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Toxicological Evaluation of Carob (*Ceratonia silique*) Bean Extracts in Male New Zealand White Rabbits

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Abstract: The carob tree (*Ceratonia silique*) grows naturally in Mediterranean region. The empiric use of carob cures by boiling the fruit of carob is very common in Turkey for its effects on asthma, diarrhea and aphrodisiac properties. Yet, there is no study in the literature available about carobs' toxicological safety evaluation. Thus, the purpose of this study was to determine if there was any toxic effect of carob bean extract >49 days period in rabbits. Male New Zealand White rabbits (6-8 months old) were divided into two groups of 8 rabbits. Rabbits in the control group received 10 cc tap water. Rabbits in the treatment group received 10 cc of carob cures by boiling the fruit of carob. The results indicated that feed intake and body weights did not changed due to treatment. Hematological parameters and enzyme concentrations were similar and within the normal range for rabbits in both groups. Liver, kidney, lung, brain and heart weights between control and treatment groups were not significant. Moreover, no apparent changes in liver, kidney, lung, brain, heart and testes were detected by gross post mortem and histopatological examination to suggest toxic effect of oral use of carob extract for 49 days. The results suggested that traditional use of carob cures to male New Zealand White rabbits did not cause any toxicological effects and can be used for human consumption.

Key words: Safety evaluation, hematological parameters, liver enzymes, heart, brain, kidney

INTRODUCTION

The carob tree (*Ceratonia silique*) is a species of flowering evergreen tree in the pea family. Carob tree is well adapted to the ecological conditions of the Mediterranean region, Cyprus, Libia and California. In Turkey, carob is common in Aegean region (4%) and all coastal area from Urla to Hatay (96%). This fruit is locally called as harnup in Turkey, it is also known as St. John's Bread in English.

The carob bean has a potential role in healthy and balanced feeding. It is an ideal nutrient source because of its high carbohydrate (40-60%) and low oil (0.4-0.8%) contents (Marakis, 1996; Karkacier and Artik, 1995). Sugar content of dry carob is 437.3 mg g^{-1} sucrose, 395.8 mg g^{-1} glucose and 42.3 mg g^{-1} fructose. Gallic acid (3.27 mg g^{-1}), the most abundant fenolic compound present in the fruit of carob (Ayaz *et al*., 2007) has a potential to induce apoptosis in cancer cells (Inouce *et al*., 1994). The carob juice is rich in electrolytes such as potassium, sodium, calcium, magnesium, iron, copper, manganese and zinc so, it is used for treatment of diarrhea. Pectin (16.2 mg dL^{-1}) and tannin (10.2 mg dL^{-1}) in carob support antidiarrheic effects and also reduce blood cholesterol levels (Wursch, 1979). Carob is also used in cough syrup because of its expectorant effects (Merzouki *et al*., 1997; Amico and Source, 1997). In addition, carob is traditionally used to increase sperm count and its aphrodisiac properties for longer terms.

Because of all these named effects, carob bean and its extract are used locally in many Mediterranean countries. Yet, there is no study in the literature available about carobs' toxicological properties. The longer term use of carob may have toxic effects. Thus, this preliminary study was conducted to evaluate whether traditional use of carob bean extract has toxic effects of in male New Zealand White rabbits.

MATERIALS AND METHODS

Animals and diets: The current experimental protocol was planned according to guidelines of the animal committee and approved by the ethics committee of Suleyman Demirel University. Total of 16 male New Zealand White rabbits (6-8 months old), weighing 2.5 and 3.0 kg were used in the current study. All rabbits were housed

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individually in galvanized cages (50×50×50 cm) and kept standard laboratory conditions (12 h dark: 12 h light and 24±4°C) during the entire experimental period.

Feed and water were provided *ad libitum*. The rabbits were fed standard commercial rabbit pellets (Ekinciler Food Company, Burdur, Turkey; 88% dry matter, 9% ash, 16% crude protein, 15% crude fiber and 2600 kcal kg^{-1} of metabolizable energy) and feed intake was measured daily.

Preparation of carob bean extract: Carob bean samples were collected from Antalya, Turkey. Extracts of carob bean was prepared daily as the way it is used traditionally for treatment of low sperm count. Carob beans (150 g) were cut into 2 cm pieces and boiled in half liter of tap water for 3 min. After cooling of the mixture for 20 min, carob fruits were removed from the water and extract was used for oral gavages.

Experiment and sampling: Rabbits were divided into two groups of eight rabbits each and were held 30 days for adaptation prior to experiment. After adaptation period, rabbits in control group received 10 cc of tap water daily for 7 weeks. Rabbits in treatment group were exposed to same amount of carob extract prepared daily. Body weights were recorded weekly. At the end of the experiment, blood samples from each rabbits were collected from the ear artery. Samples were prepared as serum and plasma. About 1 day after the blood sample collection, the rabbits were euthanized. The abdominal cavity of each animal then was opened and the liver, kidney, lung, brain, heart and testicles were excised and organ weights were recorded.

Total erythrocyte and leukocyte were counted with haemocytometers (Merk, 1974). Percent hemoglobin and hematocrit, concentrations were estimated by cynanmethemoglobin and microhematocrit methods, respectively (Merk, 1974). Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentrations (MCHC) were calculated from the values of hematocrit % and hemoglobin (Jain, 1986). Plasma protein values were measured by a refractometer (Atago, SPR-N, Japan).

Percent leukocyte distribution were determined from the blood smears that were stained with May Grunwald-Giemsa stain. *In vitro* Enzymatic Colorimetric Method was used for the determination of glucose (Glucose GOD-PAP, Biolabo Sa, Maizy, France), triglyceride (TR-F400 CH, Chema Diagnostica, Via Pellegrini Jesi, Italy) andcholesterol (CT-F400 CH, Chema Diagnostica, Via Pellegrini Jesi, Italy) in serum. Alanine aminotransferase (ALT; kinetic enzymatic method without pyridoxal activation), serum Alkaline Phosphatase (ALP; Optimized Colorometric Method) and serum Aspartate aminotransferase (AST; kinetic enzymatic method without pyridoxal activation) concentrations were measured by auto analyzer (The Roche Modular PP Auto Analyser, Roche Diagnostics, Mannheim, Germany).

Histopathology: For histological examinations, brain, heart, left kidney, left lung, left testicle and a 1 cm wide strip of the left median lobe of the liver was removed and placed into buffered formalin. All tissue samples were routinely processed into paraffin; 5 μm thick sections were stained with Hematoxylin and Eosin (H and E). The slides were examined in a single-blind fashion by a pathologist.

Statistical analyses: Results are presented as mean±SE. Proc GLM procedure was used to analyze weekly body weight changes and feed intake. All other data were analyzed by Proc t-test procedure. All statistical analyses were carried out using SAS statistical package.

RESULTS

No toxicological signs or death related to carob extract were observed throughout the 7 weeks period of the experiment. Rabbits in both groups appeared healthy and showed no unusual behavior during the entire experimental period. Body weight curves were shown in Fig. 1. No effects of carob treatment were observed on body weight. Average body weight changes were not significantly different between the groups. Moreover, no treatment related effects were apparent regarding average food intake in either group (Fig. 2).

The blood parameters are shown in Table 1. All hematological parameters showed no significant differences between control and rabbits treated with the extract. Serum total cholesterol, glucose and triglyceride concentration were not altered due to treatment (Table 2).

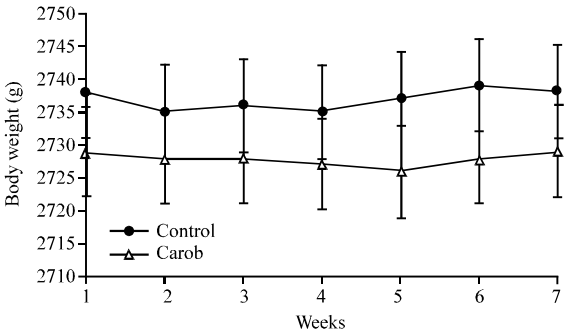

Fig. 1: Body weight changes of male New Zealand white rabbits during the experiment

Table 1: Effects of carob bean treatments on hematological parameters in New Zealand white male rabbits after oral gavages for 49 days (mean±standard error)

	Treatments		
Parameters	Control	Carob	p-value
Hemoglobin (g dL^{-1})	13.8±0.320	13.7±0.240	<0.88
Hematocrit (%)	41.0±1.590	40.8±0.960	<0.92
RBC ($\times 10^6$ μL^{-1})[1]	5.77±0.22	6.37±0.31	<0.14
MCV (fl)[2]	71.4±2.800	64.6±3.300	<0.14
MCH (pg)[3]	24.0±0.700	21.8±1.300	<0.12
MCHC (%)[4]	33.8±1.180	33.7±0.960	<0.95
WBC ($\times 10^3$ μL^{-1})[5]	6.70±0.99	7.34±0.34	<0.58
Differential leucocytes (%)			
Lymphocyte	58.7±4.730	53.8±5.140	<0.65
Pseudoeosinophil	33.6±4.540	38.4±1.200	<0.46
Eosinophil	2.5±0.890	2.4±0.200	<0.87
Basophil	0.5±0.390	0.4±0.340	<0.84
Monocyte	4.7±1.050	5.0±1.040	<0.79

[1]RBC = Red Blood Cell. [2]MCV = Mean Corpuscular Volume. [3]MCH = Mean Corpuscular Hemoglobin. [4]MCHC = Mean Corpuscular Hemoglobin Concentration. [5]WBC = White Blood Cell. [6]ALT = Alanine aminotransferase. [7]AST = Aspartate aminotransferase. [8]ALP = Alkaline Phosphatase

Table 2: Effects of carob bean treatments on biochemical parameters in New Zealand white male rabbits after oral gavages for 49 days (mean±standard error)

	Treatments		
Parameters	Control	Carob	p-value
ALT (IU L^{-1})[1]	38.28±3.240	39.57±1.70	<0.73
AST (IU L^{-1})[2]	22.57±4.000	26.14±8.27	<0.71
ALP (IU L^{-1})[3]	72.71±2.270	75.00±2.78	<0.54
Glucose (mg dL^{-1})	102.72±10.24	106.49±8.39	<0.79
Triglyceride (mg dL^{-1})	44.67±4.550	49.22±5.22	<0.53
Total cholesterol (mg dL^{-1})	25.48±2.380	24.81±2.16	<0.84

[1]ALT = Alanine aminotransferase. [2]AST = Aspartate aminotransferase and [3]ALP = Alkaline Phosphatase

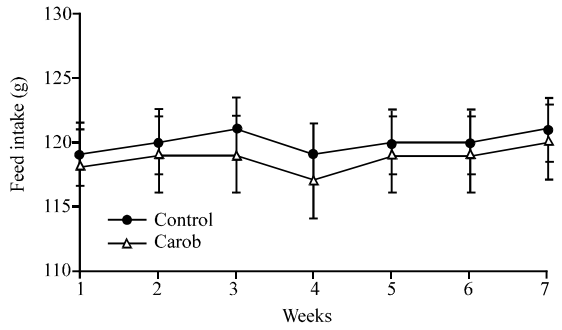

Fig. 2: Average weekly feed intake of male New Zealand white rabbits during the experiment

Moreover, no effect of carob treatment was observed on serum ALP, ALT and AST concentrations. Detailed postmortem examinations of the rabbits in both groups were performed after sacrifice. No treatment related changes were found in treated animals at necropsy. Gross examination revealed no absolute increase in organ weights tested in the current study (Table 3). No pathological lesions were observed in rabbits in different treatment groups. Histopathological parameters (liver, kidney, lung, spleen, heart and brain) also were tested to evaluate whether carob treatment resulted any organ toxicity. Including carob in the diet did not cause in any adverse toxicological effects. No microscopic changes were apparent in any of the organs tested.

Table 3: Effects of carob bean treatments on average feed intake andbody and organ weights in New Zealand white male rabbits after oral gavages for 49 days (mean±standard error)

	Treatments		
Parameters (g)	Control	Carob	p-value
Body weight	2738.00±97.0	2729.00±79.0	0.89
Feed intake	121.14±8.57	118.14±9.28	0.60
Liver weight	69.46±2.26	69.27±2.08	0.94
Kidney weight (pair)	13.46±0.20	13.46±0.29	0.99
Lung weight	11.02±0.21	10.92±0.29	0.78
Spleen	0.94±0.03	0.97±0.03	0.45
Heart weight	6.69±0.22	6.62±0.19	0.80
Brain	8.71±0.19	8.49±0.11	0.34
Testis weight (pair)	7.28±0.19	7.92±0.14	0.03

DISCUSSION

Carob bean and its' extract are commonly used in Turkish medicine and it has been used widely for many centuries to cure a whole variety of diseases. Yet there is no study in the literature available about carobs' possible toxicological properties. Thus, it is very important to evaluate any possible toxicological risk of the carob bean extract.

Overall, no treatment related changes regarding clinical sign, survival rates, body weights, food consumption, hematology, serum biochemistry and postmortem observations were noted. The oral use of extract did not appear to impede growth or affect food consumption. There was no change of body weight gain and no remarkable differences in food consumption between control and treated groups. Feed intake and body weight changes of the groups followed a similar pattern indicating a normal metabolism of the rabbits andthe utilization of nutrients was not adversely affected by intake of carob bean extract.

The hematological parameters evaluated were within the normal values reported for New Zealand White rabbits (Hein and Hartmann, 2003). There were no significant changes in the hematological parameters between the control and the treatment group suggesting that carob bean extract may be toxic as it does not affect the red and white blood cell numbers in blood. Oral administration of the extract did not cause any changes in circulating red and white blood cells andhematocrit, hemoglobin, MCV, MCH andMCHC levels indicting no toxic effects on the hematopoiesis and leucopoiesis.

Serum metabolic indices (such as ALT, AST and ALP) known to be sensitive indicators of damage to the liver. AST is found in the heart, kidney, brain, liver and skeletal muscle. Therefore, liver, heart or muscle damage can cause an increase in serum AST activity (Boyd, 1983). Accordingly, ALP levels increase due to cellular damage in numerous organs and rise in ALP activities indicates non-specific tissue irritation (Bush, 1991; Ceron *et al*., 1995).

In the current study, the results of the biochemical parameters indicated that the extract have no significant adverse effect. Serum ALT, AST and ALP levels were within the normal ranges for rabbits (Hein and Hartmann, 2003). Moreover, plasma concentrations of glucose, cholesterol, triglycerides and proteins were not affected by feeding extracts. This finding also indicates that metabolism of the animals was not affected by treatment.

The weights of the organs were similar and there were no significant differences in organ weights between control and treatment groups. On gross pathological examination, no changes were observed in any of the organs examined. The histopathological examination of the organs tested in the control and treated groups also showed no differences suggested that extract at dose tested did not result in any adverse toxicological effects on these organs.

The search of the literature failed to reveal significant information regarding the potential toxicity of carob bean extract. Most of the relevant published literature data involved studies conducted with carob bean gum. Similar to the findings with carob bean extract, carob bean gum admixed in the diet showed no evidence of a compound-related toxicity. Acute toxicity studies in rat and mice did not show any evidence of oral toxicity at doses up to 10 g kg^{-1} (Maxwell and Newell, 1972). Subchronic toxicity studies in rats showed no evidence of toxicity due to carob bean gum (Takada *et al*., 1997). Likewise, a clinical study failed to show that oral ingestion of carob bean gum has any adverse effects in humans (Behall *et al*., 1987).

CONCLUSION

The present study suggested that extract of carob bean has no toxic effect with the traditional use of the plant in male New Zealand White rabbits. To the best of the knowledge, this is the first report concerning an oral toxicity study of carob bean extract in rabbits. The findings supports that carob bean extract is nontoxic and well tolerated for the 49 days study period and therefore can be used safely in male New Zealand White rabbits.

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