

This study indicated that guava juice may have a potential to be introduced as functional food product more than tomato juice because of its highly antioxidant properties. No effect of supplementation of guava or tomato in lowering plasma levels of ALT, AST, CK, LDH and UA but still needs further investigations. Further studies are recommended to demonstrate the role of guava as a good source of vitamin C and anti oxidants in prevention or treatment of chronic degenerative diseases in humans.

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Protective Effects of Latex of *Ficus carica* L. against Lead Acetate-Induced Hepatotoxicity in Rats

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Abstract

Ficus carica L. latex is used traditionally for many therapeutic purposes. Oxidative stress may be the main reason behind most histological and cellular effects of lead. The aim of this study was to investigate the possible hepatoprotective role of *Ficus carica* L .latex against lead acetate-induced oxidative stress in rats. In the present investigation, lead acetate (500 mg Pb/L) was given orally to male rats for 28 days to induce hepatotoxicity. The *Ficus* latex was found to contain high total phenols and flavonoids. The levels of hepatic markers such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were significantly ($P<0.05$) increased in blood serum following lead acetate administration. Lead-induced oxidative stress in liver tissue was indicated by a significant ($P<0.05$) increase in the level of liver Malondialdehyde (MDA) and decreased levels of liver reduced glutathione (GSH) and superoxide dismutase (SOD). Histologically and ultrastructurally, the liver showed several histological alterations such as degeneration of hepatocytes by necrosis and apoptosis, fatty changes and inflammatory cells infiltration. *Ficus* latex (1mL of 1:50 diluted latex/kg b.wt) markedly attenuated the previous lead-induced biochemical alterations in serum and liver tissues ($P<0.05$) as well as the histological and cellular changes. From this study, it can be concluded that the *Ficus l*atex showed effective hepatoprotective and antioxidative action against lead acetate-induced hepatotoxicity in rats.

Keywords: Ficus Latex, Lead Acetate, Hepatotoxicity

1. Introduction

Lead (Pb) exposure is considered to be a major public health problem, therefore it has been paid attention by researchers in probing further into its toxicity. This heavy metal has been found to induce a wide range of behavioral, biochemical and physiological effects (Jackie *et al*., 2011).The liver, kidneys, and brain are considered to be the target organs for the toxic effects of lead (Sharma and Street, 1980; Jackie *et al*., 2011). Toxicity of lead is mainly attributed to the induction of oxidative stress by elevation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals and lipid peroxides, therefore, increased interest among phytotherapy investigators to use medicinal plants with antioxidant activity for protection against metal, especially lead, toxicity has been noted (Sharma and Street 1980; Xu *et al*.,2005; El-Nekeety *et al*., 2009; Haleagraha *et al*.,2010a; Jackie *et al*., 2011). In addition, the studies regarding protection against lead toxicity included some chelating agents (Lim 2001; Massó-González and Antonio-García 2009; Jackie *et al*., 2011) and certain antioxidants such as vitamin C, E, methionine, N-acetylcysteine, homocysteine and α-lipoic acid (Dalley *et al.,* 1989; Chaurasia and Kar, 1997; Patra et *al*., 2001; Flora *et al*., 2003; Caylak *et al*., 2008; Upadhyay *et al*., 2009) .

Ficus carica latex has been traditionally used in the treatment of gout, ulcers and warts, among other (Senapati *et al*., 2001; Hsu and Guo, 2002; Noweg *et al*., 2003; Habsah *et al*., 2005), given its proteolytic and keratolytic effects, associated with its viscosity (El-Nekeety *et al*., 2009). *Ficus* fruit latex (FFL) contains significant amounts of polyphenolic compounds (Wang *et al*., 2008; Oliveira *et al*., 2010a;Yadav *et al.,* 2011) in addition to several types of fatty and amino acids (Oliveira *et al*., 2010b). A number of chemical examinations of *Ficus carica* L. have shown the presence of psoralen, bergapten, umbelliferone (Seong-Kuk *et al*., 1995, Louis *et al*., 2000), β-sitosterol, campesterol, stigmasterol, fucosterol, fatty acids (Jeong and Lachance, 2001), 6-(2-methoxy-Z-vinyl)-7-methyl-pyranocoumarin and 9,19-cycloarlane triterpenoid as an anticancer (Weiping *et al*., 1997a, Weiping *et al*., 1997b) and antiproliferative agent: 6-O-acyl-β-Dglucosyl-β-sitosterol (Shai *et al*., 2001), calotropenyl acetate, and lupeol acetate (Saeed and Sabir, 2002). Krishna Mohan *et al*., (2007) reported that the leaf extracts of *Ficus carica* possessed a significant hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity in rats. They related this hepatoprotective activity to the presence of coumarins in the methanolic extract of their leaves. Gond *et al*. (2008) reported significant hepatoprotective activity

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with the petroleum ether (60-80˚) leaf extract of *Ficus carica* Linn. in response to rifampicin induced hepatic damage in rats.

The *Ficus* latex was found to reveal chemotherapeutic effects (El-Shobaki *et al*., 2010; Aref *et al*., 2010; Khodarahmi *et al*., 2011; Chawla *et al*., 2012) due to its antioxidant activity and several other pharmacological actions.

As far as our literature survey could ascertain, no attempt has been made to study the protective role of *Ficus* latex against lead toxicity. The aim of the present work was to investigate the hepatoprotective action of *Ficus* latex against lead acetate-induced hepatotoxicity in rats and finding the exact mechanism for this protection.

2. Materials and Methods

2.1. Latex Collection

Latex was collected directly from the neck of *Ficus carica* L. fruit before ripening in June of 2011 from Erbil city, north of Iraq. The *Ficus* latex was collected in sterile screw bottles and kept in cool boxes until transported to the laboratory. Before administration, the latex was diluted 1:50 with distilled water without any further treatment. The identity of *Ficus carica* L. was confirmed by a plant taxonomist.

2.2. Experimental Animals

The present study was conducted using 32 mature male Wistar albino rats (*Rattus norvegicus*). All rats were healthy, weighing 200 - 270 gm. and 8-10 weeks old at the time when the experiment started. The animals were bred and housed in plastic cages (56 x 39 x 19 cm) bedded with wooden chips in groups of seven rats per cage in a room with controlled temperature of 24 ± 3 ˚C, in animal house of Biology Department -College of Science-Salahaddin University-Erbil-Iraq. The animals were kept on 12/12 hours light/dark schedule during the experimental study. They were fed with standard laboratory chow containing 0.5% NaCl, 22% protein and 4-6% dietary fat and allowed to drink water *ad libitum*.

2.3. Total Phenol Content

Total phenolic compounds were determined using the Folin–Ciocalteu method (Lamien-Meda *et al*., 2008). One mL of the *Ficus* latex (without dilution) was added to 10.0 mL distilled water and 2.0 mL of Folin–Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrun, Germany). The mixture was allowed to stand at room temperature for 5 min and then 2.0 mL sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. A standard calibration curve was plotted using gallic acid concentrations at 0.02-0.1mg/mL and the results were expressed as mg of gallic acid equivalents (GAE)/mL of latex (W:V). The data obtained were the means of three determinations.

2.4. Total Flavonoid Content

For the determination of total flavonoid, modification of Lamien-Meda *et al*., (2008) was used. $AlCl_3$ (2%, w/v) was mixed with 0.5 ml of undiluted latex. After 10 min, the absorbencies were measured at 415 nm against a blank on a UV/visible light spectrophotometer (CECIL CE 2041, CECIL Instruments, England) and compared to quercetin. The calibration curve was plotted by dissolving 100 mg pure quercetin in 100 ml double distilled water then further dilution was made in five different concentration solutions such as 10µl, 20 µl, 30 µl, 40 µl, 50 µl respectively. The data obtained were the means of three determinations. The amounts of flavonoids in plant extracts were expressed as mg of quercetin equivalents (QE)/mL of latex.

2.5. Experimental design

The thirty two male rats were divided randomly into the following groups with eight animals in each group. Group 1: control rats received only 1mL of distilled water by gavage; Groups 2: received daily dose of 1mL of diluted latex/kg b.wt by gavage. Group 3: rats exposed to lead acetate (500mg/L) in drinking water Groups. 4: received lead acetate in drinking water and latex (1mL of diluted latex/kg b. wt) for 28 day. The selected dose of *Ficus* latex was based on a preliminary test for the histological effect of 1mL/kg b.wt. of different dilutions of latex in distilled water,1:30, 1:40, 1:50, and 1:60, in which the third dilution (i.e. 1:50) was more effective in normalizing liver histological features. The administration of latex in the second and forth groups was started one day earlier.

2.6. Biochemical Study

2.6.1. Preparing The Liver Homogenate

Liver was sliced into pieces and homogenized with a blender in ice-cold 0.1 M sodium phosphate buffer (pH-7.4) at 1-4 ˚C to give 10% homogenate (w/v). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 4 ˚C twice and the resulting supernatant was separated and used for various biochemical estimations such as malodialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) in various groups of rats.

2.6.2. Malondialdehyde (MDA) Determination

The method reported by Utley *et al. (*1967) was followed. Aliquots of homogenate (1 mL) were incubated at 37 ºC for 3 h in a metabolic shaker. Then, 1 mL of 10% aqueous trichloroacetic acid (TCA) was added and mixed. The mixture was then centrifuged at 800g for 10 min. Then, supernatant (1 mL) was mixed with 1 mL of 0.67% thiobarbituric acid and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 mL distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (MDA) (nmol/g wet tissue) was then calculated, by reference to a standard curve of MDA solution.

2.6.3. Determination of Superoxide Dismutase Activity

Superoxide dismutase was assayed as described by Misra and Fridovich (1972). The assay mixture contained 0.5 ml of hepatic PMS, 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µm nitroblue tetrazolium and 0.2 ml of freshly prepared 0.1 mM hydroxylaminehydrochloride. The reaction mixture was mixed quickly by inversion followed by the addition of clear supernatant of 0.1 ml of liver homogenate (10% w/v). The change in absorbance was recorded at 560 nm. Percentage inhibition was calculated using this equation:

% superoxide dismutase inhibition = [(normal activity – inhibited activity)/(normal activity)] × 100%.

2.6.4. Determination of Liver Reduced Glutathione (GSH)

Reduced glutathione (GSH) was estimated as follow: One mL of 10% of liver homogenate was taken and 1 ml of 5% TCA (w/v) was added. After 30 min, the mixture was centrifuged at 2500 rpm for 15 min. Also, 0.5 ml of supernatant was taken and 2.5 ml of 5,5'dithiobis (2-nitobanzoic acid) (DTNB) was added, mixed thoroughly and absorbance was recorded at 412 nm. The results were expressed as µmol/g tissue (Ellman, 1959).

2.6.5. Determination of Liver Homogenate Lead Level

Accurately measured samples of liver (250 mg) were digested in 10 ml concentrated nitric acid (HNO_3) by using Microwave Digestion System. After evaporation of HNO_3, dried samples were dissolved in 10 ml of distilled water. Lead content was estimated by Atomic Absorption Spectrometer (AAS, Perkin Elmer model A Analyst 100, Uberlingen, Germany) against suitable standards processed identically.

2.6.6. Determination of Liver Enzymes

For determination of liver enzymes, blood samples were collected from the heart into clean and dry tubes. Sera were obtained by centrifugation of the blood samples at 3000 rpm for 15 min at 4°C and stored at -20°C until assayed for the biochemical parameters. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using special kits (obtained from BIOLABO SA, Maizy, France) following the method described by Reitman and Frankel (1957) for AST and ALT and the method described by King and King (1954) for ALP. The absorbance for ALT and AST were measured at 505 nm, while for ALP was measured at 405nm.

2.7. Histological Examination

Liver samples were removed from the anesthetized animals, they were immediately fixed in Bouin's fluid for 24 hours, followed by a dehydration using a series of graded ethanol in ascending concentrations (50%, 70%, 95%, and 100%), immersed in xylene for clearing, infiltrated in paraffin wax, and finally embedded in paraffin wax. Four micrometer thick paraffin sections were obtained by using rotary microtome (Bright, MIC) and stained by hematoxylin and eosin (H&E) (Bancroft *et al*., 1977). The specimens were examined and photographed under light microscope (digital binocular compound microscope 40x-2000x, built-in 3MP USB Camera).

2.8. Electron Microscopy

Samples of liver ($1mm^3$) were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 - 7.4 for 24 hours, washed by cacodylate buffer 0.1M, postfixed in 1% Osmium tetroxide for one hour, dehydrated through a graded series of ethanol (50%, 70%, 95%, and 100%), and then cleared in propylene oxide for 15 minutes (twice), infiltrated with propylene oxide plus resin mixture and finally embedded in resin. The semi-thin sections were stained by 1% toluidine blue in 1% borax, while the ultrathin sections were mounted on copper grids and stained by uranyl acetate and lead citrate (Hayat 1974) and examined by TEM (LE1CA/ EM FC6, CM12 Philips).

2.9. Data Analysis and Statistics

All data were expressed as means ± standard error of mean (M ± SE) and statistical analyses were carried out using statistically available software of statistical package for social science (SPSS) version 11.5. One-way analysis of variance (ANOVA) was performed to test for significance followed by Duncan's multiple range comparison tests for comparisons between the groups. *P* values ≤ 0.05 and 0.01 were considered statistically significant.

3. Results

The present work revealed that the latex of *Ficus* had significant quantity of phenolic compounds and flavonoids. (Table1). The total phenolic content was 149.33 mg GAE/mL of the latex, while the total flavonoid content was 43.23 mg QE/mL of the latex.

Table 1. Total phenol and flavonoid in *Ficus* latex

Chemical content	latex
Total phenol mgGAE/ml	149.33±7.04
Total flavonoidmg QE/ml	43.23±3.35

Total phenol was expressed as mg of gallic acid equivalents (GAE)/mL of latex.

Total flavonoids were expressed as mg of quercetin equivalents (QE)/mL of latex.

Values given are means of 3 determinations ± SEM

It was found that the mean serum AST, ALT, ALP levels in control animals were 66.8, 31.1 and 445.3 IU/L respectively whereas in lead acetate treated rats, the level rose to 319.6, 192.8 and 809.3 IU/L respectively. When *Ficus* latex was administered to the lead acetate treated group, it reduced the AST, ALT and ALP levels to 55.5, 36.5 and 489 IU/L respectively (Table 2).

Table 2. Serum liver enzymes in different groups of treated rats

Biochemical analysis	Control	Latex	Lead acetate	Lead plus latex
ALT(IU/L)	31.1 ± 2.0^{b}	20.62 ± 1.11^{a}	192.8 ± 16.0^{c}	36.5 ± 3.6^{b}
AST(IU/L)	66.8 ± 4.2^{b}	51.72 ± 0.22^{a}	319.6 ± 22.7^{d}	55.5 ± 5.13^{ab}
ALP (IU/L)	445.3 ± 23.1^{b}	320.9 ± 0.68^{a}	809.3 ± 65.3^{c}	489.6 ± 43.9^{b}

As shown in Table 3, the liver mean values of MDA, GSH and SOD in control rats were 7.11 nmol of malondialdehyde/ g of liver, 2.13 µmols of GSH/g of the liver and 13.25 IU of SOD/gm of the liver respectively. In lead acetate treated animals, the MDA, GSH and SOD levels were 15.5, 1.02 and 6.27 respectively. *Ficus* latex reduced MDA to 8.84 nmol MDA/g of the liver and increased GSH and SOD levels to 1.91 and 15.62 respectively. The liver tissue of the control was found to contain 0.59 µg of lead/g of the liver. This quantity was significantly raised to higher amounts in the other groups in comparison to the control. The highest amount was recorded in the lead acetate treated group (3.79±0.17). The *Ficus* latex was found to cause a significant reduction of

the quantity of lead when administered to the lead acetate group (Table 3).

Table 3. Biochemical analysis in liver homogenates of different treated groups of rats

Biochemical analysis	Control	Latex	Lead acetate	Lead plus latex
lead μg/g liver	0.59± 0.06[a]	0.98± 0.01[b]	3.79± 0.17[d]	1.34± 0.09[c]
MDA nmol /gm liver	7.11 ± 0.17[b]	5.25± .34[a]	15.5± 0.59[d]	8.84± 0.23[c]
GSH(µmols/gm liver)	2.13 ± 0.13[b]	2.73± 0.32[d]	1.02± 0.12[a]	1.91 ±0.44[c]
SOD(U/g protein)	13.25± 1.14[c]	10.61± 0.07[b]	6.27± 0.16[a]	15.62 ±0.13[c]

Different letters in the same row refer to significant changes, while similar letters refer to non- significant changes at (p<0.01) and (p<0.05).

The most characteristic feature of lead toxicity after 28 days of lead acetate exposure as revealed by the present investigation was the fatty degeneration of hepatocytes, pyknosis of nuclei and dilatation of blood sinusoid lumen in comparison to control sections (Fig 1.A-D). In the plastic sections, slightly split nuclei were also detected in addition to infiltration of few inflammatory leucocytes. The liver sections of *Ficus* latex treated rats were showed approximately attenuated histological alterations (Fig 1E &F).

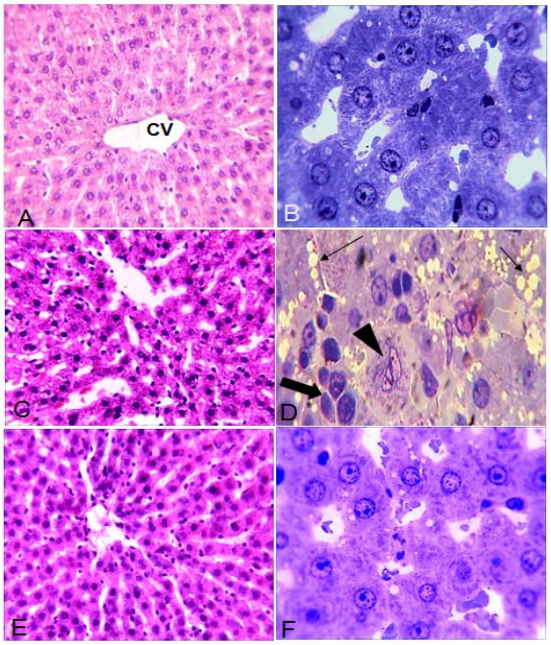

Figure 1. Sections through rats livers: A) showing normal structure,H&E,400X, B) control, plastic section, toluidine blue,1000X, C) lead acetate treated rat liver showing nuclear pyknosis, fatty change, H&E,400X, D) Plastic section revealing large lipid droplets (thin arrows) and early fragmented nuclei(arrow head), notice the appearance of few infiltrated inflammatory leucocytes(thick arrow), toluidine blue,1000X, E) Ficus latex plus lead acetate treated rat liver appears close to the control structure, H&E, 400X, F) Plastic section of Ficus latex plus lead acetate treated rat liver, notice the very low quantity of lipid droplets and the approximately normal nuclei appearance, Toluidine blue,1000X, CV means central vein.

The ultrastructural figures (Fig 2A-D) confirmed the histological observations and gave further information. The lead acetate treated rat liver showed accumulation of a high quantity of lipid droplets in comparison to a normal hepatocyte cytoplasm of the control. Furthermore, characteristic apoptotic cells containing a fragmented nucleus were detected (Fig 2C&D). Electron micrographs of the liver of Ficus latex plus lead acetate treated rats showed approximately normal ultrastructural feature of nucleus and cytoplasmic organelles especially mitochondria(2E&F).

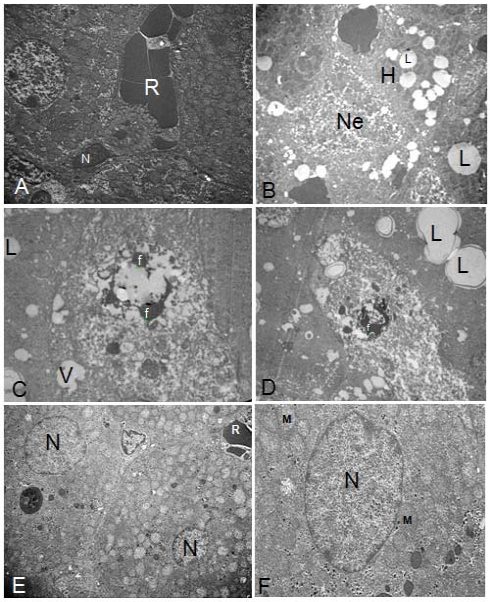

Figure 2. Ultrastructure of the liver of rats: A) Control section showing healthy hepatocyte containing normal mitochondria structure, 2000 X, B) Lead acetate treated rat liver section showing necrotic hepatocyte (Ne) adjacent to lipid accumulated hepatocytes(H), 2000X, C) An apoptotic hepatocyte containing a fragmented nucleus (f), 2000 X, D) Another apoptotic hepatocyte condensed and is going to fragment, 2000 X, E) & F) Healthy hepatocytes in the latex plus lead acetate treated group in two different magnifications, 2000 X and 5000 X respectively. L: lipid droplet, N: hepatocyte nuclei, n: blood sinusoid lining cell nuclei, M: mitochondria, R:RBC, V:vacuole**.**

4. Discussion

The present investigation revealed increase of MDA and decrease of GSH and SOD levels in the liver of lead acetate treated rats in comparison to control. This means that it increased the oxidative stress in the treated rats. It is known that lead-induced oxidative stress tissue damage could be caused by two mechanisms: increased generation of ROS, and by causing direct depletion of antioxidant reserves (Gurer *et al*.,1998; Upasan *et al*.,2001; Ercal *et al* 2001; Hamadouche *et al*.,2009). Intense lipid peroxidation caused by lead exposure may affect the mitochondrial and cytoplasmic membranes causing more severe oxidative damage in the tissues and consequently releasing lipid hydroperoxides into circulation (Shabani and Rabbani 2000; Abdel-Wahhab *et al*.,2008) which reflects the induction of oxidative stress (Dringer 2000; Newairy and Abdou 2009; Bokara *et al*., 2009).

The histological study of the present investigation showed several changes such as degeneration of liver cells especially hepatocytes, fatty changes, blood sinusoidal lumen dilatation and the appearance of infiltrated inflammatory leucocytes. Several publications have mentioned the lead-induced liver damage (Sharma and Pandey 2010; Sharma *et al*., 2011; Liu *et al*., 2011). Liver steatosis includes macrovesicular and microvesicular steatosis. Macrovesicular steatosis is characterized by a single large cytoplasmic vacuole of triglycerides within the hepatocyte that displaces the nucleus peripherally, while microvesicular steatosis is characterized by the presence of multiple small droplets of triglycerides within the hepatocyte, which do not displace the nucleus (Sturgill and Lambert 1997). It appeared that the microvesicular steatosis was more prominent in the present work. Lead was recently found to cause increase of serum triglycerides (Liu *et al*.,2011).

The current work revealed an increase in the level of ALT, AST and ALP in lead acetate treated rats in comparison to control and this may be due to the degeneration of hepatocytes by necrosis which causes leakage of these enzymes into blood circulation (Jensen *et al*., 2004). This elevation was attenuated after giving latex to the lead acetate treated rats. Gond *et al*., (2008) have reported a significant reduction in serum ALT and AST levels in rifampicin treated rats after administrating *Ficus carica* Linn. leaf extract.

Despite its chemical, biological, and ecological importance, *Ficus carica* latex is still poorly studied.(Oliveira *et al* 2010). In the present work, *Ficus* latex was found to contain high phenol and flavonoid contents. Recently, bioflavonoids and polyphenols of plant origin have been used extensively for free radical scavenging and to inhibit lipid peroxidation (Xu *et al*., 2005; Newairy and Abdou 2009). These antioxidant compounds could have played a major role in scavenging the reactive oxygen species induced by lead acetate in the serum (Haleagrahara *et al* 2010b).

Treatment with *Ficus* latex along with lead acetate treatment decreased the lead induced changes in MDA and antioxidant enzyme levels. Results on liver lead levels (LLL) showed that lead acetate alone showed a significantly higher LLL compared to all other groups. The detected decrease in LLL in latex plus lead acetate treatment group may refer to the possible chelating effect of *Ficus* latex. However, this property of *Ficus* latex requires further study. Relatively few data were available regarding the antioxidant effect of *Ficus* latex (Oliveira *et al*., 2010a; Joseph and Justin 2011; Yadav *et al*., 2011). Histologically, the *Ficus* latex when administered to lead acetate treated rats attenuated the previous histological changes.

It has been previously reported that zinc and ascorbic acid treatment showed moderate therapeutic efficacy when administered individually, whereas more pronounced protective effects were observed after combined therapy of zinc and different doses of ascorbic acid (Upadhyay *et al*., 2009). As compared with latter work, it seemed that *Ficus* latex gave better therapeutic results. Cylak *et al*.,(2008) have obtained good antioxidant results when methionine, alpha-lipoic acid, N-acetylcysteine and homocysteine were administered individually, although the present investigation used higher dose of lead (twice and half time). Haleagrahara and his co-workers (2011) detected significant antioxidant effect of alpha-lipoic acid against lead-induced oxidative stress when lead was given at same present dose but with shorter duration. It can thus be concluded that, the *Ficus* latex used in the present work for attenuating lead hepatotoxicity introduces an easiest antioxidant agent compared with other antioxidants.

Electron micrographs revealed the appearance of apoptotic hepatocytes with its characteristic feature, fragmentation of the nucleus (Ziegler and Groscurth, 2004).This type of programmed cell death was induced due to the oxidative stress (Buttke and Sandstrom 1994) caused by lead acetate as revealed by the current results.

5. Conclusion

Lead is considered as a strong hepatotoxic agent, caused its toxicity, at least in part, through inducing oxidative stress. The *Ficus* latex was highly successful in attenuating lead hepatotoxicity due to its high total phenol and flavonoid contents. Three mechanisms were suggested for this attenuation: first: lowering the oxidative stress, second: increasing the oxidant enzymes level and third: acting as chelating agent for lead ions.

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Evaluation of the Reproductive Toxicity of Dietary Fumonisin B_1 in Rats

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Abstract

The toxicity of dietary fumonisin B_1 (FB_1), a mycotoxin from the common maize fungus *Fusarium verticillioides*, on serum gonadotropins, ovarian histopathology, and fertility were examined in female Wistar rats. Thirty-nine female rats were randomly assigned to two test diets containing 10.0 and 20.0 mg FB_1/kg and a control diet. After 14 days of feeding, blood samples were obtained by intracardiac puncture from 4 rats in each treatment for gonadotropins evaluation, and were then killed by cervical dislocation to collect samples of ovaries for histopathology. Also, each of the remaining nine females in each treatment was mated to one healthy adult male rat. The serum LH concentrations of rats fed diets containing 10 and 20 mg FB_1/kg were significantly lower (P<0.05) than those fed the control diet, while the serum FSH level of rats fed diet containing 20 mg FB_1/kg only was significantly lower (P<0.05) compared with the controls. Dietary FB_1 however failed to induce histopathological changes in the ovaries of the rats. Fertility, gestation lengths and foetal weights of the rats decreased significantly (P<0.05) with increased dietary FB_1. The concentration of daily dietary FB_1/kgBW of 1.74 in this study is less than five times the estimated probable daily fumonisin intake of 355 μg/kg BW for person eating 'mouldy' maize in the high oesophageal cancer area of the Transkei region, South Africa. The apparent significant "safety factor" of about five times relative to human FB_1 exposure may be a cause for concern in areas where maize is a dietary staple.

Keywords: Fertility, Fumonisin B_1, Gonadotropins, Mycotoxin, Rats, Toxicity.

1. Introduction

Mycotoxins are natural contaminants of cereals and other food commodities throughout the world and they significantly impact human and animal health. Animals, as well as humans, are exposed to mycotoxins through consumption of contaminated diets, which can be considered the gateway to cases of natural intoxication by these fungal secondary metabolites (Gutema, 2000; Hennigen, 2000).

The economic consequences of mycotoxin contamination are profound, and exposure of people and livestock to mycotoxin-contaminated foods is particularly a serious problem in the tropics (Reddy and Raghavender, 2008). According to Lawlor and Lynch (2001), 25% of the global crop is contaminated with mycotoxins. Crops with large amounts of mycotoxins often have to be destroyed. Alternatively, contaminated crops are sometimes diverted into animal feeds. Giving contaminated feeds to susceptible animals poses a serious threat to the health and productivity of the animals and cause great economic losses (Griessler and Encarnação, 2009), by acting directly or indirectly on fertility. Reproductive inefficiency is recognized as the most costly limiting constraint to efficient animal production as reproduction is the bedrock of animal production (Gbore, 2009; Ewuola and Egbunike, 2010). This makes the assessment of the effects of mycotoxins on livestock reproduction a unique challenge in an effort to improve livestock production.

Fusarium verticillioides (Sacc) Nirenberg (= *F. moniliforme* Sheld.), one of the most prevalent mycotoxigenic fungi reported to be associated with dietary staples such as maize intended for human and animal consumption throughout the world (Nelson *et al*., 1991; Kedera *et al*., 1992), produces the mycotoxin, fumonisin. *F. verticillioides* is present in virtually all maize samples (Marasas *et al*., 2001). Maize is the major cereal utilized in the formulation of livestock feeds in several parts of the world, hence, the potential for fumonisins to be found in feeds and feedstuffs is high. Several naturally occurring fumonisins are known; FB_1 has been reported to be the most abundant and most toxic which represents approximately 70% of the total concentration in naturally contaminated foods and feeds, followed by fumonisins B_2 (FB_2) and B_3 (Murphy *et al*.,

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1993; Norred, 1993). Consequently, toxicological studies on the fumonisins have been concentrated on FB_1.

The carcinogenicity, hepatotoxicity and mutagenicity as well as the effects on feed intake, live weight gain and blood abnormalities of fumonisin in animals have been well documented (Marasas *et al*., 1988; Harrison *et al*., 1990; Kellerman *et al*., 1990; Gelderblom *et al*., 1991, 1994; Colvin and Harrison, 1992; Voss *et al*., 1998; Ewuola and Egbunike, 2008; Ewuola *et al*., 2008; Gbore and Egbunike, 2008, 2009; Gbore, 2009). However, studies on depression of fertility and reproductive processes in rats by the toxin are rare.

Flynn *et al*. (1996) reported growth inhibition of rat embryos exposed *in vitro* to FB_1 on gestation day (GD) 9.5. Although useful as screens, *in vitro* methods allow direct foetal exposure and avoid maternal gastrointestinal absorption, pharmacokinetics, placental transfer, and other potential barriers of *in utero* exposure. *In vivo* assessment of the effects of fumonisin is therefore necessary.

Consumption of lesser amounts of fungal toxins at levels below those that cause overt toxicity may result in impaired fertility and decreased reproduction in animals. In a preliminary study, Gbore and Olorunfemi (2009) reported significant concentration-dependent decline conception rates in rabbits exposed to dietary fumonisin prior to mating. Fumonisins inhibit sphingolipids metabolism, and a variety of biological activities for sphingolipids have been reported (Wang *et al*., 1992). It was therefore hypothesized that fumonisin could alter the release of gonadotropins from the pituitary because the brain contains high levels of sphingolipids. Studies on the alterations of gonadotropins and subsequent fertility in rats exposed to dietary FB_1, to our understanding, are scarce. It is essential, therefore, to assess the gonadotrophic and ovarian histopathological effects of *F. verticillioides*-contaminated maize-based diets and subsequent fertility and reproductive processes in rats.

2. Materials and Methods

2.1. Experimental Site and Animals

Eight weeks old male and female Wistar rats (*Rattus norvegicus*) obtained from a commercial breeder of Wistar rats in Benin City, Nigeria were used. Females weighed 168.92 ± 1.41 g. Male rats (202 ± 2.21 g) were used as sires only and were not exposed to dietary fumonisin. Thirty-nine mature females were individually housed under standard housing conditions (22°C, light/dark cycle 12/12 hours) in wire mesh rat cages at the Animal House of the Department of Biochemistry, Adekunle Ajasin University, Akungba Akoko, Nigeria. Further laboratory analyses were carried out at the Department of Chemical Pathology of the University College Hospital, and Department of Veterinary Pathology, all of the University of Ibadan, Ibadan, Nigeria. This study was approved by the local Institutional Animal Ethics Committee and was performed in accordance with "Guide for the care and use of Laboratory Animals" (National Research Council, 1996).

2.2. Fumonisin B_1 Production and Experimental Diets

Maize grits in 500 g quantities were placed into autoclavable polypropylene bags and soaked with 200 ml of distilled water for 2 h, then autoclaved for 1 h at 121°C and 120 kPa. The autoclaved maize grits were then cultured with a toxigenic strain of *F. verticillioides* (MRC 286) obtained from the Plant Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to produce FB_1 as described previously (Nelson *et al*., 1994). Uncultured maize grits and the cultured maize grits were used to formulate three diets. Samples of homogenously mixed diets were quantified in replicates for FB_1 and other common *Fusarium* mycotoxins including deoxynivalenol (DON, vomitoxin), T-2 toxin, and zearalenone using mycotoxin quantitative CD-ELISA test kits (Neogen, Lansing, MI, USA) and reconfirmed by using HPLC analyses as described by Shephard *et al*. (1990). The concentrations of FB_1 in the diets were adjusted to 0.2, 10.0 and 20.0 mg/kg constituting diet 1 (= control diet, which had no *Fusarium*-contaminated maize grits), diets 2 (medium FB_1-contaminated diet) and 3 (high FB_1-contaminated diet), respectively. The concentrations of all other common *Fusarium* mycotoxins screened were below the detection limit of 0.2mg/kg for the toxins. The dietary FB_1 doses in this study were based on a preliminary dose-range-finding study by Collins *et al*. (1998a). Lower doses were selected for this study, the highest dose being 20 mg/kg. The pelleted diets provided ~20% crude protein, 5% crude fibre and 2.9 kcal of digestible energy/g.

2.3. Experimental Model

After 3 weeks of physiological adjustment period, the rats were randomly allocated to each of the three diets (n = 13 rats per treatment). The rats were provided with fresh clean water and appropriately weighed feed daily, and the weights of feed portions given and left uneaten after 24 h were determined. The body weight was determined weekly on a weighing scale (Ohaus Corp., Pine Brook, NJ, USA) with a precision of 0.05 g. The body weight gain of each rat was determined weekly as the weight difference in comparison to the weight in the previous week.

After 14 days of feeding, blood samples were obtained by intracardiac puncture from 4 rats from each treatment and were killed by cervical dislocation to collect samples of ovaries. Blood samples were collected into vacutainer tubes, covered and centrifuged at 4000 rpm for 10 minutes. The separated sera were decanted and deep-frozen for serum gonadotropins analyses at the Chemical Pathology Unit of the University College Hospital, Ibadan, Nigeria.

2.4. Evaluation of Reproductive Performance

To evaluate reproductive performance, after 2 weeks of feeding the respective experimental diets, each of the remaining nine females in each treatment was placed in a cage with one healthy adult male. The animals were kept together overnight, and then separated the following morning. Immediately after each separation, a vaginal smear examination was carried out to determine if sexual intercourse had occurred. When intercourse was positive (presence of spermatozoa in the vaginal smear), the night/day routine was discontinued and the female was housed individually during the estimated period of

gestation. At parturition, percentage of fertility with respect to the number of positive smears, gestation length, number of litters, mean litter size, number of live foetus per litter, and foetal weights were determined.

2.5. Examination of Ovaries

For examination by light microscopy, ovary samples collected were fixed in 10% neutral buffered formalin (pH 7.2) before dehydration in ten changes in ethanol of different concentrations ranging from 70 to 100% at 1-hr interval. After dehydration, the tissues were cleared in two changes of chloroform before infiltration and embedding in molten wax (60°C) for 12 h. Thereafter, the tissues were blocked in paraffin wax and later sectioned using a microtome. Paraffin sections (4µm) of the ovary samples were stained with haematoxylin and eosin. Photomicrographs were taken with a Zeiss Axiophot instrument using Kodak Plus-X pan (PX 135 to 24) film.

2.6. Determination of Serum Gonadotropins

Serum levels of gonadotropins; follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were measured using a commercial ELISA kit (Habersham, Buckinghamshire, UK). All samples were run in duplicate in a single assay.

2.7. Statistical Evaluation

Data from this study were analyzed by one-way analysis of variance procedure of SAS (2001). The treatment means were compared using the Duncan procedure of the same software and results giving *P* values of <0.05 were considered significantly different.

3. Results

3.1. Feed Consumption and Body Weight Gain

Daily observation through the 35 days did not indicate detectable alterations in the general state of any of the animals. The average food consumptions were 17.64, 16.03, and 18.77 g for rats on diets 1, 2, and 3 respectively. Significant ($P<0.05$) differences in feed consumption and final weights were not observed (Figures 1 and 2), although the mean weights of the dams given 10 and 20 mg FB_1/kg (216 ± 17.6 to 218 ± 15.0 g) were 2.5 - 3.4% lower than that of the controls (223.56 ± 11.0 g) on day 35. Based upon feed consumption and body weight data, the diets provided 0.08, 0.74 and 1.74 mg FB_1/kg BW per day to the rats on diets 1, 2, and 3, respectively.

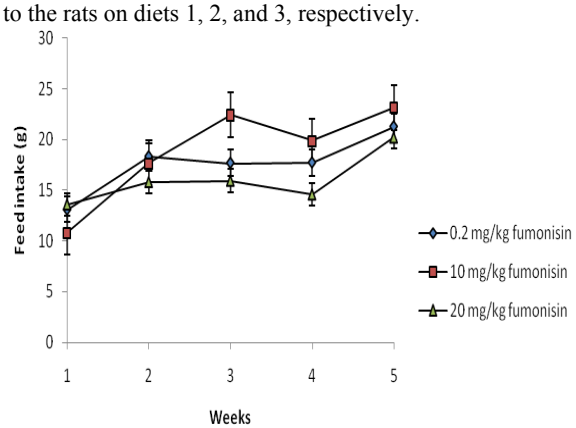


Figure 1. fed intake of rats fed different concentrations of dietary fumonision B1.

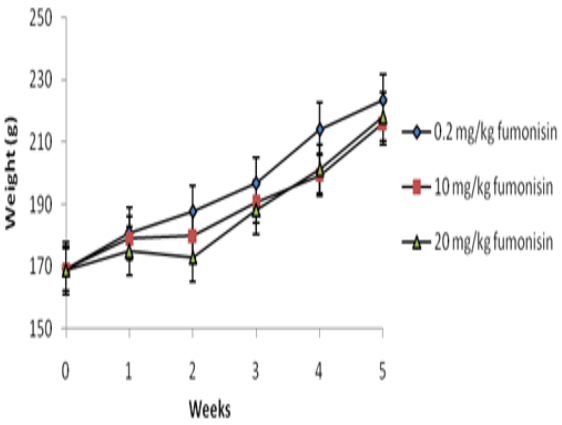


Figure 2. weights of rats fed different concentrations of dietary fumonision B1.

3.2. Serum Gonadotropins Levels

Dietary FB_1 significantly depressed serum gonadotropins in rats (Table 1). The serum LH concentrations of rats fed diets containing 10 and 20 mg FB_1/kg were significantly lower ($P<0.05$) than those fed the control diet. Similarly, the serum FSH levels of rats fed diet containing 20 mg FB_1/kg were significantly lower ($P<0.05$) than those fed the control diet and diet containing 10 mg FB_1/kg.

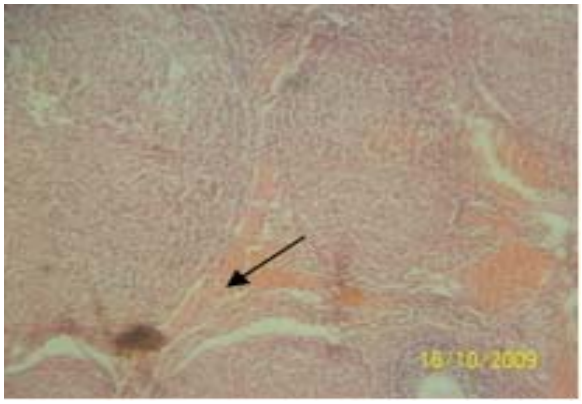
Table 1. Serum Gonadotropins (ng/ml) of Rats Exposed to Different Concentrations of Dietary Fumonisin B_1 (Mean ± SEM)

	Dietary fumonisin B_1 concentrations (mg/kg)		
Parameters	0.2 (Control Diet)	10.0 (Diet 1)	20.0 (Diet 2)
Luteinizing Hormone	23.00 ± 0.01[a]	10.90 ± 2.50[b]	10.03 ± 2.80[b]
Follicle Stimulating Hormone	215.00 ± 21.01[a]	214.90 ± 20.07[a]	199.80 ± 22.16[b]

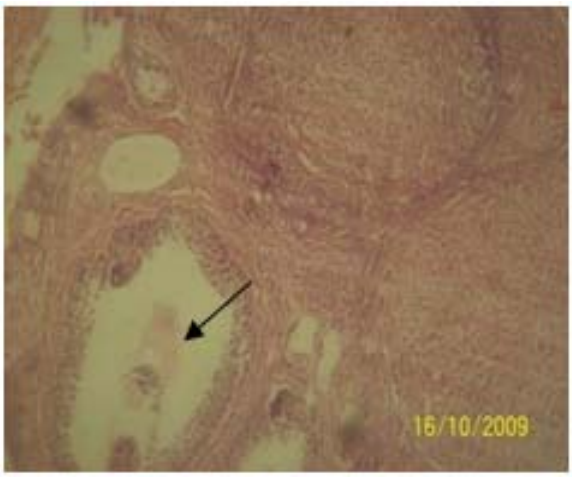
[ab]: Means on same row with different superscripts differ significantly ($P < 0.05$)

3.3. Histopathology of the Ovaries

The histopathological examination of the ovaries showed no modification by the dietary FB_1 concentrations. No visible lesion was observed in the ovaries (Figures 3a, b and c) for the control rats, those fed 10 and 20 mg FB_1/kg diets, respectively.



a



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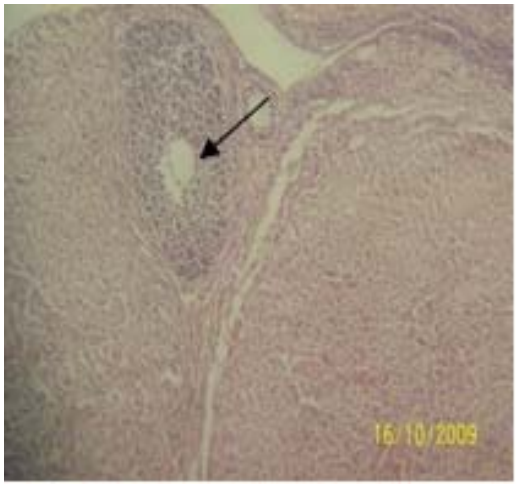

c

Figure 3. Haematoxylin-and-eosine stained sections of rat ovaries exposed to diets containing different concentrations of dietary FB_1. No visible lesion was observed in the ovaries of rats fed diets containing different concentrations of FB_1 as shown in Figures 3a, b and c for the control animals, those fed 10 and 20 mg/kg dietary FB_1 respectively. Note the oocytes (arrowed). Mag. X 200.

3.4. Fertility Assessment

The summary of the reproductive indices of female rats exposed to different concentrations of dietary FB_1 are shown in Table 2. The results revealed that fertility, gestation length and foetal weight/litter were dose-dependent. These parameters decreased with increased dietary FB_1, which were significant (P<0.05) at both feed contamination levels. Out of the 9 rats mated in each group (as determined using the vaginal smears), only 5 had pups in the group fed diet containing 20 mg FB_1/kg as against 8 and 7 for rats fed the control diet and diet containing 10 mg FB_1/kg, respectively. No statistically significant difference was found across the treatment groups for number of live foetus/litter. The total number of pups obtained from the dams resulted in relative number of pups/dam of 6.78 for those fed the control diet as against 6.11 and 3.78 for those fed 10 and 20 mg FB_1/kg diet, respectively.

Table 2. Reproductive Performance of Rats Exposed to Different Concentrations of Dietary Fumonisin B_1 (Mean ± SEM)

	Dietary fumonisin B_1 concentrations (mg/kg)		
Parameters	0.2 (Control Diet)	10.0 (Diet 1)	20.0 (Diet 2)
Fertility (%)	88.89 ± 33.33[a]	77.78 ± 44.10[b]	55.56 ± 52.70[c]
Gestation length (days)	21.60 ± 0.53[a]	23.33 ± 0.51[b]	23.00 ± 0.74[b]
No of live foetus/litter	7.67 ± 0.33	8.00 ± 0.33	7.25 ± 0.38
Foetal weight/litter (g)	5.33 ± 0.01[a]	5.23 ± 0.04[b]	5.22 ± 0.04[b]
No of litters	8	7	5
Total no of live foetus	61	55	34
No of dead foetus	0	1	2
Rel.* no of live foetus/dam	6.78	6.11	3.78

*Relative to total no of rats with positive scrapes in each treatment.

[abc]: Means on same row with different superscripts differ significantly (P < 0.05).

4. Discussion

Although, initial studies using laboratory animal (Voss *et al*., 1996; LaBorde *et al*., 1997; Collins *et al*., 1998a, b) provided no evidence that FB_1 is teratogenic. However, more recent observations and experimental findings have again drawn attention to FB_1 as a possible risk factor for birth defects (Merrill *et al*., 2001, Marasas *et al*., 2004, Voss *et al*., 2006). In this present study, the decrease in fertility of 77.78 and 55.56% for the rats fed diets 2 and 3 respectively compared with 88.89% for the control rats revealed adverse effect of the dietary fumonisin on fertility processes in the rats. The total number of pups obtained from the 9 mated female rats in each treatment showed that the 34 pups obtained from rats fed 20 mg/kg dietary FB_1 was only 55.74% of the pups obtained from the controls. This is quite significant in breeding programmes.

Effects of fumonisin on developing foetuses were expressed most often by foetal death and resorption (Floss *et al*., 1994). Reduced weight gain is often the earliest indicator of maternal illness in developmental toxicity studies, and would be expected as a sign of maternal effect. While the cause of foetal deaths in rats exposed to diets containing 10 and 20 mg FB_1/kg was not evident in this study, it was clear that the dose-dependent significant decline in foetal weight observed in this study was as a result of developmental toxicity produced by FB_1 and not secondary to maternal toxicity. In a study (LaBorde *et al*., 1997), the observed reduced foetal weight from rabbits gavaged daily on GD 3 – 19 with purified FB_1 at 0.5 – 1 mg/kg/day was ascribed to maternal toxicity, rather than any developmental toxicity produced by FB_1. Dose-responsive significant decrease in foetal weight observed in this study correlates with reports of other studies. Pregnant rats dosed by gavage on GD 8 – 12 with a

semipurified extract of culture material containing FB_1 with a purity of 80% resulted in lower foetal weight at dose of 60 mg/kg (Lebepe-Mazur *et al*., 1995). Similarly, decreased body weight of live foetuses obtained from pregnant Syrian hamsters gavaged with FB_1 in a dose-dependent manner was reported in a study (Penner *et al*., 1998). Voss *et al*. (1996) reported lower litter weights from rats fed *F. moniliforme* culture material providing 1 – 55 ppm FB_1 from two weeks before mating compared to the control group.

The dose-dependent significant decrease in fertility observed in this study may be due to an increase in gonadal steroid inhibition or suppression of the hypothalamus and/or pituitary gland resulting in a decline in serum FSH and LH levels. These two gonadotropins are the most important regulatory hormones of ovarian and uterine function (Everett, 2006). Suppression in secretion of hypothalamic gonadotropin releasing hormone (GnRH) causes reduced secretion of LH and FSH from pituitary (Rai *et al*., 2004). Fumonisins are structurally similar to sphinganine and sphingosine and inhibit sphingosine metabolism in tissues, leading to an accumulation of sphingoid bases, which are intermediates in sphingolipid biosynthesis (Wang *et al*., 1991). Because the brain contains high levels of sphingolipids, the disruption of sphingosine metabolism was speculated to be the mechanism behind the degeneration of neuronal cells seen in equine leukoencephalomalacia (Wang *et al*., 1991). A variety of biological activities for sphingolipids have been reported (Wang *et al*., 1992). Alterations in the amounts of any of these by fumonisins could potentially result in a variety of biological and pathological effects (Penner *et al*., 1998). These may be responsible for the significant decline in serum gonadotropins resulting in reduced fertility with increased dietary FB_1 observed in this study. The mechanisms involve is not clear further investigations are therefore necessary to elucidate the mechanism.

The elongated gestation lengths observed in this study further leads credibility to the fact that FB_1 potentially affect reproductive development. As in this study, seemingly longer gestation length from 21.9 ± 0.35 days was observed in rats fed control diet to 22.7 ± 1.00 days in rats fed *F. moniliforme* culture material providing 10 ppm FB_1 from two weeks before mating (Voss *et al*., 1996). Adverse effects of mycotoxins on sexual and reproductive developments have been reported. Green *et al*. (1990) and Rainey *et al*. (1990) observed that 1.5 mg zearalenone/kg diet disturbed the hypothalamo-hypophyseal function of prepubertal gilts, but after withdrawal of the contaminated diets, the animals attained puberty without delay, and their fertility was unimpaired. Also, dietary zearalenone levels as low as 0.05–0.06 mg/kg DM have been shown to increase the number of ovarian follicles and to decrease the serum concentration of the gonadotropic hormone FSH in female piglets (Döll *et al*., 2003), thus potentially affecting their sexual development. Recently, dietary FB_1 have been reported to delay attainment of sexual maturity in growing pigs (Gbore, 2009) and rabbits (Ewuola and Egbunike, 2010).

Impaired action of FSH and LH on the ovary has as a primary consequence, a concomitant alteration in the capacity of this organ to synthesize ovarian reproductive hormones; mainly estrogens from follicular cells and progesterone from luteal cells (Everett, 2006). Since the histopathological examination of the ovary did not reveal any leision, it is suggested that the decline in fertility rates could not have resulted from impaired ovaries but impaired action of the gonadotropins on the organs.

The data from this study suggest that dietary FB_1 of ≥10 mg /kg significantly reduced serum gonadotropin levels and lowered fertility without adverse effect on the ovarian histology of rats. The concentration of daily dietary FB_1/kgBW (1.74) which resulted in significant decline in fertility of female rats in this study is less than five times the estimated probable daily fumonisin intake of 355 µg/kg BW (Gelderblom *et al*., 1996) for person eating 'mouldy' maize in the high oesophageal cancer area of the Transkei region, South Africa. The apparent significant "safety factor" of about five times relative to human FB_1 exposure may be a cause for concern in areas where maize is a dietary staple. However, further studies on whether the toxin disrupts the hypothalamic production or release of GnRH or the absorbed FB_1 acts directly on the pituitary, at doses that are not maternally toxic, to lower serum gonadotropins are warranted.

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Morphological Diversity among *Corchorus olitorius* Accessions Based on Single Linkage Cluster Analysis and Principal Component Analysis

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Abstract

Corchorus olitorius L. is a leaf vegetable grown in Africa as well in the Middle East. Fifteen accessions of leaf *C. olitorius* were collected from two National Research Institutions and Biotechnological Center and were evaluated for genetic variability using Single linkage cluster analysis (SLCA) and Principal Component Analysis (PCA). The experiment was carried out at Babcock University Teaching and Research Farm during 2009. The experiment was laid out using a Randomized Complete Block Design (RCBD) with three replications. The contribution of Number of leaves per plant, plant height at maturity, Fresh leaf weight, total plant weight and harvest index in the PCA leads to the conclusion that these traits contributes more to the total variation observed in the fifteen accessions of *C. olitorius* and therefore can be used in discriminating among the accessions of *C.olitorius.* BUCor 24 and BUCor 31, with high potential for number of leaves per plant, plant height at maturity, fresh leaf weight and total plant weight would make good parental stock material when breeding for *C. olitorius* leafy vegetative yield, while BUCor 31, and BUCor 05 are more distinct and diverse of all the accessions and can serve as sources for variability in character for *C. olitorius* improvement. The accessions were grouped in four major cluster by the SLCA.

Keywords: Accessions, *Corchorus*, *malukhiyah,* Nijeria and variability

1. Introduction

Corchorus species are known in Arabic as *malukhiyah* and used as green leafy vegetables. *Malukhiyah* is eaten widely in North Africa and the Middle East, such as Lebanon, Palestine, Israel, Syria, Jordan and Tunisia. In Turkey and Cyprus, the plant is known as *molohiya* or *molocha* and is usually cooked into a kind of chicken stew. (Schippers, 2000). *Corchorus olitorius* L. is a leaf vegetable with Africa as the primary center of origin due to wide diverse plant types found in the continent (Benor *et.al*., 2011, Kundu, 1951) and it is widely cultivated for the sliminess of the leaves in local dishes. It is one of the leading leaf vegetables in West Africa (Grubben, 1977). The Egyptian traditional food *Malachia* is made with *C. olitorius* and the slimy property has made it popular beyond the North African origin. Large morphological and physiological variation exist among the leaf *C. olitorius* found on farmers plots in Nigeria (Nath and Denton,1980) and distinct types based on variation in leaf shapes were separated from local cultivars at NIHORT, Nigeria (Denton,1997). Apart from the variation in the leaves, considerable variation in other morphological traits still exists within the various local morphotypes (Akoroda, 1985). Similarly cultivated *Corchorus* with distinctly different leaf shapes are known by different names in Cameroon and Benin (Schippers, 2000; Westphal Stevels, 1986). Soliman *et al.* (2010) found variation in a number of vegetative characters among three Egyptian cultivars of *C. olitorious.* Several studies conducted used molecular marker techniques to detect the genetic variation. Ogunkanmi *et al.* (2010) demonstrated the presence of inter and intra genetic variability among 40 accessions each of *C. incisifolus* and *C. olitorius,* respectively base on Random Amplified Polymorphic DNA (RAPD) markers**.** The study of the genetic diversity of the many African indigenous leaf vegetables, including *Corchorus olitorius* (Chweya and Eyzaguirre, 1999) will aid early and adequate exploitation of the desirable nutritional properties of the crops for better nutrition and good health especially among the rural communities in Africa (Oguntona and Akinyele, 1995). Adequate analysis of germplasm diversity is essential for proper understanding and utilization of genetic variability among accessions and their characters.

Using fuzzy cluster analysis, Yu and Li, (1991), recognized six cluster groups containing varying numbers

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of accessions with up to 16 accessions in one cluster of *C. olitorius* based on observations obtained from 12 morphological characters. There are very few reported improved varieties of leaf *C. olitorius* in Africa and as such the cultivars found on farmers plots are mostly traditionally inherited mixed populations and locally sourced types. This has aided the protection of the genetic variation and the immediate erosion of the crop genetic resources (Fondio and Grubben, 2004). In addition to farmers' conservation of the genetic variability of C. *olitorius*, various other steps had been taken to collect and conserve the germplasm of *Corchorus* sp. in national and institutional gene banks in Nigeria, Ethiopia, Kenya, Sudan and Zambia (Attere,1997). This experiment was set up to assess the genetic variability among fifteen accessions of *Corchorus olitorius*, using Single linkage Cluster and Principal Component analyses based on six morphological characters

2. Materials and Methods

Fifteen accessions of leaf *C. olitorius* were collected from two National Research Institutions and Biotechnological Center namely, National Horticultural Research Institutes (NIHORT), Institute of Agricultural Research and Training (IART) and National Center for Genetic Resources and Biotechnology (NACGRAB), in Nigeria. The accessions represent part of the institutional germplasm which are locally grown by farmers in Nigeria. The experiment was carried out at the research and teaching experimental plot of the Horticultural Unit of Babcock University which is located in the southern rainforest belt of Nigeria with an annual average rainfall of 1500mm and mean daily temperature of 25-27^{o}C between May and December 2009.

The seed dormancy for each accession was first removed with hot water treatment for ten seconds before planting. *C. olitorius* is a small seeded crop and in order to ensure that the seeds are evenly spread, the seeds of each accessions were mixed separately with fine river sand (1gm seed :10kg sand) and then drilled in rows on raised beds. Each row was 5.0 meters long with spacing of 50cm between two rows and within rows, the seedlings were thinned to a spacing of 2.0cm between plants. The trial was set up in a Randomized Complete Block Design (RCBD). There were four rows of plants per plot for each accession and each plot was replicated three times.

Table 1. Accession codes and their sources

Serial Number	Accession Name	Source
1	BUCor 02	IAR&T
2	BUCor 04	NIHORT
3	BUCor 05	NIHORT
4	BUCor 08	NIHORT
5	BUCor 10	IAR&T
6	BUCor 12	NIHORT
7	BUCor 13	NIHORT
8	BUCor 14	IAR&T
9	BUCor 15	NACGRAB
10	BUCor 18	NIHORT
11	BUCor 19	NIHORT
12	BUCor 23	NIHORT
13	BUCor 24	NIHORT
14	BUCor 31	IAR&T
15	BUCor 40	NIHORT

NIHORT: National Horticultural Research Institute, Ibadan, BU: Babcock University Ilishan-Remo, NACGRAB: National Centre for Genetic Resources and Biotechnology, IAR&T: Institute of Agricultural Research and Training, Ibadan.

Before drilling the seeds, a pretreatment of cured poultry manure was applied on all the plots at the rate of 20t/ha. The plants were raised under rain fed conditions and manual weeding was carried out to maintain weed free plots. Regular insecticidal control measure was maintained during the investigation. After eight-weeks of sowing when the accessions were fully established and revealed distinct variations in plant morphological characteristics, ten competitive plants from the two middle rows in each plot were harvested and observations were taken on following morphological characteristics and yield on each plant

1. Plant height at maturity(cm),
2. Number of leaves per plant
3. Fresh leaf weight per plant(gm),
4. Stem weight(gm),
5. Total plant weight (gm).
6. Harvest index (%) :determined by dividing the fresh leaf weight by total plant weight and expressing the value in percentage

The mean value for each character was calculated as the average for the ten harvested plants and was used for the statistical analysis.

2.1. Data analysis

Data were analyzed using SAS Microsoft windows 8.0 (SAS, 1999) adopting the method of Steel and Torrie (1980). The PCA and SLCA were used to determine the extent of genetic variation and percentage similarity within accessions. Eigen-values and factor scores were obtained from PCA, which were used to determine the relative discriminative power of the axes and their associated characters. FASTCLUS procedure was used to group the fifteen accessions based on their genetic relationship. A dendrogram is generated from SCLA to display position of accessions and their percentage similarities.

3. Result

The result of the PCA showed that two of the six Principal Component Axes (PCA) had Eigen-values greater than two and all together accounted for over 80% of the total variability (Table 2). The first Principal Component Axes (PCA accounted for 56.80% of the total variation while the second Principal Component Axes (PCA 2) accounted for 23.60% of the total variation. The PCA 1 and PCA 2 are loaded with characters such as number of leaves per plant (0.46), plant height at maturity (0.40), Fresh leaf weight (0.52), total plant weight (0.53) and harvest index (0.74). The relative discriminating capacity of the PCA is shown by their Eigen-values. The PCA 1 had the highest discriminating power as revealed by its highest Eigen -value of 3.40 followed by PCA 2 with Eigen value of 1.42. The accessions were classified

into four distinct cluster groups using the FASTCLUS procedure (Table 3).

Table 2. Principle Component Analysis among Fifteen *Cochorus olitorus* accessions

Character	PC Axis 1	PC Axis 2	PC Axis 3
Number of leaves per plant	0.46	0.26	-0.20
Plant Height at Maturity	0.40	-0.52	-0.03
Stem weight	0.25	-0.29	0.87
Fresh leaf weight	0.52	0.17	-0.15
Total plant weight	0.53	-0.01	-0.14
Harvest index	0.11	0.74	0.41
Eigen-Value	3.40	1.42	0.88
% Variance	56.80	23.65	14.69
Cumm. % Variance	56.80	80.45	95.14

Table 3. Mean, Standard Deviation (sd) in parenthesis of four clusters with major characteristic patterns of 15 *Corchorus* accessions

Character	I	II	III	IV
Accession	10,12,13,14, 18,19,2,4,8	24,31	5	15,23, 40
Number of leaves per plant	11.32 (2.19)	19.072 (4.53)	10.2 (0.0)	9.65 (2.04)
Plant Height at maturity	29.10 (1.5)	31.02 (1.25)	31.27 (0.0)	19.7 (2.02)
Stem weight	2.94 (0.55)	4.34 (0.8)	11.73 (0.0)	1.75 (0.29)
Fresh leaf weight	1.93 (0.55)	3.27 (0.57)	1.7 (0.0)	1.3 (0.17)
Total plant weight	4.87 (1.06)	7.6 (1.32)	4.73 (0.0)	2.92 (0.29)
Harvest index	0.39 (0.04)	0.43 (0.0)	0.42 (0.0)	0.45 (0.02)

The highest number of accessions was located in cluster I with nine accessions, clusters IV and II had three and two accessions respectively whereas cluster III had only one accession. Cluster II contained accessions with the highest number of leaves per plant followed by accessions in cluster I, while accessions in cluster IV had the least number of leaves per plant. Clusters II and III contain plants with the highest plant height at maturity. This was closely followed by accessions in cluster I, whereas, the lowest value was recorded in accessions found in cluster IV.

Stem length was found to be the highest in accessions located in cluster III and the lowest in accessions presented in cluster IV. Meanwhile, fresh leaf weight was highest in accessions found in cluster II and lowest in accessions found in cluster IV. Total plant weight was highest in accessions found in cluster II, but lowest in accessions found in Cluster IV. The highest harvest index was recorded in accessions found in cluster IV, followed by cluster II, whereas the least was recorded in accessions found in cluster I.

The plot of (PCA 1 and 2) is shown in Figure 1. Result shows that accessions BUCor 31(31), BUCor 40(40) BUCor 15(15), BUCor 23(23) and BUCor 05(5) were the most dispersed and diverse of all the accessions considered in this study. BUCor 23, BUCor 15 and BUCor 40 are mostly described by characters in PCA 2, whereas characters in the PCA 1 best described BUCor 05 and BUCor 31. The dendrogram drawn from the SLCA shows the relationship between the 15 accessions (Figure 2). The dendrogram revealed four distinct clusters that joined to form one big cluster at 1% level of similarity. At 100% level of similarity all the accessions were totally

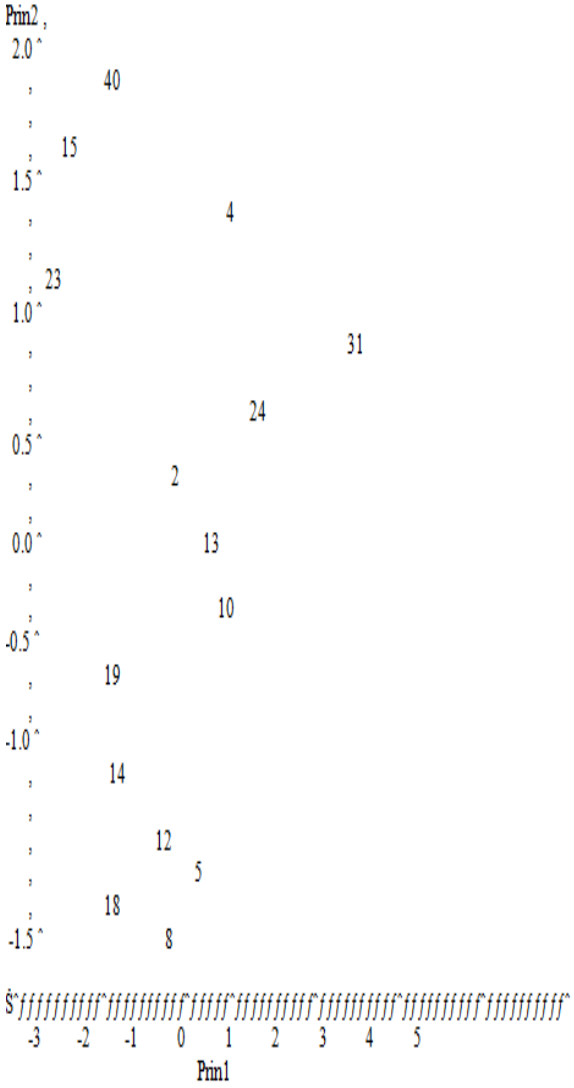

Figure 1. Configuration of fifteen *Corchorus* genotypes under PCA 1and 2.

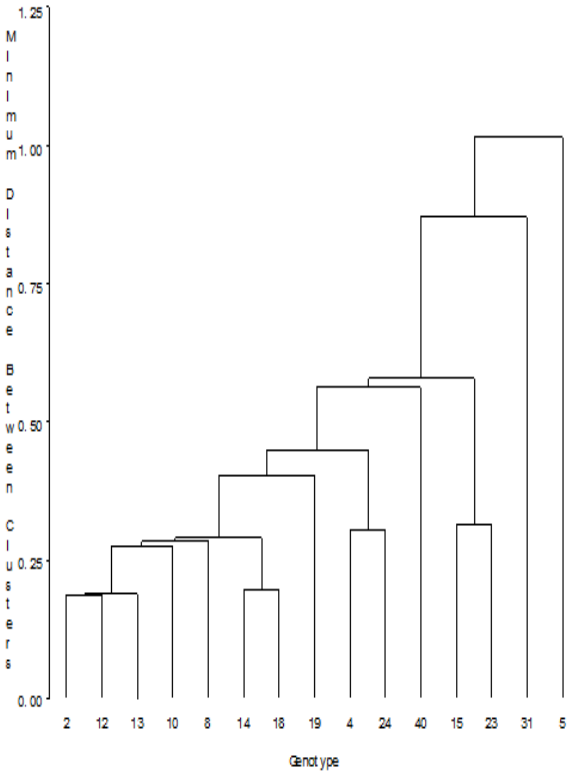

Figure 2. Dendrogram from SLCA of fifteen *Cochorus olitorus* accessions.

distinct from each other and had formed one single cluster at 1% similarity level. At 68% level of similarity eight of the accessions BUCor 2 (2), BUCor 12 (12), BUCor 13 (13), BUCor 10 (10), BUCor 8 (8), BUCor 14 (14), BUCor 18 (18) and BUCor 19 (19) had joined together to form a cluster. These accessions also formed another major cluster joining with three other accession at 52.5%.

At 43.5% similarity level these accessions had join with three others to form one major cluster. At 25% level of similarity all the accessions have joined to form one major cluster except accession BUCor 31 (31) and BUCor 05(5), which were still distinct from all the others. However, these two accessions later joined the other accessions to form one major single cluster at 1% level of similarity.

4. Discussion

The relatively high mean contribution of number of leaves per plant, plant height at maturity, fresh leaf weight, total plant weight and harvest index confirm the individual contributions of these traits to the total variation observed in the fifteen accessions of *C. olitorius*, hence these characters can be used in discriminating between the accessions of *C. olitorius*. This suggests that any selection from any cluster group for leaf vegetable yield must take into consideration these traits. This agrees with the report of Islam *et al*., (2002) in their report on *C. olitorius*

Accessions in cluster II with high potential for number of leaves per plant, plant height at maturity, fresh leaf weight and total plant weight would make good parental stock material when breeding for *C. olitorius* leafy vegetative yield which is the economic yield of *C. olitorius* in Nigeria and some other African countries. The clustering scores among the PCA suggests that there is a strong relationship amongst individuals in a cluster (Nwangburuka *et al*., 2011). The range in the similarity indices (1 – 82%) among the accessions is large enough to suggest sufficient variability among the accessions (Tokpol *et al*., 2006; Morsy, 2007 and Rawashdeh, 2011). BUCor 31, and BUCor 05, which were more distinct and diverse can serve as sources for variability in characters for the improvement of the accessions studied. Meanwhile, crosses between accessions in a cluster may not produce meaningful improvement in the offspring's, since these accessions are expected to have similarities in gene and therefore may not introduce reasonable variation. This agrees with the report of Torkpol *et al*., (2006).

BUCor 12 (2) and BUCor 12(12) were the closest accessions with (82%) similarity, high percentage of similarity could be related to these accessions may have similar ancestral origin with a common gene.

5. Conclusion

The contribution of number of leaves per plant, plant height at maturity, fresh leaf weight, total plant weight and harvest index leads to the confirmation of the individual contributions of these traits to the total variation observed in the fifteen accessions of *C. olitorius* and therefore can be used in discriminating among the accessions of *C. olitorius*.

Furthermore, BUCor 24 and BUCor 31, with high potential for number of leaves per plant, plant height at maturity, fresh leaf weight and total plant weight would make good parental stock material when breeding for *C. olitorius* leafy vegetative yield.

The clustering scores among the PCA suggested that there is a strong relationship amongst individuals in a cluster. The accessions were grouped in four major cluster by the SLCA. The range in the similarity indices (1– 82%) among the accessions as shown in the dendrogram drawn from SLCA is large enough to suggest sufficient variability among the accessions. Finally, BUCor 31, and BUCor 05, are more distinct and diverse of all the accessions and can serve as sources for variability in character for *C. olitorius* improvement.

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Crop Loss Assessment of *Lixus incanescens* Boh. (Coleoptera: Curculionidae) on Sugar Beet, *Beta Vulgaris L.*

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Abstract

Sugar beet weevil, *Lixus incanescens* Boh., is one of the most important pests of sugar beet in many parts of Iran and neighbor countries. The leaf and petioles of sugar beet are attacked by adults and larvae. The economic losses due to *L. incanescens* damage have not been estimated in Iran. Moreover, chemical application is currently the conventional control method. Therefore, it was necessary to assess crop losses. This project was done in a sugar beet field during 2006 and 2007 in Tehran (Iran) using fenvalerate EC20% (1 L/ha). The number of infested petioles in 50 plants was counted in treated and untreated plots once a month until the harvesting time. Then, the weight (kg), sugar content (%), sugar extraction coefficient (%) and white sugar content (%) of roots were measured. The results showed that in both years, there was significant difference between the mean number of infested petioles in each plant in treated (0.47 ± 0.03 and 0.047 ± 0.003 in first and second years, respectively) and untreated (6.53 ± 0.33 and 4.82 ± 0.52 in first and second years, respectively) plots. But, there were not significant differences regarding the indices including weight, sugar content, sugar extraction coefficient and white sugar content. The cost-benefit ratio was 2.65- 3.7, when the field had an average of 5-6 infested petioles per plant.

Keywords: Chemical control, Crop loss Assessment, *Lixus incanescens*, Sugar beet**.**

1. Introduction

Sugar beet weevil, *Lixus incanescens* Boh., is the major pest of sugar beet. It has been reported from many parts of Iran and other countries like south of Ukraine, east south of Russia, Caucasia, Kazakhstan, Turkmenistan and Turkey (Davatchi and Kheyri, 1960; Aleeva, 1953). It has three generations per year in Iran. The leaf and petiole of sugar beet are attacked by adults and larvae of *L. incanescens.* The adults prefer plants which have well developed four to six leaves and feed on petioles and new leaves. The larvae attack the petiole of sugar beet and petiole vessels are torn and broken. In each petiole, 1 to 10 larvae can be found. The damage reduces leaf green area, root weight and nutrient movement rate in plants. As Ocete *et al.* (1994) reported, the larvae can cause up to 75% root weight loss. Adults hibernate under plant debris and stones. Severe damage happens in the second generation in August. The percentage of damage is related to date of planting. Hence damage in early-planted sugar beet is less than late-planted ones (Parvizi and Javanmoghadam, 1988). It also feeds on common purslane (*Poryulaca oleraceae* L.), common orache (*Atriplex patula* L.), common goosefoot (*Chenopodium album* L.), *Amaranthus retroflexus*, *Salsola kali* and *Atriplex hortensis.* So, destroying the host weeds can reduce its population (Kheyri, 1966; Parvizi and Javanmoghadam, 1988). It was a key pest in Turkmenistan during 1970-1973 (Gold *et al*., 2004) and in 1983 in south west of Romania (Manole, 1990). Also, it is one of the most important pests of sugar beet fields in Uzbekistan (Rashidov and Khasanov, 2003).

Assessment of crop losses was investigated for several pests and diseases (Hills *et al*., 1980; Shane and Teng, 1983; Campbell *et al*., 1998; Hull, 2007). There are several methods for estimating crop losses by insect pests. In the direct method, actual crop losses are measured in the field. In the indirect methods, crop losses are estimated by relation between insect density or damage symptoms and yield index (Walker, 1991 a), e.g. the relation of number and length of holes caused by stem borers and yield index (Walker, 1991 b).

The most precise method of estimating crop losses is through direct measurement of actual losses. Crop loss can be defined as the difference between the potential yield (the yield that would have been obtained in the absence of the pest and the actual yield (De Groote *et al*., 2001).

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A reliable analytical frame for plant health decision making is essential. Cost-benefit analysis provides such a frame, typically by projecting a stream of predicted costs and benefits for managing options, expressed in financial terms, and setting present values on these streams. Further detailed analysis may deliberate the distribution of benefits in time and space, risk attitudes can be combined, and non-monetized elements can be integrated. The aim is to provide an obvious and objective frame in which managing options can be compared on common economic criteria (Mumford *et al*., 2000).

Cost-benefit analysis is an organized frame to analyze the efficiency of projects, programs, policies or regulations. It can be used to improve the quality of public policy decisions by recognizing all the costs and benefits of a policy, and evaluating them using as a metric a monetary measure of the aggregate change in individual well-being resulting from the policy (Boardman *et al*., 1996). Cost-benefit analysis is based on the economic theories of prosperity and can be used to assess how rare resources should be assigned to the avoidance and control of pests and unwanted species in the agricultural part.

Sugar beet weevil has become a key pest in Iran. Farmers apply insecticides a lot against this pest because all stages except adults develop in the petiole, and adults emerge gradually. The objectives of this study were a) to assess the crop losses and b) to determine the cost- benefit ratio to finally reduce application of insecticides.

2. Materials and Methods

The project was done in an unsprayed sugar beet field (2 ha) during 2006 and 2007 in Tehran, Iran. Distance between rows was 50 cm. Sugar beets (variety Universe) were planted at a distance of 25 cm from each other. The field was irrigated by a center pivot sprinkler. Percentage of damaged plants (plants with symptoms on their leaves and petioles) was calculated. Because, damaged plant percentage depended on *L. incanescens* abundance. Then, 15 plots, each including five 15-m rows, were selected. The middle row of each plot was selected for sampling. Each row had 50 sugar beet plants. Six plots were treated once every two weeks by fenvalerate EC20% (1L/ha). The rest of the plots were not treated (considered as check).

2.1. Sampling Method of Crop Loss Assessment

The number of infested petioles in 50 plants was counted once per month (The initial sampling showed that variation in infested petioles was significant during a month) until the harvesting time. Then, the tubers of the middle row of each plot were collected separately and transferred to the laboratory of Sugar Beet Seed Institute in Karaj, Iran. The samples were weighed after washing. A cossette was prepared using all 50 glands of each row (van der Poel *et al*., 1998). Sugar content (%), sugar extraction coefficient (%) and white sugar content (%) of roots were measured (Kunz, 2004).

2.2. Cost-Benefit Ratio Calculation

Cost- benefit ratio was calculated by the following formula (Ponnusamy, 2003):

$$\frac{\text{Treated benefit (\$) - Untreated benefit (\$)}}{\text{Cost of Protection}}$$

Cost of protection must be calculated. Cost of protection ($) is sum of insecticide cost ($), labour cost ($), sprayer rent cost ($), and crop loss compensation ($).

In Iran sugar factories buy sugar beet on a basis of sugar content using by the following formula (Sheikholeslami, 2003):

$$\text{Value of Sugar Beet (\$)} = \frac{\text{Sugar content (\%) - 3}}{13 \times \text{price (\$)}}$$

'Sugar content' as a grade is measured in sugar factories. Here, the average grade in each treatment was calculated. The value '3' is the rate of yield loss or rate of sugar is not extractible. The value '13' is the minimum acceptable grade that lower than it, is not purchased.

'Price' is value of sugar beet per kilogram that is determined annually by the Agricultural Ministry. Price of sugar beet was 4.6 cent per kg during the two years of our research.

After calculating income per one kilogram, the average gland weight of 50 plants was multiplied by the average number of sugar beet plants per hectare (100000 plants), then the total income was calculated per hectare.

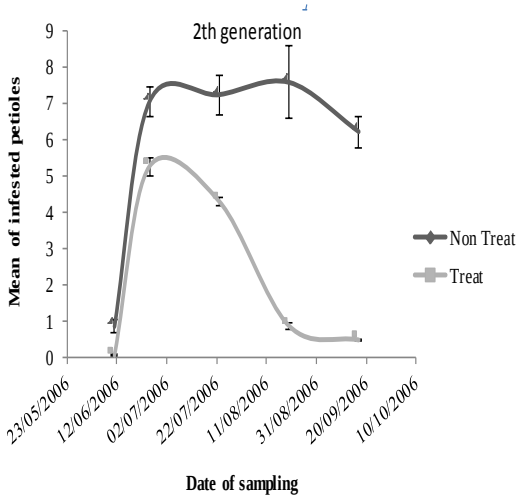
2.3. Statistical Analysis

Data were analyzed using SAS Var. 9.1 (SAS Institute Inc., Cary, NC). Means were compared by T-test (PROC TTEST). Correlation between traits such as infested petioles and indexes (sugar content, sugar extraction coefficient, white sugar content and weight) was estimated (PROC UNIVARIATE, PROC CORR).

3. Results

3.1. Comparison between means number of infested petioles in treated and untreated plots

In both years, the highest mean number of infested petioles was observed in August, during which, the second generation emerges (Figure 1).



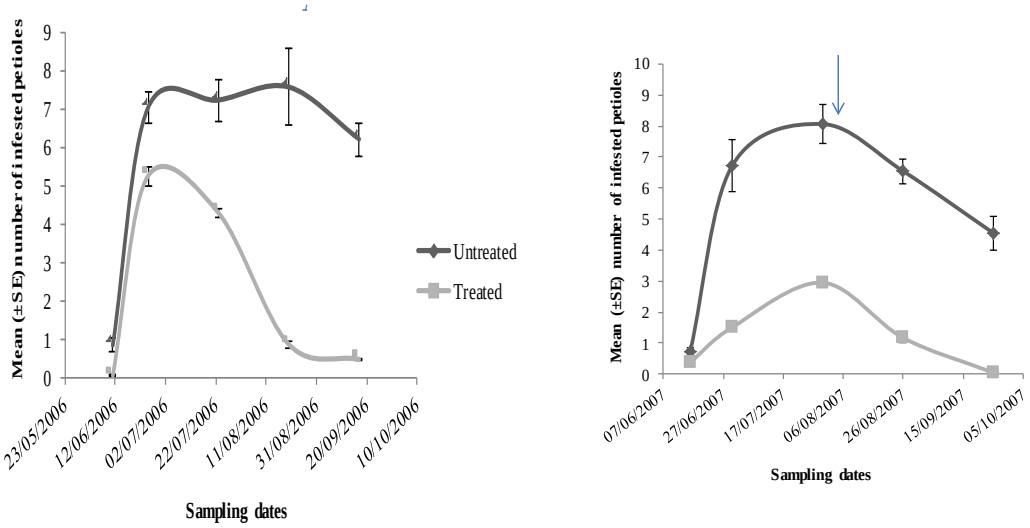


Figure 1. Mean (± SE) number of infested petioles at different sampling dates in (up) 2006 and (down) 2007. The arrows indicate the start date of the second generation.

Results showed that in both years, there was a significant difference between the mean number of infested petioles in each plant in treated and untreated plots (2006: T= 18.36, DF(7,5), *P*<0.0001) (2007:, T= 9.09, DF(7,5), *P*<0.0001). However differences were not significant in the indices including sugar content (2006, T= -2.23, DF(7,5), *P*>0.0517) (2007, T= -2.22, DF(7,5) *P*>0.0464) sugar extraction coefficient (2006, T= -1.62, DF(7,5), *P*>0.1311) (2007, T= -1.34, DF(7,5), *P*>0.2047) white sugar content (2006, T= -1.73, DF(7,5) *P*>0.1090) (2007, T= -2.02, DF(7,5), *P*>0.0666) and weight (2006, T= -0.87, DF(7,5) *P*>0.4109) (2007, T= 1.14, DF(7,5), P>0.2897). Table 1 shows the mean of infected petioles and indexes in treated and untreated plots in 2006- 2007.

Results showed that, when the number of infested petiole increased, sugar content decreased (2006: rs = -0.6733, *P*= 0.0083; 2007: rs= -0.6811, *P*= 0.0073). On the other hand, sugar contents between treated and untreated plots were not different.

Table 1. Mean (± SE) number of infested petioles and the indices including sugar content, white sugar content, sugar extraction coefficient and weight in treated and untreated plots in 2006- 2007.

Treatments	Infested petioles/plant		Sugar content/plant (%)		Sugar extraction coefficient /plant (%)		White sugar content/plant (%)		Weight/plant (Kg)	
	1st year	2nd year	1st year	2nd year	1st year	2nd year	1st year	2nd year	1st year	2nd year
Treated	0.47±0.03	0.047±0.003	14.57±0.09	16.41±0.27	80.28±0.43	74.11±0.73	11.70±0.14	12.18±0.30	1.47±0.02	1.12±0.03
Untreated	6.53±0.33	4.82±0.52	14.04±0.21	15.64±0.23	78.87±0.67	72.70±0.73	11.11±0.27	11.33±0.28	1.37±0.11	1.28±0.13

Number of infested petioles was negatively correlated with sugar extraction coefficient (2006: rs= -0.6835, *P*= 0.0070; 2007: rs= -0.3827, *P*= 0.1768) and white sugar content (2006: rs= -0.7011, *P*= 0.0052; 2007: rs= -0.6239, *P*= 0.0171). The amount of impurities and sugar molasses had positive correlation with infestation rate.

This study showed direct relation between the number of infested petioles and root weight in two years (2006: rs= -0.2706, *P*=0.3494; 2007: rs=0.5201, *P*= 0.0566).

3.2. Cost- Benefit Ratio Calculation

Cost-benefit assessment shows (tables 2 and 3) when all plants of the farm are infested with *L. incanescens* and infection mean rate is 5-6 petiole per plant, insecticide application can increase yield, sugar content percentage and benefit while reducing damage.

Table 2. Calculation process of income rate per hectare.

Year of experiment	1st year		2nd year	
Treatments	Treated	Untreated	Treated	Untreated
Mean sugar content (%)	14.57	14.04	16.41	15.64
Income rate of selling 1 kg sugar beet ($)	0.041	0.039	0.047	0.045
Mean weight of 50 plants (Kg)	73.63	68.72	56.42	54.52
Mean plant weight per hectare	147260	137440	112840	109040
Income rate per hectare ($)	6028.82	5369.09	5354.37	4876.92

Table 3. Calculation of cost-benefit ratio between treated and untreated plots in 2006- 2007.

Main factors in calculation	First year		Second year	
	Treated	Untreated	Treated	Untreated
Chemical control cost ($/ha)	180	-	180	-
Income of selling sugar beet ($/ha)	6028.82	5369.09	5354.37	4876.92
Benefit ($/ha)	479.73		477.45	
Cost Benefit ratio	3.7		2.65	

4. Discussion

Sugar beet has two growth stages: 1) from germination to tuber formation, and 2) sugar production in tubers. Incidence of pests and diseases in the second growth stage may reduce sugar content and storage in tubers. The reduction of green area of sugar beet fields by pests may also decrease sugar storage in roots. Moreover, sugar content depends on different factors such as depth of plowing, seeding date, time and amount of nitrogen fertilizers, planting density per hectare, shoot appear, pests and diseases incidence, incomplete crown beater and delay in harvest and transferring to factory (Amini, 1988).

The amount of sugar content and impurities such as potassium, sodium, and harmful nitrogen in sugar beet tubers are the main factors in quality assessment (Smith *et al*., 1977). The quality of crop increases with high rate of sugar content and low rate of impurities, because impurities prevent crystallization of sucrose and decrease efficacy of extracted sugar and increase the amount of molasses in the factory (Eck *et al*., 1990; Dunham and Clark, 1992; Kerr and Leaman, 1997).

The second generation of *L. incanescens* causes severe damage because this period is synchronized with sugar storage in the roots which is so important in sugar beet development. So, if active leaves decrease, it can reduce sugar storage. Then sugar content percentage and value of sugar beet would be dropped. Results of the present study demonstrated that when the number of infested petioles with *L. incanescens* increased, sugar content, sugar extraction coefficient and white sugar content decreased however root weight did not change. As Jadidi *et al.* (2010) indicated, the main effect of defoliation stage was significant on quality traits of sugar beet such as sugar content, white sugar content and sugar extraction coefficient, but its effect on quantity traits such as root yield was not significant. However, different levels of defoliation affected both quality and quantitative traits of sugar beet. Different experiments on defoliation showed that defoliation in early spring had negligible effect on yield loss, but defoliation in the summer causes more yield reduction (Dunning and Winder, 1972; Jones *et al.*, 1955).

Stallknecht and Gilbertson (2000) stated that date and severity of defoliation, are more important than the sugar beet stage of growth, regarding reduction of root yield and sucrose content of sugar beet.

Parvizi and Javanmoghadam, (1988) compared percentage of plant infestation to *L. incanescens* in different dates of planting and different generations of pest. They showed that percentage of plant infestation was higher in late-planted fields than early-planted fields in all three generations. So, the mean percentage of plant infestation in the second generation in the late-planted fields (%46.25) was almost double of early-planted fields (29.25%). Arbabtafti *et al.* (2008) found that if there were five to six infested petioles per plant, which is equivalent to 20% infestation per square meter, spraying could be done. The economic injury level for other defoliator pests of sugar beet such as armyworm, beet webworm, variegated cutworm and grasshoppers were estimated about 25% of damaged leaves (DiFonzo *et al*., 2006). Lilly and Harper (1962) indicated that sugar beet could recover from light to moderate defoliation with little or no decrease in yields of roots and sugar. It showed that insect infestation causing 25% or less defoliation of beets resulted in no economic importance. During late June, July, and early August pests should be controlled if the beets were defoliated 50% or more. Even when the leaves have been defoliated to 75% it was still possible to obtain a reasonably healthy crop.

Throughout this study, the cost-benefit ratio was calculated 2.65- 3.7. If it is above 1, chemical application can be economic. Results of the present study were similar to the results of experiments conducted in England and India. It was calculated for some pests like Colorado beetle which is a serious pest of potatoes in many countries but has never become established in England because of peripheral English climate for pest. Climate change might make Colorado beetle a greater hazard in future. The benefit- cost ratio of the current policy of elimination and suppression is estimated at 7.5 : 1, and the Net Social Benefit at 3.35 million pounds (Mumford *et al.*, 2000). *Thrips palmi* also has an extensive range of crop plants but it is only a threat to protected crops in England. The benefit-cost ratio was estimated at 7.4 : 1 and the Net Social Benefit was 2.2 million pounds (Mumford *et al*., 2000). Tobacco Whitefly has more than 500 plants which it is known to eat but in the English climate it is a potential pest of only protected crops. The Benefit-Cost ratio was 3.1 : 1 and the Net Social Benefit was 11.1 million pounds

(Mumford *et al.*, 2000). In assessment of neem-based insecticide, in controlling the ear head bug on rice, high cost benefit ratio was obtained from the treatment plot (2.74) compared to control plot (2.55). These results showed that application of neem-based insecticide (Azadirachtin 0.03%) at 500 ml/ha decreased the occurrence of ear head bug and increased the grain yield of rice and thus offer an appropriate approach to pest management (Ponnusamy, 2003).

Sugar beet weevil decreases sugar content, sugar extraction coefficient and white sugar content. Sugar content percentage has a key role in acceptance and rejection or determining price of sugar beet crop. Therefore, it is necessary to manage this pest. So when there was 20% infestation per square meter, spraying would be done.

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Properties and Antibiotic Susceptibility of *Bacillus anthracis* Isolates from Humans, Cattle and Tabanids, and Evaluation of Tabanid as Mechanical Vector of Anthrax in the Republic of Chad

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Abstract

Anthrax is a zoonose caused by the organism *Bacillus anthracis.* Anthrax affects mainly animals, particularly cattle, but also humans. The economic loss caused by Anthrax in the Republic of Chad are very important. In this study, the potential role of tabanids in anthrax transmission has been evaluated. Tabanids were collected in infested areas of the Chari-Baguirmi Province in Chad and examined using standard bacteriological and biochemical methods. Hundreds of anthrax bacilli were isolated: in this, about 89% of the 1499 tabanids examined were contaminated by anthrax organisms. *B anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%). Anthrax Bacilli were also isolated from cattle and humans, including nine cutaneous cases associated with tabanids bites. All *B. anthracis* isolates displayed similar biological properties whatever their origin. They were non motile, sensitive to penicillin, non hemolytic and fully virulent as they can elicit the disease in experimental animals. All but one biochemical character were identical out of the 49 tested. Isolates were sensitive to meticillin, tetracycline, nitrofurantoin, piperacillin, oxytetracycline, sulphathiazol, benzathien-penicillin and chloramphenicol. Resistance was found against polymixin, fusidic acid, clindomycin and sisomycin. Given together, the results suggested that tabanids could be an important vector of Anthrax in Chad.

Keywords: Chad, Anthrax, *B. Anthracis*, Biology, Biochemistry, Antibiotics, Tabanids, Vector, Mechanical Transmission.

1. Introduction

Bacillus anthracis, the ethiological agent of anthrax, is a large encapsulated Gram-positive rod. It grows vegetatively within an infected host animal and sporulates when it is exposed to the atmosphere or harsh environments (Hunter (1989), and Turnbull (1990)). Spores show a high degree of physical resistance, and can survive for years in soil (Manchee (1981), and Wilson (1964)). The longevity of the spores in the environment is an important biological factor in the distribution of Anthrax.

In the Chari-Baguirmi Province of Chad, temperature usually higher than 40°C and alternance of heavy rains (from May to October with an average 650 mm) and windy dust and dry weather (from November to April), create optimal conditions for the occurrence of Anthrax outbreaks. Soil covering the infested area has a pH ranging 6,5-7,0 that can sustain the persistence of Anthrax. Moreover, the disease transmission can be further complicated by the hatching period of various flies, mainly tabanids.

The role of insects in the spread of *B. anthracis* during anthrax outbreaks has been reported. Several Hematophagous diptera are able to transmit *B.anthracis* mechanically. Some other diptera have the ability to contaminate different surfaces either with spores or bacilli (Kozel (2007), and Nazil (2005)). Flies may transmit Anthrax from animal to animal or from animals to man (Sen, 1944). Herren (1989) pointed out the importance of bitting flies in the epidemiology of Anthrax, included tabanidae and Lipoptena. It has been reported that blood-sucking flies (e.g. Hippobosca and Aedes), as well as flies that do not suck blood (like *Mousca domestica* and Flesh eating flies), could transmit Anthrax (Blackburn, 2010; Fasanella *et al*., 2010). The mouthparts of tabanids are adapted for both blood-sucking and lapping, and particularly well suited to animal. The labella are retracted to expose the fascile which pierces the skin and lacerates the tissues for pool-feeding. The mandibles move with a scissor-like action and the maxillae move forwards and backwards (Dickerson and Lavoipierre, 1959b). When feeding ceases, the fascile is withdrawn and as the labella come together, they trap an enclosed film of blood of about 10 microliters that are protected from drying (Foil,

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1987). Ttabanids are able to be mechanical vectors of many pathogens (Foil, 1989) including *B. anthracis*. They can carry anthrax bacilli from anthrax-infected animals and disseminate spores by means of their wings and legs. Structurally, the tabanid is well adapted for collecting pathogens. Its stout legs end in three pads-like hairs that readily collect spores and disseminate them by contact. The well developed tabanid's wings t can also be used for spore spreading. Tabanids have an intrinsic flight range of over 50 km (Hocking, 1953) and may play a role in the dissemination of spores and bacilli in a wide area, contributing to the epidemic of Anthrax.

Lack of rapid and efficient control measures, as well as irregular vaccination programs against the disease, could also contribute to occurrence of large outbreaks in the Chari-Baguirmi Province, sick animals are slaughtered and meat usually consumes with or without prior veterinary inspection, even in case of confirmed Anthrax cases. Part of the meat is eaten fried or dried for further consumption. The skin is removed for sale. Carcass is neither disposed nor burned as having fuel is often problematic in the area. This further can help to diffuse the disease agent by various flies.

The Direction de l'Elevage et des Ressources animales (DERA, 2000) have reported Anthrax in the following villages: Chilo, Balarye, Abardjl, Ousmanari, Ambague, Kiessa-Cherif and Kiessa-Hassan.

Both animals and humans have been seriously affected (WHO, 1998, N'Gamiandje, 1984, Lamarque, 1990, Aba, 1997 and DERA, 1996, 1998). In 1998, the DERA reported cases of mortality among the population. This makes *B. anthracis* an important public health issue in a country where bitting flies populations are varied and numerous.

Although, *B. anthracis* can be transmitted through bitting flies, none bacillus has been so far isolated from them. This study aimed to determine the role of tabanids in the transmission of Anthrax in a region known as enzootic Anthrax zone. The second purpose of the present work was to examine the different isolates of *B. anthracis* collected from human, cattle and tabanid origin using various techniques. A biological and biochemical characterization of each isolates was carried out based on standard protocol. Susceptibility to antibiotics was also determined for each isolates.

2. Materials and Methods

2.1. Materials

Nine films of blood from suspected anthrax-infected cattle, 4 swabs from inflammatory fluid from 4 humans bitten by tabanids and 1499 tabanids collected from the infested area using fly nets were used for this study.

2.2. Methods

2.2.1. Bacteriology

Biological characterization was done in triplicates using the following procedure :

Direct microscopic examination of the suspected materials (swabs, films and dissected tabanid tissues) was performed using the methods of Gram, Soltys (1960) and Morris (1955).

These materials were grown in nutrient broth, selective medium of Morris, aired solid nutrient agar and 5% Columbia sheep blood agar and incubated at 37°C, for 48 hours; stained by Gram as well as Soltys and examined by direct microscopy.

The pure isolates were inoculated subcutaneously with 0.5 ml, 1.0 and 0.25 ml into each laboratory animal, i.e. 8 guinea pigs, 6 rabbits and 10 mice, respectively, to determine the pathogenicity amongst other characteristics of the isolates.

2.2.2. Biochemistry

Biochemistry characterization was done using the commercial biochemical reaction typing tools API 50 CH and API 20 £ containing together 49 substrates according to the manufacturer's protocols (API system S.A – Montialieu Vercieux – France).

2.2.3. Sensitivity Test

Antibiotic susceptibility testing was carried out using the antibiogramme BIO-DISC (Biomerieux- France) according to the manufacture's instructions. The results were interpreted according to the international guidelines as described by Maho, 2006.

2.2.4. Mobility Test

Semi-solid agar (nutrient broth with 0.3% agar) in culture tubes have been inoculated by the isolates. Inoculations were made by the stab method with a straight needle. Incubation was carried out at 37°C, for 6 days.

3. Results

About 89% of the 1499 tabanids examined were contaminated by anthrax-like organisms. *B. anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%) (Table 1).

Inoculation of the nutrient broth cultures by the humans inflammatory fluid, cattle blood and tissues from tabanids showed floccular growth on the surface of the cultures which sinks to the bottom within 24 hours of incubation. Isolates were non hemolytic on 5% Columbia sheep blood agar. Dull opaque, grayish-white colonies, with an irregular border was observed on aired solid nutrient agar media. Upon Gram-staining, long chains encapsulated, gram positive bacilli with rod shape were observed. These isolates were non motile and sensitive to penicilline (Table 2).

When the isolates were stabed into Motility Test medium, growth occurs only along the line of inoculation.

The 30 laboratory animals inoculated subcutaneously with isolates died within 24-72 hours post-inoculation, i.e. guinea pigs –(n = 10, 100% mortality), mice (n = 9, 90% mortality) and rabbits (n = 9, 90% mortality), indicating that isolates could be fully pathogenic to laboratory animals.

Autopsy carried out on guinea pigs showed that the tissues were swarming with gelatenious infiltration beneath the skin of the abdomens. The spleens were hypertrophied with dark uncoagulated blood.

Edematous areas on the livers, spleens, hearts and kidneys were detected. While staining these tissues,

regularly encapsulated bacilli were observed as single cells, in cluster or short chains. The spread of blood from guinea heart on solid agar medium showed a colony of B. *anthracis*-like within 24 hours of incubation at 37°C.

All isolates were sensitive to meticillin, tetracycline, nitrofurantoin, sulphatiazol, piperacillin, benzathiene-penicillin, chloramphenicol and oxytetracycline, and resistant to fusidic acid, polymyxin, sisomycine and clindomycin. Intermediate reactions were found against netilmicin, streptomycin, rifampicin, pipemidic acid, cefalotin and sulfamids.

Biochemical characterization of the strains yielded similar results for all isolates with 48 out of 49 tests. Both human and cattle isolates showed negative reaction for Arbutine in contrast to those of tabanids (which tested positive) (Table 3).

Table 1. Distribution of spores and bacilli of *B. anthracis* in different tissues of dissected tabanids(-, neither spores nor bacilli)

Villages from which tabanids were collected	Total and Percent (%)	Number of tabanids examined	Number of tabanids showing spores on their wings and legs		Number of tabanids showing Bacilli in their mouthparts and midguts		Number of tabanids showing neither spores nor bacilli
			wings	Legs	mouthparts	Midguts	
Chilo		824	351	206	24	113	130
Balarye		205	-	49	112	34	10
Abardjil		207	74	-	75	52	6
Ousmanari		97	-	-	56	37	4
Ambague		109	49	52	-	-	8
Kiessa- Cherif		26	-	11	4	9	2
Kiessa-Hassan		31	5	13	11	-	2
	Total	1499	479	331	282	245	162
	Percent (%)	100%	31.9	22.1	18.8	16.3	10.8

Table 2. Biological properties of isolated B. anthracis from humans, cattle and tabanids (-, negative reaction; +, positive reaction)

Biological properties	Reactions		
	Humans	**Cattle**	**Tabanids**
Shape	rod	rod	rod
Capsulation	+	+	+
Gram staining	+	+	+
Mobility	-	-	-
Hemolysine	-	-	-
Pathogenicity	+	+	+
Growth on nutrient broth and aired solid nutrient agar medium	+	+	+
Sensitivity to penicilline	+	+	+

Table 3. Similarities and differences in Biochemical properties of isolated *B. anthracis* from humans, cattle and tabanids, by using different substrates

REACTIONS			
Substrate	Humans	Cattle	Tabanids
D-raffinose	-	-	-
Galactose	-	-	-
Salicine	-	-	-
D-glucose	+	+	+
Maltose	+	+	+
Ribose	+	+	+
Saccharose	+	+	+
Arbutine	-	-	+
Amidon	+	+	+
Glycogene	+	+	+

Only the main differences and similarities between human, bovine and tabanids are indicated: -, negative reaction; +, positive reaction.

4. Discussion

During the past few years, *B.anthracis* has been one of the main bacterial zoonosis reported in Chad. High cattle density and abondant tabanids population present in N'Djamena rural area favour the occurrence of large outbreaks in the province.

The potential role of tabanids in the spread of B. anthracis to humans and domestic animals during an anthrax outbreak has been highlighted in a few studies (Lamarque, 1990; Sirol,1971; Davies, 1985). However vegetative cells or spores have never been isolated from these vectors. Choquette (1983) and Davies (1983) reported that, anthrax spore may spread within a geographic region through insects feeding on infected animals.. Tabanids as well as *Stomoxyis calcitrans*, *Musca domestica*, and *Calliphora erythrocephala* have been evaluated as transmitting agents (De Vos, 1998 and Dragon, 1999).

In this study *B. anthracis*-like strains were isolated from Tabanids and human bitten by tabanids. About 89% of the 1499 tabanids examined were contaminated by both spores and bacilli. *B. anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%). This result suggests that tabanids may play a role in the dissemination of anthrax spores and bacilli (either mechanically or through bite) in a wide area and contribute to the epidemic of anthrax within the Chari-Baguirmi Province of Chad. The infected areas can present a persistent public health risk to surrounding population.The different isolates collected throughout this work from humans, cattle and tabanids were identified as *B. anthracis* strains based on their biological and biochemical properties. They were distinguished from closely related species such as *Bacillus cereus*, *Bacillus pumilus* and

Bacillus stearothermophilus, as they are non motile and show positive reactions to glycogen and starch. They also differed from other non-pathogenic, aerobic spore-former species such as *Bacillus mesentericus*, *Bacillus mycoides*, *Bacillus brevis*, *Bacillus coagulans* and *Bacillus megaterium* which are mobile and haemolysin producer. Curiously, all B. anthracis-like isolates showed similar biochemical properties, except for Arbutine. 20-80% variability in Arbutine profile are reported for *B. anthracis* (Logan, 1984).Although isolates from tabanids tested positive to Arbutine, in contrast to those from humans and cattle, we believe that this discrepancy might be due to some artifacts. However, additional PCR confirmation and typing would be helpful to ascertain the species identification and to point out the route of transmission.

5. Conclusion

To diminish the risk associate with anthrax for humans, in N'Djamena rural area, we recommended wide spread animal vaccination (Marty, 2001).

Fly control should also be considered a part of an Anthrax control program, along with appropriate measures to promptly eliminate infected animals and carcasses. In addition, Health officials should use the set of antibiotics evaluated in this study, as recommended in other studies (Aubry, 1980, Frean, 2003 and Lamarque, 1990).

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Carob Fruit as Source of Carbon and Energy for Production of *Saccharomyces cerevisiae*

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Abstract

Carob (*Ceratonia siliqua L.*) aqueous extracts were prepared from carob pods and kibble (pods minus seeds) particles of 0.5 to 1.0 cm diameter, at 45 °C for 2hr. *Saccharomyces cerevisiae* cells were grown on extracts by fed-batch method at 32 ºC, an optimal pH 5.5, and shaking at 300 rpm for 10 h. The cell yield and yield coefficient were 12.6 % and 0.27 g /g sugar utilized, respectively. Aeration effect and continuous feeding on the yield of *Saccharomyces cerevisiae* were studied on extracts prepared from carob pod powder or from chopped kibble. The biomass yield coefficient was about 0.27 g- yeast/g sugar utilized for both the pod and the kibble extracts. The cells maintained the availability of reducing sugars on the expense of non-reducing sugar present in carob extract; therefore, reducing sugars were maintained at their initial level. However, aeration and continuous feeding increased the yield to 113 %. Instantaneous growth rate constant after 10 h-period was 0.18 h^{-1} which decreased with time. Supplementation of Ca, Mg, N, and P salts to extract in a continuous fed-batch culture did not significantly increase the cell mass above the yield in the control. These nutrients were important in fixed batch culture. This indicates that, in cell division, the carbon source in the extract is not limiting factor, while the above additives become the limiting factor. Comparing the yield of the yeast grown on carob kibble extract with other substrates, previously and currently employed in the industry, reveals that the carob kibble extract is more economical substrate for industrial production of baker's yeast.

Keywords: Baker's Yeast, Carob, Carob Pod, *Ceratonia siliqua.* Yeast, *Saccharomyces cerevisiae*

1. Introduction

Carob tree (*Ceratonia siliqua L.* Leguminosae family) is widely cultivated in the Mediterranean regions, and in areas of North America (Blendford, 1979; Manso *et al.*, 2010). The tree is considered an important flora for economic and environmental reasons (Batlle and Tous, 1997). The world annual production carob fruit is > 315000 tons, which is distributed among Spain (42 %), Italy (16 %), Portugal (10 %), Morocco (8 %), Greece (6.5 %), Cyprus (5.5 %), and Turkey (4.8 %) (Santos *et al.*, 2005). Carob fruit, or pod, consists of kibble (pulp or locust bean) and the seeds, or locust kernel gum. The seeds which contain the polysaccharides, galactomannans, are used in gum production (Davies *et al.*, 1971). The chemical compositions of carob kibble are compiled from various published reports (Yousif and Alghzawi, 2000; Calixto, 1987; Petit and Pinilla, 1995; Avallone *et al.*, 1997) and represented in Table 1. The sugar contents in carob pods, 48 % - 56 %, (Santos *et al.*, 2005; Ahmed, 2001) were reported to be higher than those in sugar cane, 14 % -18 %, (Sugarcane, Wikipedia). However, the profiles of carbohydrates which were determined in g /100 g carob pod were: Fructose at 10.2 – 11.5, glucose at 3.3 – 3.68, and sucrose at 29.9 – 38.4 (Biner *et al.*, 2007).

Table 1. Chemical composition of carob kibble.

Composition	Concentrations g /100g	composition	Concentration mg /100g
Total sugars[a]	45.0±0.30	Potassium[c]	802.00
Tannins[b]	18.5 ±0.20	Sodium[c]	10.10
Ash[a]	2.79±0.22	Magnesium[c]	66.89
Proteins[a]	5.54±0.33	Calcium[c]	440.00
Fats[a]	0.3±0.04	Iron[c]	2.34
Crude fiber[a]	10.99±0.51	Zinc[c]	0.70
		Copper[c]	0.62
		Manganese[c]	0.56
		Manganese[c]	0.56
		Phosphorus[c]	31.58

[a]Tannins were reported at 3.15±0.03 (Yousef and Alghzawi, 2000), [b](Calixto, 1987), [c](Petit and Pinilla, 1995).

These three main sugar components were also reported as 11.6 %, 12.8 %, and 37.2 %, respectively by other investigators (Santos *et al.*, 2005). Tannins in carob pod

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were identified and quantified (Papagianonopoulos *et al.*, 2004), and the chemical structures of major individual polyphenols in carob fiber were studied (Owen *et al.*, 2003). In order to increase the availability of free sugars for the production of protein from carob pod extracts using certain microorganisms, the media were supplemented with ammonium salt. The organisms of interest to produce protein through that process were *Aspergillus niger* (Imrie & Vitos, 1975), *Fusarium moniliforme* (Drouliscos *et al.*, 1976; Macris and Kokke, 1977, 1978), *Rhizopus oligosporus* and *Monascus ruber* (Righelato *et al.*, 1976). Carob pod is used in various processes of food technology, medicine, and other industrial processes. At the industrial level, the pod was employed in the production of ethanol by *Saccharomyces cerevisiae* (Turhan *et al.*, 2010; Roukas, 1995; Roukas, 1994a, b, Roukas, 1993), by *Zymomonaras mobilis* (Vaheed *et al.*, 2011), the production of citric acid by *Aspergillus niger* (Roukas, 1998), the production of mannitol by lactic acid bacteria (Carvalheiro *et al.*, 2011), and the production of biocontrol agent *Pantoea agglomerans* PBC-1 (Manso, 2010). The economical and industrial aspects of carob products were reviewed by (Davies *et al.*, 1971; Ayaz *et al.*, 2009). The nutritional and health beneficial aspects of the carob fruit products have been reviewed (Ayaz *et al.*, 2009; Zunft *et al.*, 2003, 2001; Makris and Kefalas, 2004). Many countries of the third world are carob-cultivating, and rely heavily on bread and pastry products that utilize great amounts of baker's yeast, *S. cerevisiae*. Because of the cheaper price of carob fruit compared to other sources of sugars utilized in the production of yeast, and the higher content of the sugars that are consumable by the yeast than that of the sugarcane, it is highly feasible to utilize carob fruit as source of carbon and energy to produce the yeast, *Saccharomyces,* which is the scope of this study.

2. Materials and Methods

2.1. Materials

Carob fruit and sucrose were purchased from local grocery markets. Anthrone was obtained from Merck, Germany. Bovine serum albumin and sodium potassium tartrate were obtained from British Drug House, UK. Coomassie Blue-G250, ethanol, Folin-Denis reagent, glucose monohydrate, phosphoric acid, and tannic acid were obtained from Fluka, USA. Malt broth and nutrient agar were obtained from HiMedia Laboratories, Ptv. Limited, Bombay, India. Yeast extracts were obtained from Oxoid, England. Bacteriological peptone was obtained from Mast Laboratories, UK. 3,5-Dinitrosalycylic acid was obtained from Sigma, USA. Baker's yeast was obtained from Astrico, Yeast Industries Co. LTD, Jordan. All other reagents were analytical grade, and double distilled water was used when required.

2.2. . Methods

2.2.1. Inoculum Preparation

Pure colony of *S. cerevisiae* was prepared as follows: Dry commercial yeast (one to two pellets) was added to 2 ml of sterile malt broth and shaken in a water-shaker bath for two h at 32 ºC. Then, 0.1 ml of the yeast malt broth was diluted to 10 ml with sterile physiological saline solution. Finally, one drop of diluted yeast suspension was placed on culture medium composed of: malt extract (0.5 %), yeast extract (0.5 %), sucrose (2 %), peptone (0.5 %), agar (2 %) (MYSP), and incubated at 32 °C for 48 h (Campbell, 1988; Ahmed, 2001). Thirty ml of YPS-medium (yeast extract, 1 %; peptone, 2 %; sucrose, 2 %) were inoculated with this colony to produce inoculum for larger cultivation culture.

2.2.2. Preparation Of Carob Extract

Three carob pod extracts (CE) were prepared at different conditions and were used to grow *S. cerevisiae.* The extracts were prepared as follows:

Carob Extract-1 preparation (CE-1). Carob pods were cut into small pieces with particle size of 0.5 to 1.0-cm. The pieces were packed into a column (30-cm x 2-cm). The column was eluted with water at a rate of 2 ml /min using peristaltic pump. The pod to water (p:w) ratio was 1:4 by weight. The eluant, which was centrifuged at 3000 x g and 0 °C for 30 min, was designated carob extract-1 (CE-1).

Carob Extract-2 preparation (*CE-2*). Carob pods were milled to fine powder using Moulinex miller. The powder was mixed with water at a ratio of 1:4 by weight at 45±3 °C for 2 h. The extract, which was centrifuged at 3000 x g and 0 °C for 30 min, was designated carob extract-2 (CE-2).

Carob Extract-3 preparation (*CE-3*). The preparation of CE-3 was similar to CE-2 but kibble (pods minus seeds) with particle size around 0.5 to 1.0-cm was used.

2.2.3. The Effect of Ph On the Growth of S. Cerevisiae

CE aliquots at various pH values (30 mL in 100-mL arm-flask) that contain 2 % sugar (expressed as glucose) were inoculated with the cells. The flasks were shaken at 250 rpm in a water bath at 32 °C for 24 h.

2.2.4. Determination of Water-Extractable Materials

Total protein was determined according to Bradford (1976) using bovine serum albumin as the standard. Tannins were determined by Folin-Denis reagent as described (AOAC, 1990) using tannic acid as the standard. Total sugars were determined by the Anthrone method using glucose as the standard (Graf *et al.*, 1951). The reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). Non-reducing sugars were calculated as the difference between total sugars and the reducing sugars.

2.2.5. Effect of Aeration and Continuous Feeding

Diluted CE-2 (at 28 mmol /L sugar, pH 5.5) was added continuously to 2-L arm-flask using peristaltic pump. The rate of feeding was calculated according to the following equation:

$$F_s = [\mu/y_{xs}+m_s][(C_{xo}.V_o)/C_{si}].e^{\mu t},$$

where F_s, L /h, is the flow rate of the feed medium; μ, μh^{-1}, is the specific growth rate constant; y_{xs}, g cells /g substrate, is biomass yield on the substrate; m_s, g substrate/g cells /h, is maintenance coefficient; V_o, L, is initial culture volume; C_{xo}, g /L, is initial biomass concentration; C_{si}, g /L, is substrate concentration in the

feed; and t, h, is time after starting the feed (Van Hoek *et al.*, 2000). The working volume was 1L. The air was provided by air pump that is connected to the arm of the flask. The rate of air pumping was 2 m^3 /min. Olive oil, 0.13 ml /L, was used as antifoam (Drouliscos *et al.*, 1976).

2.2.6. Cultivation of Cells

Two doses (200 mL each) of diluted CE were added to 2L arm-flask containing 200 ml diluted CE to give 28 mmol /L sugar as glucose equivalent and inoculated with the yeast at 3 %, (3.31±0.16) x10^7 cells /mL of the initial volume. Sugar concentration was maintained at 28 mmol /L. The first dose was added after 3.5 h, and the second dose was added after 7.0 h. The final volume was 600 ml, pH 5.5. The inoculated media were shaken at 32 °C and 300 rpm. Aerobic condition was provided by passing the air through Millipore filter.

2.2.7. Determination of Cell Number and Weight of Yeast

The growth of yeast was monitored by the change in absorbance at 550 nm using Bausch and Lomb spectrophotometer, and by counting the number of viable cells on MYSG-medium. The dry weight of the yeast was determined as follows: Cultivation medium was centrifuged at 2500 x g for 20 min, and 0 °C, and the pellet was washed with saline solution. The yeast was filtered on filter paper and dried to constant weight in an oven at 105 °C for 12 h.

2.2.8. Purity of the Culture

The yeast culture was examined microscopically using methylene blue as stain and bacterial contamination was examined by Gram stain (Benson, 1985).

2.2.9. Bread Preparation

Bread was prepared in two ways: one is using *Saccharomyces cerevisiae* that was produced in this study and the other one was using commercial yeast that was from the local bakeries, which is sold in local grocery markets. The method of preparation was based on that applied in the local bakeries. A flour quantity, 100 g, was mixed with 55 mL water and 1g yeast. The mix was let stand at room temperature for 45 min, cut into small pieces and let stand fort 15 min followed by baking the dough in the oven at temperature used by the bakeries.

2.2.10. Statistical analysis

Data averaging was performed on samples of three or more runs. The standard deviation (±) was calculated from a computer-run program.

3. Results

3.1. Chemical Composition of CE-

The concentrations of reducing and non-reducing sugars, protein, and tannins as g /100g, and % of total sugars extracted are shown in Table 2. The CE-1, CE-2, and CE-3 contained reducing sugars as 2.1, 2.6, and 1.8 g/100 ml, respectively. CE-2 and CE-3 have essentially equal concentration of non-reducing sugars, 12.5 and 14.6 g /100ml, respectively. However, the total sugar contents, as g /100 ml, were 23.2, 17.7, and 16.4, in CE-1, CE-2, and CE-3, respectively. Protein and tannin contents were low and almost equal in these extracts, except CE-2 showed 0.11g / 100mL, which is high if compared to the other values.

Table 2. Protein, sugar, and tannin contents in carob extracts (CE) prepared under variable conditions. The values are g /100ml extract.

	CE1	CE2	CE3
Total sugar	23.2±4.5	17.7±1.9	16.4±0.4
Reducing sugar	2.1±0.2	2.5±0.1	1.8±0.0
Non-reducing sugar	21.1±4.3	12.5±1.8	4.6±0.4
Protein	0.0±0.0	0.06±0.0	.02±0.0
Tannin	0.06±0.0	0.11±0.14	.07±0.0

3.2. Growth Kinetics of S. cerevisiae on CE.

The growth kinetics was studied by following the increase in turbidity at light wavelength of 550 nm which is related to cell growth. At the same times, changes in the levels of reducing and non-reducing sugar were measured. The pattern of growth and the consumption of reducing and non-reducing sugar using CE-1, CE-2, and CE-3 media are presented in Figure 1. The non-reducing sugar values were deduced from the total and the reducing sugars values were determined experimentally (see Methods). In this study, the cells were grown in the fed-batch culture. CE was added to maintain initial sugar concentration at ~ 28 mmol /L as glucose at time intervals, 3.5 h each.

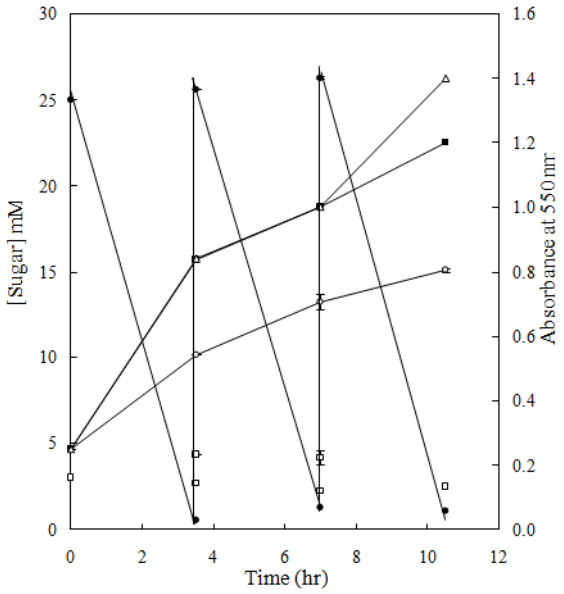

Figure 1. Yeast growth kinetics on CE. S. cerevisiae cells were grown in CE fed-batch medium, at pH 5.5, 32 °C, and shaking at 300 rpm for 10 h. At 3.5 h intervals (i.e. after 3.5 h and 7 h) two CE doses of each extract were added to maintain sugar concentration at ~ 28 mM . -□- Reducing, -●- non-reducing sugar, A550nm cell growth -○- : on CE-1, -■- on CE-2, -Δ- on CE-3.

Instantaneous growth rate constant (μ_i, h^{-1}), biomass yield (Y_{Ns} CFC /g sugar utilized), cells biomass yield on substrate ($Y_{x/s}$, g yeast /g sugar utilized), and specific biomass production rate (q_x, CFC /g sugar utilized /h) are presented in Table 3. The level of reducing sugar was

maintained at ≅ 3 mM, while non-reducing sugar level decreased to almost zero at the end of the interval period. The yield of cellular mass values were 0.04, 0.15, and 0.27 as g of dry cells per one g sugar utilized using CE-1, CE-2, and CE-3, respectively (Table 3).

Table 3. Kinetic parameters of yeast growth on carob extracts (CE) prepared under variable conditions (for abbreviation in the table, see footnotes below).

	CE1			CE2			CE3		
Time, h	3.5	7	10	3.5	7	10	3.5	7	10
μ_i, h^{-1}	0.22	0.22	0.21	0.53	0.28	0.19	0.4	0.24	0.06
Y_{Ns}[a]	-	-	0.53	-	-	1.46	-	-	3.7
q_x[b]	-	-	0.52	-	-	1.46	-	-	3.7
$Y_{x/s}$	-	-	0.04	-	-	0.15	-	-	0.27

Abbreviations: CFC is colony forming cells, instantaneous growth rate constant (μi, hr^{-1}), biomass yield (YNs CFC/g sugar utilized), cells biomass yield on substrate (Yx/s, g yeast/g sugar utilized), and specific biomass production rate (qx, CFC/g sugar utilized/hr). [a]The values are multiplied by 10^{-9} i.e. number of colony-forming cells is $x10^9$).[b]The values are multiplied by 10-8 (i.e. colony forming cells is $x10^8$)

Apparently, the parameter μ_i was constant at 0.22 h^{-1} for CE-1 at times of growth, 3.5, 7, and 10 h. For CE-2 and CE-3, the values were 0.53 h^{-1}, 0.28 h^{-1}, and 0.19 h^{-1} and 0.4 h^{-1}, 0.24 h^{-1}, and 0.06 h^{-1}, respectively.

3.3. Effect of Ph on S. Cerevisiae Growth on CE

The pattern of yeast growth on CE-2 at pH range of 3 to 7 was studied. The sugar level was kept at 2 %. The profiles show an optimal pH for yeast growth at 5.0 to 6.0 (figure is not shown).

3.4. Effect of Aeration and Continuous Feeding on Yeast Growth

The effect of aeration and continuous feeding of CE on cell growth is presented in Table 4. The biomass yield was, somehow, improved by 113.2 %. Typical results of such experiments are presented using CE-2. Table 2 shows the reducing sugar concentration increased and non-reducing sugar decreased with increasing feeding rate. Also, it is observed that reducing sugar concentration was maintained around 3 mM. The values of μ_i at 2, 4, 6, 8, and 10 h were 1.41 h^{-1}, 0.502 h^{-1}, 0.369 h^{-1} , 0.308 h^{-1}, and 0.184 h^{-1}, respectively. $Y_{x/s}$, q_x, and Y_{Ns} were $6.37x10^8$, $6.37x10^7$, and 0.27, respectively, at 10 h growth period.

Table 4. Aeration effect on parameters of yeast growth on continuous feeding of CE-2. Aeration rate was $2m^3$ / min, and feeding flow rate was 26 mL /h. The cellular growth was at pH 5.5, 32 °C, and shaking at 150 rpm (abbreviations of parameters are the same of those in table 3).

Time, h	2	4	6	8	10
μ_i, h^{-1}	1.41	0.502	0.369	0.308	0.184
Y_{Ns}[a]	-	-	-	-	0.637
q_x[b]	-	-	-	-	0.637
$Y_{x/s}$	-	-	-	-	0.27

[a]The values are multiplied by 10^{-9} (i.e. number of colony-forming cells is $x0^9$).

[b]The values are multiplied by 10^{-8} (i.e. number of colony-forming cells is 10^8).

The effect of growth limiting nutrients (i.e. Ca, Mg, N, P, and S as the salt forms) that may be depleted before the depletion of the carbon source is presented in Table 5 which summarizes the yield (g dry cells /100g carob kibble) of yeast grown on the carob extracts prepared under various conditions. The yield from CE-1, CE-2, and CE-3 were 2.0±0.2, 7.2±0.6, and 12.6±1.8, respectively. These values were obtained from cultures without aeration and continuous feeding. Up on aeration and continuous feeding, the yield increased by 20 % (15.4±2.2) for CE-3. The additives increased the yield to the level obtained from the aeration and continuous feeding (Table 5).

Table 5. The yield of yeast (g of dry cells /100g carob kibble) using CE prepared by different procedures, and the effects of certain exogenous nutrients added to the extract.

Extract	Yield
CE-1	2.0±0.2
CE-1[a]	11.9±0.6
CE-2	7.2±0.6
CE-2[a]	12.6±0.1
CE-3	12.6±0.8
CE-2, aeration, continuous feeding	15.4±2.1
CE-3[a]	12.4±.1.2

[a] plus 22.7 mM $(NH4)_2SO_4$ + 13.2 mM KH_2PO_4 + 0.9 mM $MgSO_4$ + 0.1 mM $CaCl_2$

3.5. The bread which was prepared using the produced yeast in this study and that prepared using commercial yeast for leavening the dough were tasted by seven persons. Five persons said that there is no difference in the two types of bread, and two persons said that the bread prepared using CE yeast had a better taste and a distinct flavor.

4. Discussion

4.1. Procedure to Prepare CE

In this study, the best procedure for preparation of CE from carob kibble was to mix kibble particles of 0.5-1.0 cm diameter in size with water in 1:4 p:w ratio for 2 h at 45 °C. This was based on information in the literature and this work. The CE-1 and CE-3 extraction methods were mainly developed to reduce the extraction of tannins. It was suggested that tannins have an inhibitory effect on yeast growth; although tannins have antioxidant property by their antiradical-scavenging effect (Yoshida *et al.*, 1989). Since the smaller the size of the particle the larger the extractable material, CE-1 lacks a number of nutrients such as nitrogenous substances. The protein in carob is associated with tannins (Calixto, 1987). Thus, CE-1 lacks the protein that is important as nitrogen source for yeast growth. Therefore, for this reason preparation procedure of CE-2 was developed. Applying temperature at 45°C improved the extractability of nutrients. Hence, a combination between CE-1 and CE-2 was designed to give CE-3 that has lower tannins level than CE-2 and more nutrients than CE-1.

CE-1 contained the highest sugar level among the carob extracts obtained in this study, and this may be explained in two ways. In one way, heating during preparation of CE-2 and CE-3 causes degradation of some sugars. In another way, tannins, proteins, and other nutrients are present in higher concentrations in CE-2 and CE-3 than in CE-1. These nutrients may compete with sugars for water in the extraction process. Because degradation of sugars starts at temperature above 50 °C (Mulet *et al.*, 1988) and CE-2 and CE3 have higher concentration of protein and tannin (Table 2), it was suggested that the nutrients - water competition assumption is more reasonable. Tannins concentration was lower in CE-1 and CE-3 than in CE-2. This is because tannins in carob are in granules (Mulet *et al.*, 1988). When the particle size is small the granules are degraded, and this event frees tannins.

4.2. Growth Kinetics of S. Cerevisiae on CE

The growth of yeast gave the highest yield on CE-3. This might be due to higher content of nitrogen source and lower tannin concentration compared to CE-1 and CE-2, respectively. Furthermore, *S. cerevisiae* consumed sugars from CE-1, CE-2 and CE-3 in the same pattern. Apparently, nonreducing sugars decreased to zero level, while reducing sugars remained around 3 mM. It means that 10% of the sugars remained not consumed. This observation is in agreement with (Roukas, 1993). Also, this may suggest that consumption of reducing sugars becomes concentration-dependent >3 mM. This was seen in the growth abrupt upon the addition of CE. Alternatively, accumulation of reducing sugars could be explained in another way. Sucrose, the main sugar present in carob, is converted by the yeast to fructose and glucose. So in order to consume all reducing sugars, it may require longer time than 3.5 h. Since carob has small concentrations of free glucose, fructose, xylose, and others (Calixto, 1987), the accumulation of the reducing sugars could be due to a combination of reasons. Furthermore, previous studies showed that *S. cerevisiae* cannot utilize xylose because it lacks the enzyme xylose isomerase (Gong *et al*., 1981). Xylitol and penititol are poor carbon source for yeast due to its limited permeability into cells (Singh and Mishra, 1995).

The parameter μ_i decreased within 3.5 h-interval, and this was in agreement with reported values. The yield coefficient was highest with CE-3 because it might be of low tannins and high protein contents.

S. cerevisiae growth parameters obtained from batch versus continuous feeding media of CE were lower in batch. Y_{xs} and q_x were 1.8 x 10^9 CFC /g sugar utilized and 5 x 10^7 CFC /g sugar utilized /h, respectively, in batch-culture (Roukas, 1993). The yield coefficient for molasses and sorghum hydrolysates was found 0.28 (Reed and Nagodawithana, 1991; Konlana *et al.*, 1996), which is comparable to 0.27 for CE-3 in this study. Molasses yield coefficient could not be compared with that for CE-3 because molasses yield coefficient was under optimal condition of nutrient supplements, that requires further investigated on carob fruits.

4.3. Effect of pH

The optimal pH for growth of baker's yeast was examined in batch-culture for 24 h at 32 °C. It appears that at pH values, 5.0 to 6.0, the yeast has the same growth rate. This indicated that the optimal pH for yeast growth under the conditions of this study is between 5.0 and 6.0. This range of pH value is consistent with that for molasses (Reed and Nagodawithana, 1991) and for whey (Champagne *et al.*, 1990).

4.4. Effect of Aeration, Continuous Feeding, And Ph

Aeration and continuous feeding increased the biomass yield. Under strict aerobic conditions the best yield was 54 g yeast solid per 100 g of glucose (Reed and Nagodawithana, 1991). So if carob kibble has 50 % sugar, it suggests that the maximum yield would be 27 %. The obtained result, 26 %, is essentially the theoretical value. The parameter μ_i (0.18) after 10 h is in agreement with van Hoek *et al.*, 2000, equation. This high value indicates that ethanol essentially was not produced. Its production would lower the availability of carbon source for cell growth.

Therefore, the optimal method to prepare CE was to use carob kibble with particle diameter between 0.5-1.0 cm, and 1:4 p:w ratio for 2h. Accordingly, continuous (column extraction) and discontinuous methods both gave the same results. But, to automate the carob extraction for large-scale production, continuous extraction may be the method of choice (Petit and Pinilla, 1995).

Comparing carob with other substrates used for baker's yeast production, carob gave equal biomass yields in some cases, and higher values in others.

The seeds of carob pods contribute more than 60 % of the pod market price (Makris and Kefalas, 2004) and carob kibble, that is left, mostly, is thrown away as waste. Thus, carob kibble is a cheap substrate for production of baker's yeast. The present results point out that carob kibble is an excellent economical substrate for industrial production of baker's yeast. The production of yeast from carob fruit may be another motive to enhance the cultivation of vast areas of land that are suitable for forestry.

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