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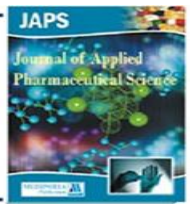

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White Bean Flour (*Phaseolus vulgaris*): Therapeutic and Toxicological Research in Wistar Rats

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ABSTRACT

The objective was to evaluate the possible therapeutic and toxic effects resulting from administration of white bean flour (WBF) in Wistar rats for 21 days. The assay was performed to assess the consequences of using WBF crude, at a the concentration corresponding to the recommended daily dose to cause weight loss and hypoglycemic effect, which is 1,000 mg of phaseolamin/day. The administration of WBF at 1 mg/g body weight in Wistar rats with diabetes induced by STZ in a period of 21 days, did not alter physiological parameters (consumption of food and water, urine volume), biochemical markers (total cholesterol, HDL cholesterol and triglycerides), liver enzymes (gamma-GT, AST and ALT), and weight of organs (liver and pancreas) as well as fecal excretion of proteins and lipids. An increased excretion of carbohydrate fecal confirms the results of inhibition of α-amylase. Despite the tendency to decrease blood glucose from 20 days of treatment, there was no significant reduction during the period evaluated in the treated group. It can be concluded that despite the decreasing tendency of hyperglycemia and possibly aid in weight loss by decreasing absorption of carbohydrates in the diet, high doses of WBF may have deleterious effects on the body resulting from chronic use.

Keywords: Diabetes, phaseolamin, enzymatic inhibition, white bean flour.

INTRODUCTION

The term "low-carb" has become part of the current vocabulary, and the number of foods low in carbohydrates and dietary supplements with starch blockers for the weight loss increased dramatically in recent years. Starch blockers are used in order to promote weight loss and decreased blood glucose, interfering with the digestion of carbohydrates by inhibiting α-amylase, a digestive enzyme responsible for breaking down complex carbohydrates (like starch) into simple sugars, which can then be absorbed in the small intestine. The end result is a potential reduction in calories, carbohydrate availability and derivatives (Chokshi, 2006). The white bean has been considered the best source of an α amylase inhibitor, also known as phaseolamin. Extracts of bean (*Phaseolus vulgaris*) as well as some of its individual components have been reported by the effects in reducing appetite and body weight and blood glucose in rats (Fantini *et al.*, 2009). Studies have reported that repeated daily administration of raw white bean extract markedly reduced the daily food intake in rats with access to a starch-enriched diet, an effect that is associated with a reduction in body weight gain, and a steady reduction of glucose (Tormo *et al.*, 2006, Fantini *et al.*, 2009).

For their properties, white bean was placed on most wanted list for those who want to lose weight and facing a restricted diet or have high blood sugar. However, besides of α-amylase inhibitors, raw beans has a variety of antinutritional factors and potentially toxic substances that can cause a reduction in feed efficiency and histopathological changes (Chokshi, 2006). In this context, the objective was to evaluate the possible therapeutic and toxic effects resulting from administration of white bean flour in rats with streptozotocin-induced diabetes in the period of 21 days. The biological assay was performed to assess the consequences of using white bean flour (WBF) at a the concentration corresponding to the recommended daily dose to cause weight loss and hypoglycemic effect, which is phaseolamin 1000mg / day.

MATERIALS AND METHODS

The evaluation of the efficacy and toxicity of white bean flour (WBF) was performed by means of biological tests with rats, conducted at the Animal Laboratory of Physiology and Pharmacology, Department of Veterinary Medicine, Federal University of Lavras (UFLA).

Material

The biochemical analysis were performed with the Veterinary Automatic Biochemical Analyzer thermoplate. The blood glucose was determined weekly using reactive strips and the Accu-Chek Active®. For analysis of glucose, amylase, total cholesterol, HDL cholesterol, triglycerides, AST, ALT and Gamma-GT were used commercial kits Labtest ® (Brazil).

White bean flour (WBF)

The shell grains were washed with distilled water, dried in an oven with air circulation at 30 ° C to constant weight and then ground to obtain a flour of about 60 mesh. The flour obtained was stored in tightly closed container, protected from light until the time of use.

Animals

The animals used were albino Wistar rats, SPF (Specific Pathogen Free) provided by the Biotherium of Federal University of Lavras (UFLA). The temperature of the bioterium was maintained at 21 ± 2 ° C with automatic light-dark periods of 12 hours. The animals were fed a diet (chow species, Nuvilab ® CR1) and water ad libitum. The experiment lasted 21 days, counted from the confirmation of the induction of diabetes. Throughout the experiment the animals were in individual metabolic cages. The care and food hygiene were performed daily by single handler. The project was submitted prior to the assessment of the Bioethics Committee of the Dean of the Federal University of Lavras, under protocol n ° 037/2010.

Study of efficacy and toxicity

Biological assay was conducted for 21 days to evaluate the efficacy and subchronic toxicity of WBF. In the planning and execution of the research were adhered to the recommendations of the Brazilian College of Animal Experimentation (COBEA) and Resolution RE 90 of Anvisa, which determines that the publication of a guide for conducting toxicity studies pre-herbal clinic.

Experimental procedures

Induction of diabetes

We used 20 rats divided into two groups - control and treated - both diabetics. Diabetes was induced with a solution of streptozotocin (STZ) Sigma ® at a dose of 60 mg / kg dissolved in sodium citrate buffer 0.01 M pH 4.5 at the time of intraperitoneal inoculation. After 15 days, blood glucose was measured to exclude possible early spontaneous reversals of induced diabetes mellitus.

Management of treatment

After induction of diabetes and the adjustment period, animals were weighed and divided into two groups of ten animals each, separated according to the treatment to be administered by gavage: Group 1 (control) - water and Group 2 (treated) - WBF. The administration was performed daily by gavage using stainless steel tube and syringes at the same time, by the same individual, respecting the maximum volume of 1 mL per animal. The WBF was given daily in the treatment group, diluted in water by gavage at a dose that contains the amount of inhibitory protein α-amylase (phaseolamin) recommended for humans (1g/day). Order to calculate the dose, was considered the protein content of 13% in the WBF (Pereira *et al.*, 2010). The content of α-amylase inhibitor (phaseolamin) is from 9 to 11% of the proteins of the WBF (Obiri *et al.*, 2008). Considering the daily recommendation for phaseolamin, 1 gram / day and an average body weight of 70 kg adult human individual, can calculate the dose per gram of body weight, equivalent to 1 mg g-1 body weight of the animal. To this end, the solution of WBF in the treated group was administered daily prepared at a concentration of 0.2 g mL-1, and the volume administered in accordance with the calculated weight of each mouse. Thus, each animal was administered by gavage, a volume of sample solution corresponding to the calculated dose as a function of their body weight measured daily. The same procedure was performed for the control group which was given only water.

Data Collection

The weights of the animals, as well as feed and water consumption (difference between the quantity supplied and the amount remaining after 24 hours) were recorded daily for assessment of parameters such as weight gain, growth curve and change in appetite and thirst. It also measured the volume of urine excreted and the dry weight of feces that were collected. In feces, we determined the factors of moisture, protein (% N x 6.25), lipids, and carbohydrates. The food efficiency ratio (FER), were calculated by the direct relationship between the average weight gain and feed intake by the groups. Weekly, the same day and time, the glucose levels were measured and blood collected by the technique of amputation of the tail. After completing 21 days of experiment, the animals were fasted for 12 hours prior to sacrifice,

which was carried out prior anesthesia with thiopental 25mg/kg intra venous (IV), followed by exsanguination by cardiac puncture. After sacrifice, necropsy was also performed with removal of internal organs (heart, liver, kidneys, pancreas and duodenum), preparation of histological slides and weighing of the liver and pancreas.

Laboratory testing of blood

Using the collected blood from the heart, laboratory measurements were performed for evaluation of biochemical or metabolic parameters (glucose, total cholesterol, HDL cholesterol, triglycerides and gamma-GT, ALT, AST and amylase). All tests were performed at the Laboratory of Physiology and Pharmacology, Department of Veterinary Medicine, Federal University of Lavras.

Laboratory testing of feces

The feces of control and treated groups were collected from the 2nd to the 21th day. Samples from each group were diluted in water to measure pH. The rest of the feces of both groups, was dried at 50 ° C until constant weight and then ground in a knife mill (TE 631 Tecnal) to obtain homogeneous powders, which was used to determine the levels of lipids, proteins and carbohydrate. The crude protein content was determined by semi-micro Kjeldahl, according to AOAC (1995), becoming the total content of N protein by using the factor 6.25. The lipid content was determined by extraction with methanol and chloroform, according to the method described by Folch et al. (1957), and carbohydrates were determined by anthrone method proposed by Trevelyan & Harrison (1952).

Histopathology

For histopathological analysis, histological slides were prepared from fragments of organs removed at necropsy. In the histological processing, the organs were cut into small pieces, fixed with buffered formalin within 24 hours and then stored in 70% alcohol for preservation until the procedure for inclusion. Subsequently, the fragments were processed for inclusion in paraffin blocks, then, were submitted to microtomy, resulting in cuts of about 3 mm thick. The sections obtained were then stained with hematoxylin-eosin (HE) and mounted on slides / glass slides for histopathologic examination with light microscopy.

Experimental design and statistical analysis

The experimental design was completely randomized with two treatments (control and treated) in 10 repetitions. For data processing, analysis of variance was performed and, for comparison of means was used Scott-Knott test at 5% probability. The results were subjected to analysis of variance, using the software SISVAR (Ferreira, 2000).

RESULTS AND DISCUSSION

Induction of diabetes and hypoglycemic effect

The operational definition used in the diagnosis of diabetic state was, weight loss associated with two blood glucose levels above 400 mg / dl (22.1 mmol / L), 14 days after intraperitoneal injection of STZ (Delfino *et al.,* 2002). The most relevant clinical signs were observed in these animals: polyuria, weight loss and changes in the coat, common in animals with elevated blood glucose levels (Lerco *et al.,* 2003). After induction the 20 animals were divided into two groups with good homogeneity regarding blood glucose levels at time zero (immediately before treatment).

Regarding the severity of hyperglycemia observed after intraperitoneal administration of 60mg/kg of STZ, there was a significant increase in blood glucose ranging between 397 and 487 mg / dL. Similar values were obtained with the same procedure by other authors (Delfino *et al*., 2002, Santos Junior, 2006, Rodrigues *et al.*, 2010).

Fasting blood glucose checked on the last day of the experiment was 488 ± 64 mg / dL in the control group and 492 ± 19 mg / dL in the treated group. There was no reduction in hyperglycaemia in the treated group. However, it is noteworthy that the hypoglycaemic effect of phaseolamine refers to postprandial glycemia, as the α-amylase inhibitor interferes spot in the digestion of carbohydrates from the diet, without the need for absorption into the bloodstream to exert their share.

The mean blood glucose groups at baseline and after 7, 14 and 21 days was 424, 450, 464 and 487mg/dL in the control group and 397, 447, 460 and 412mg/dL in the treated group, respectively. Thus, after 21 days decreased by 15.4% in blood glucose levels in the treated group compared to control Despite the trend toward a decrease in blood glucose from 20 days of treatment, no significant decrease during the study period in the treated group. A longer period of assessment would be necessary to obtain more conclusive data. However, the state of health prevented the observation for a longer time.

Physiological parameters

In Figure 1 shows the growth curve of control and treated animals, every 3 days during the entire period of the experiment.

It was found that the groups, control and treated, not significantly different ($p > 0.05$). However, given the high blood glucose levels of animals, weight gain was impaired, even in the control group.

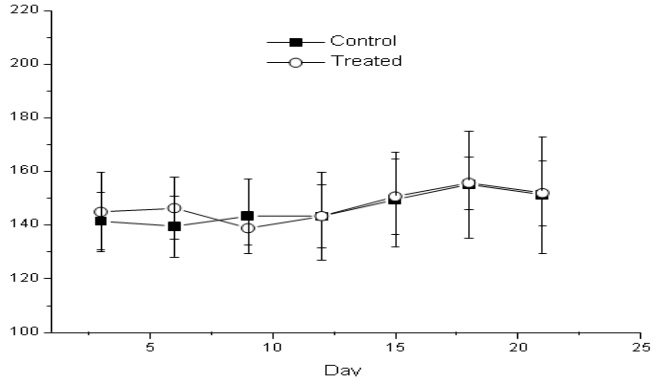

Fig. 1: Growth curve of rats during the 21 days of experiment. The points represent the mean ± standard deviation of the mean weight of the control group and treated every 3 days of experiment. There was no significant difference between the groups evaluated all week.

Table. 1: Average daily consumption of food, water, urine volume, and food efficiency ratio during the 21 days of the experiment for the control and treated groups.

Parameters	Control	Treated	CV
Average consumption of food (g day $^{-1}$)*	32,10±2,5c	29,10±1,9c	10,1 1
Average water consumption (mL day $^{-1}$)*	93,82±6,8a	100,76±10,6a	9,65
Urine volume (mL day $^{-1}$)*	76,20±7,5b	72,60±4,9b	8,86
Coefficient of feed efficiency	0,34±0,02 d	0,22± 0,01 e	7,59

* Values are the mean of each animal per day during the 21 days of experiment.
* Values with the same letters do not differ in level of 5% significance by Scot Knott test.

The performance of experimental animals in relation to the average consumption of water, food, feed efficiency and average daily volume of urine is summarized in Table 1. In relation to the consumption of diet, was not observed, effect of administering white bean flour, since the daily intake of food by control and treated groups was statistically the same. Additionally, there was no change in daily water consumption and urine volume between the groups. Fantini *et al.*, 2009, on the other hand, had decreased body weight, blood glucose and food intake in rats. However, such results were due to administration of 500 mg / kg of dry extract of *P. vulgaris* once daily for 10 days, not the white bean flour used in our study.

Table 2 contains results of pH, and moisture content of carbohydrate, lipid and protein in the feces excreted by animals throughout the experiment.

Table. 2: pH, moisture and proportion (%)* of nutrients in dried feces of control and treated groups.

Parameters	Control	Treated	CV
pH	6,93±0,2 a	7,11±0,4 a	4,86
Moisture (%)	62,5±1,9 b	64,4±3,5 b	8,63
Carbohydrate (mg.g^{-1})	24,26±2,4 c	29,04±2,8 d	15,95
Lipid (mg.g^{-1})	46,04±6,2e	45,21±3,9 e	18,58
Proteín (mg. g^{-1})	32,25±1,5 f	33,37±1,4 f	4,33

* Values with the same superscript letters do not differ in the 5% level of significance by Scot Knott test .

There is no difference between the treated group and the control of pH, moisture, and the total quantities of lipid and protein. The excretion of carbohydrates was higher in the treated group, reinforcing the evidence of inhibition of α-amylase in vitro with decreased absorption, and consequent increase in the excretion of carbohydrates in the faeces. The increased excretion of carbohydrates may explain the lower feed efficiency of the treated group compared to control (Table 1). In long-term, this inhibition may aid in weight loss by decreasing the availability of calorie intake from carbohydrates and decreased postprandial glycemia in diabetic subjects. It should be noted that an affective action requires a balanced diet and physical activity daily. Surveys have shown that different cultivars common bean varies widely as to the presence of heat-stable trypsin inhibitors, and that the disulfide bonds between cysteines contribute to the stabilization of protein tertiary structure and therefore there is greater thermal stability of these inhibitors (Lujan at al. 2008). However, despite the high in vitro inhibition of trypsin, the amount of protein excreted in the feces did not differ significantly between the control and treated groups. It can be suggested that the inhibition of trypsin in the duodenum may induce a greater production of this enzyme by the pancreas, in order to keep intact protein digestion. The protease inhibitors accelerate the secretion of enzymes by a mechanism of "feedback" through the cholecystokinin (CCK). Normally, the amount of enzymes secreted by the pancreas is regulated by protein content existing in the intestinal lumen. Trypsin binds to proteins until it is too much, and when this happens, the free trypsin sends a signal to the pancreas to reduce the synthesis of trypsinogen. But when the inhibitor binds to trypsin, the secretion of trypsinogen by the pancreas is greater. This results in hypertrophy of the pancreas, a reversible biological response does not cause damage to the organ or its function (Duarte *et al.*, 2010). Until recently no one knew how the inactivation of trypsin inhibitor stimulated the production of CCK. It has been isolated from a rat pancreatic juice "monitor peptide", which is sensitive to trypsin. This peptide acts as a signal to release the hormone CCK in the intestine. The inactivation of this peptide by trypsin leads to blocking the release of CCK, however, when trypsin is complexed with the inhibitor, the peptide is free to induce the release of that hormone, whose effect is the increase of the pancreas with consequent increase in the secretion digestive enzymes (Duarte *et al.*, 2010).

Biochemistry

Table 3 presents the results of some biochemical indicators measured in the blood of animals under study.

Table. 3: Average blood levels of total cholesterol[1] and fractions in control and treated groups.

Indicators	Control	Treated	CV
Colesterol (mg dL^{-1})	66,6±8,7	59,8±10,2	15,39
Triglycerides (mg dL^{-1})	48,2±14,2	49,4±13,2	28,42
HDL (mg dL^{-1})	31,3±6,4	28,1±4,36	19,03
VLDL + LDL (mg dL^{-1})	35,3±7,5	31,7±7,2	17,3

1 Data are the mean ± standard deviation. There was no significant difference between groups.

The measurement of blood lipids and liver enzymes provides important parameters in determining the safety of functional ingredients or final products derived from plants tested for toxicity (Patel *et al.*, 2008). Elevated levels of total cholesterol and triglycerides are directly associated with the prognosis of risk of coronary atherosclerosis, ischemic heart disease and stroke, while HDL cholesterol is protective against these disorders. The results of measurements of total cholesterol, triglycerides and HDL cholesterol were statistically the same for treated and control groups and indicate that the WBF did not interfere with lipid metabolism, being devoid of any effects related to changes in rates of blood lipids, whether beneficial or deleterious. However, it can be observed (Table 3), which although not significant, there was a 10% decrease in serum cholesterol in the treated group. Similarly, we found that a diet of beans White Gold administered to adult Wistar rats for 28 days, decreased 7% serum cholesterol, this difference also did not show significant (at Lujan al., 2008). The cholesterol lowering effect of beans has been reported and, although the mechanisms of action are not yet fully understood, suggests the possible effects of soluble fiber, saponins, tannins and proteins. (Lujan at al., 2008).

Enzyme markers

Liver enzymes

Liver enzymes commonly included in serum chemistry screening profiles are alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (gamma-GT). The results of measurements of these enzymes in the blood of animals after 21 days of the experiment are shown in Table 4.

Table. 4: Average blood levels[1] of enzyme markers in control and treated groups.

Indicators	Control	Treated	CV
Gama-GT (U L^{-1})	220,1±13,4a	220,9±13,3a	6,06
Amylase (U L^{-1})	503,8±153,4b	295,9±60,8c	32,44
ALT (U L^{-1})	176,1±40,5d	158,06±31,5d	24,06
AST (U L^{-1})	159,9±31,0e	127,5±25,4e	28,97

1 Data are the mean ± standard deviation.
Values with the same letters do not differ in the 5% level of significance by Scot Knott test.

The elevation of the serum-enzyme levels attributed to liver dysfunction may result from rupture of hepatocytes, resulting in necrosis or changes in cell membrane permeability (Kaneko, 1989). Damage or destruction of liver cells release transaminases into the circulation. ALT (SGPT) is found mainly in the cytoplasm of hepatocytes, while 80% of AST (SGOT) are present in the mitochondria. This difference has aided in the diagnosis and prognosis of liver diseases. In mild hepatocellular injury, the predominant form in serum is cytoplasmic, whereas in serious injury, a release of the mitochondrial enzyme, raising the ratio AST / ALT (Motta, 2009). Based on the data in Table 4, shows that the values of AST, ALT and amylase were statistically the same for both groups. This finding suggests that there was no damage to the liver and pancreatic functions in accordance with the biochemical parameters analyzed. Also in relation to the enzyme gamma-GT, there were no significant differences between the groups. The gamma-GT has principal application in the study of hepato-biliary disorders, with greater specificity than other enzymes, such as alkaline phosphatase and transaminases (Motta, 2009). Thus, the results of gamma-GT suggests positively that WBF has no hepatotoxic effect (Table 4). The elevation of serum gamma-GT observed in the two groups is related to diabetes. Studies show that, for reasons not well established, diabetic patients present high activity of gamma-GT (Arza *et al.*, 2009).

Pancreatic enzyme

The serum amylase, in turn, shows significant variation between groups, being lower in the treated group (Table 4). The amylase is a protein molecule involved in the breakdown of dietary starch and glycogen into maltose, with clinical application in the diagnosis of acute pancreatitis. Considering that the pancreas is the organ most frequently affected by antinutritional factors present in raw white bean flour, there was an expectation that any damage to the pancreas could reflect elevated serum amylase in the treated group, which did not occur at all. However, the correlation between damage and pancreatic hyperamylasemia must be carefully considered. A study of induction of moderate and severe pancreatitis in rats, it was observed that amylase values were significantly lower in rats with pancreatitis group compared with the control, and returned to baseline levels within 2 weeks, following regeneration pancreatic, as evidenced by histological patterns in this period of evolution. Thus, the significant reduction in the synthesis of pancreatic enzymes by damage to the cells of the pancreas, may result in decreased plasma levels (Ramos *et al.*, 2005). These findings allow us to infer that the plasma amylase levels do not correlate directly with the severity of the injury. The inverse correlation between this parameter and the severity of pancreatic injury was reported and attributed to the decline in secretory capacity when the pancreatic cell damage is severe. This analysis is consistent with the observation that higher levels of serum amylase are found in experimental models of pancreatitis characterized by interstitial edema and minimal cell death. Pancreatic edema, which is not indicative of severity, showed a better correlation with hyperamylasemia, and the usual indices for the diagnosis of severe pancreatitis, acinar necrosis as inappropriate or even correlate inversely with the level of serum amylase (Ramos et al. , 2005). In a study evaluating the performance and serum parameters of rats fed diets containing raw soybean, the serum amylase showed significant variation between the experimental groups, being lower in the treatment with 100% substitution of egg white protein by soy protein (Campello *et al.*, 2009). In this case, the authors attributed the absence of hyperamylasemia with maintenance of pancreatic integrity of the structure without triggering the inflammatory response. In contrast, in our studies, we suggest that the significant decrease in the level of serum amylase in the group treated with WBF, may be related to damage to cell structure by hypersecretion of pancreatic trypsin possibly to compensate for the inhibitory action of this enzyme. We agree that although it should consider the difficulties in estimating the percentage of extent of tissue necrosis, low levels of serum pancreatic enzymes suggest significant necrosis of acinar cells (Ramos *et al.*, 2005).

Weight of organs

The main anti-nutrients found in beans affect the cells of the pancreas and liver may cause hyperplasia and hypertrophy of these organs. Thus, the weight of these organs in animals treated compared to untreated was used as a parameter to check for anatomical abnormalities. There was no difference between control and treated groups. The average weight (g ± sd) of the liver was 0.5 ± 6.12 and 6.39 ± 1 and the pancreas: 0.11 ± 0.04 and 0.12 ± 0.03 for the control and treated groups, respectively.

Histological examination

Figure 2 shows microscopic images captured from the organs submitted to histological processing. There were no macroscopic changes in organs analyzed by dissection: kidney, intestine, pancreas, heart and liver. The histological processing occurred similarly in all groups. For each organ / animal / group, we performed a most significant area of photomicrography. There were no injuries or significant microscopic changes (hematoxylin-eosin, HE) among animal tissues evaluated between the control and treated groups (Figure 2).

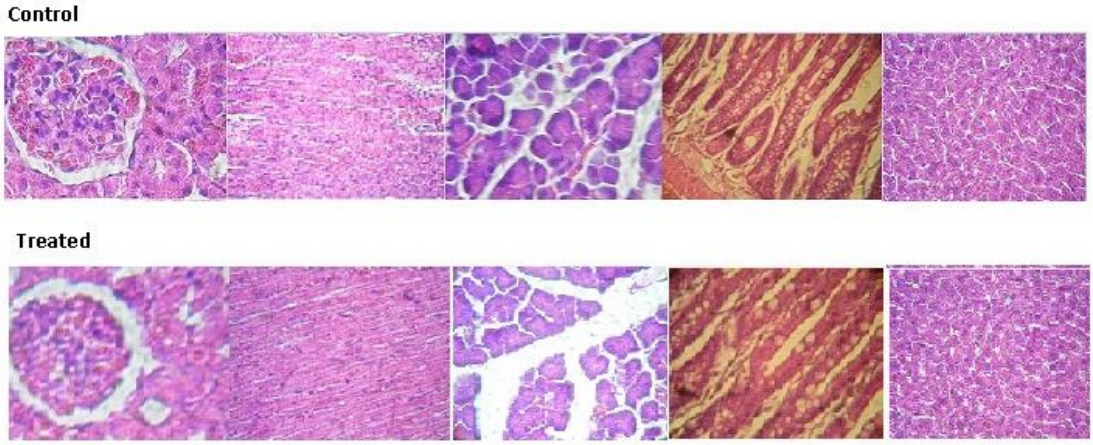

Fig: 2 Photomicrographs (reading from left to right) in the order: kidney, heart, pancreas, intestine and liver, using HE staining with 400x magnification.

The possible change denoted by pancreatic hipoamilasemia was not verified histologically. However, the time period of the experiment may not have been enough to reveal such damage by light microscopy

CONCLUSIONS

The administration of the WBF dose of 1mg / g body weight in rats with STZ-induced diabetes in a period of 21 days, caused no change in physiological parameters (water consumption, feed and urine volume) in biochemical markers (total cholesterol, and triglycerides), liver enzymes (gamma-GT, AST and ALT), and organ weights (liver and pancreas) as well as fecal excretion of proteins and lipids. An increased excretion of carbohydrate in the stool confirms the results of inhibition of α-amylase. Serum amylase has been able to contribute to the diagnosis of pancreatic injury, but did not correlate with the severity of injuries. Thus, the decline of serum amylase suggests the occurrence of cell damage in the pancreas, not confirmed by histological analysis with optical microscopy. Despite the trend toward a decrease in blood glucose from 20 days of treatment, there was no significant decrease during the study period in the treated group. It can be concluded that despite the tendency to decrease hyperglycemia and possibly aid in weight loss by decreasing absorption of carbohydrates in the diet, high doses of FFB may have deleterious effects to the body, resulting from chronic use.

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