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Original Research Article

Evaluation of the Toxic Effect of Ethanolic Extract of Saffron in Male Mice after Subchronic Exposure

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This study is conducted to investigate the toxicity of saffron (*Crocous Sativa L*) in male Balb-C mice. The range finding study revealed that ethanolic extract of saffron is practically not acutely toxic. The subchronic exposure of mice to two different doses 4000, 5000 mg/Kg.BW orally for five weeks exhibited a significant decrease in WBC, RBC count and low hemoglobin level. Kidney function impairment was observed through a significant increase in serum level of BUN and creatinine, which was more significant after high dose (5000 mg) exposure, histopathological changes like degeneration of epithelial cells, lining the proximal and distal convoluted tubules was also observed. Some liver injury enzyme tests ALT and AST revealed a significant increase in serum activity of AST only after exposure to both doses of plant extract, mild histopathological changes in liver tissue included dilation of the central vein and vacuolar degeneration were observed. There was a significant decrease in body weight of mice proportional to the dose and period of exposure during the time of study.

Keywords: Toxic effect, Ethanolic extract, Saffron, Male mice, Subchronic

1. INTRODUCTION

Since the past decades, people have been interested in folk and recently herbal medicine for the treatment of a variety of diseases (1). They don't care about the possible toxic effects of medicinal plants. Saffron (powder of deride stigma of ***Crocous Sativus L***) is habitually used in many nations like Iraq and Iran as a spice by sprinkling it on cooked rice. Pharmacological studies have revealed the extract of saffron has different therapeutic effects like anticonvulsant (2), antidepressant (3), antinociceptive (4) and antioxidant (5). Saffron as a medicinal plant has a wide range of therapeutic and adverse effects (6). Nowadays, there is the tendency for studying the plant's adverse and toxic effects (7). Toxicological studies had shown that the LD_{50} of saffron stigma in mice is 1.6g/K.g.BW, but certain studies of sub-acute exposure showed a decrease in hematocrit, erythrocyte and hemoglobin values, while it didn't cause pathological lesion in different organs (8). Nikethamide is a CNS stimulant which induces convulsion (9).

Since saffron is habitually used by many people worldwide, we are concerned to study if there are possible toxic effects, because the safety of saffron is very important in relation to their habitual consumption and medicinal use.

2. MATERIALS AND METHODS

2.1. Plant

The powder of saffron stigma was purchased from Novin Zeferan Co (Mashhad, Iran).

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2.2. Preparation of Ethanolic Extract of Saffron by Maceration Method.

Briefly, 10 gm of stigma powder was macerated in 500 ml of 80% v/v of ethanol for 72 hours. The solution was subsequently filtered and concentrated under reduced pressure at 40°C. The extraction yield was 51%.

2.3. Animals

Male Balb-C Mice with a body weight ranged 25-30 gm were procured from the animal house of the college of veterinary medicine, University of Baghdad and were maintained in an air-conditioned room (25° C ±1° C) with a 12 hours light: 12 hours dark cycle. Standard pellet diet and water were provided *ad – labitum*.

2.4. Experimental Design

This work was performed with the approval of the College of Veterinary Medicine/Baghdad University in accordance with international ethical standards of research for work with laboratory animals.

2.5. Saffron Dosage Solution

Each mouse was dosed 0.1 ml/10 gm body weight, which contained the estimated and calculated doses in all experiments included in this study.

2.6. Acute Toxicity assessment

Range finding study (10) is carried out for the evaluation of acute toxicity of saffron. Twenty male Balb-C mice were divided equally into four groups and given different ethanolic extract solution doses subsequently (2000, 3000, 4000, 5000) mg/Kg.BW by gastric gavage needle. The criterium which is considered for determining the acute toxic effect of plant ethanolic extract is death after 7 days of dosing.Three animals were further dosed with 5000 mg/Kg.BW of the plant extract subsequently and observed for the same period.

2.7. Assessment of Effective Dose of Ethanolic Extract of Saffron Stigma as Anticonvulsant

At the first we had conducted a pilot study to choose the dose of Nikethamide, (N,N-Diethylnicotinamide) a package of 25 ml contained 25gm obtained from SIGMA-ALDRICH that only resulted in convulsion without death. Seven subsequent doses (100 ,120,140,160,180,200,220) mg/Kg.Bw of Nikathamide dosed orally by gastric gavage, each single dose was given to two mice. The effective dose (ED50) of ethanolic extract of saffron stigma was determined by Up & Down method (11).

Five male Balb-C mice were dosed with 200mg/kg.BW of Nikethamide orally to induce convulsion and then treated simultaneously with ethanolic extract of saffron stigma orally with initial dose 500mg/kg.BW for the first mouse, depending on the outcome, the dose of plant extract was increased by 20% when the signs of convulsion didn't show any relief but the dose was decreased also by 20% in case of recovery. The ED50 was calculated with the following equation:

$ED_{50} = XF + Kd$

XF = the last dose
d = difference between doses. It is 100 mg in this experiment.
K = constant. It is 0.70 in this experiment.

2.8. Sub Chronic Exposure of Ethanolic Extract of Saffron Stigma in Mice

Fifteen male Balb-C mice were divided equally into three groups. The first group (T1), the second group (T2) were dosed respectively, with 4000 and 5000mg mg/Kg.BW of ethanolic extract of saffron stigma orally by gastric gavage for five weeks, the third group was considered control group (C) dosed with distilled water for the same period. Body weights were recorded before treatment and weekly after treatment till the end of the exposure. At the end of exposure, some hematological parameters were measured which included red blood cells count (RBC) according to (12), white blood cells count (WBC) according to the method mentioned by Coles (13), Hemoglobin by converting to methemoglobin using drabkin's reagent. Values were determined by the method described by Varely and Bell (14), also some biochemical parameters included Serum uric acid was assessed using a commercially available kit (Bio System-SPAIN).

The principle of this method depends on the coupled quinone imine reaction results that are measured by spectrophotometry [15]. Blood urea nitrogen (BUN) was assessed using a commercially available kit (RANDOX-UK). The modified urease—Berthelot method was used, and is based on the formation of a green complex of 2-2 dicarboxylindophenol [16]. Serum creatinine was assessed using a commercially available kit (BioSystem-SPAIN). The method depends on the reaction of serum creatinine with picrate in an alkaline medium, forming a colored complex, which is then measured by spectrophotometry (17).

Liver enzymes included alkaline phosphatase by a commercial kit of LiNEAR Chemicals-SPAIN which is depended on the formation of free 4-nitrophenol and inorganic phosphate, the test has been formulated according to (18), alanineaminotranseferase (ALT) by commercial kit of BioSystems-SPAIN by means of the Lactatedehydrogenase coupled reaction (19), aspartateaminotraseferase also by commercial kit of BioSystems-SPAIN but the test depends on coupled reaction of Malatedehydrogenase (20) which were measured. Blood was collected from the animals in both two groups by heart puncture after anesthesia with 0.1 ml/100 gm BW of ketamine 10% post treatment. The serum was obtained from blood samples by centrifugation at 5000 r per minute for five minutes.

2.9. Histopathology

The animals in both groups were sacrificed at the end of the dosing period. Liver and kidney were obtained and preserved in 10% formalin, then sent to the MEDICINAL CITY HOSPITAL laboratory for histopathology processing as previously described (21).

2.10. Stastical Analysis

Stastical analysis was performed using SPSS version 13.00. A one-way ANOVA was used for the assessment of the body weight. RBC count, WBC count, Hb, serum ALT, AST, BUN, serum uric acid and serum creatinine differences between the groups. A P-value < 0.05 was considered statistically significant. LSD multiple range tests were performed for comparisons between the means.

3. RESULTS AND DISCUSSION

3.1. Acute Toxicity

The result of range finding study conducted to evaluate acute toxicity of ethanolic extract of saffron stigma in male Balb-C mice revealed there are no signs of toxicity and death in all mice of the four groups that were given the plant extract orally during the time of this experiment and till the end (after 7 days) of dosing as shown in Table (1). The three additional animals that were dosed with 5000mg/Kg.BW also didn't show any signs of toxicity and death.

The range finding study is more favorable instead of LD_{50} estimation (22) for expectation toxicity of saffron, which is considered as aspic and is being added to cooked rice and beverage in many countries all over the world. The EPA (1994) recommended that 5 gm/Kg.BW of any substance that doesn't cause death to roll out any acute toxic effect and no need for LD_{50} estimation. So ethanolic extract of saffron stigma is considered practically not to have any acute toxic effect. We thought that the purpose of this study is to aid in the selection of the proper starting dose for other studies.

3.2. Effective Dose of Ethanolic Extract of Saffron Stigma as Anticonvulsion

The result of pilot study that was conducted in Male Balb-C mice with suitable dose of Nikethamide that produced convulsion without death, is summarized in the table 2 which revealed that 0.2 ml/Kg.BW of Nikethamide orally is the best one.

The estimation of ED_{50} of ethanolic extract of saffron stigma which was conducted in male Balb-C mice through relieving the convulsive effect of Nikethamide. The result of Up and Down method that used for this purpose is summarized in the Table 3, which revealed the ED_{50} is 570 mg/Kg.BW orally in male Balb-C mice according to the below equation.

The anticonvulsion effect of saffron also was ensured by (2) who found that both watery and ethanolic extract of saffron exhibited anticonvulsive effect in electroshock seizure and pentylentetrazoline induced convulsion in mice. We thought the anticonvulsive effect of the ethanolic extract of saffron stigma is due to the safranal, the colouring agent of saffron, which was found relieving both minimal clonic seizures and generalized tonic-clonic seizures following pentylenetetrazolen administration in rats (23). The effect of safranal is mediated, through the GABA (A)-benzodiazepine receptor complex (24).

3.3. Clinical Study of Subchronic Exposure of Ethanolic Extract of Saffron Stigma in Male Balb-C Mice

3.3.1. Some hematological test (Total white blood cells count WBC, Red blood cells count RBC and hemoglobin)

Ethanolic extract of saffron stigma with both doses 4000 and 5000mg/Kg.BW showed significant increase $P < 0.05$ of total white blood cells count, significant decrease $P < 0.05$ in red blood cells count and blood hemoglobin in male Balb-mice in comparison with mice of control the group which received distilled water. There were no significant differences in WBC count and RBC count between both treated groups with saffron, but there was a significant decrease in blood hemoglobin of mice that were treated with 5000mg/Kg.BW in comparison with mice dosed with 4000mg/Kg.BW, table (4). Daryoush and colleagues (25) found the intraperitoneally doses of 0.35, 0.70 and 1.05 g kg^{-1} of stigma ethanolic extract for two weeks to male wistar rats caused significantly decrease in RBC count and hemoglobin blood level and increase in WBC count in a dose dependent manner, which are nearly coordinated with our present study findings. The elevation of WBC count may be due to inflammatory effect of saffron ethanolic extract revealed in histopathological changes in kidney of both dosed groups, represented by dilation and congestion of interstitial blood vessels with perivascular lymphatic cuffing, figure (1), and infiltration of inflammatory cells within the lumina and interstitial tissue. Furthermore, there is severe mononuclear interstitial infiltration with slight fibrosis and the formation of eosinophilic hyaline, figure (2) and figure (3).

The anemic effect of the extract is may be due to saffron alkaloid colchicines (26) which has antimitotic i.e inhibitory role in cell division of rapidly dividing cells (27). Since stem cells of bone marrow are rapidly dividing cells and included precursor of red blood cells (erythroblast), we supposed that ethanolic extract of saffron stigma with both 4000 and 5000 mg/Kg.BW exerted its anemic effect may be through this mechanism.

3.3.2. Some Kidney Function Tests (Serum creatinine, blood urea nitrogen and uric acid)

The highest dose 5000mg/Kg.BW of ethanolic extract of saffron stigma showed significant increase $P < 0.05$ serum creatinine, blood urea nitrogen (BUN) and serum uric acid incomparison with both mice treated with 4000mg/Kg.BW and control group, while the mice treated with 4000 mg/Kg.BW of ethanolic extract of saffron stigma showed significant increase $P < 0.05$ of serum uric acid and no significant differences in serum creatinine and blood urea in comparison with control group only, table (5).

It is axiomatic that creatinine blood level inversely correlated with kidney function, so when the kidney function declines, serum creatinine rises. Also the increase of blood urea nitrogen may be seen with decreased kidney function and some other status like dehydration and high dietary protein intake. We could conclude the last two reasons because there were no signs of diarrhea and dehydration in mice of the treated and control groups, and also they had been fed with the same food. The histopathological changes which included degenerative changes of epithelial linings of the proximal and distal convoluted tubules with atrophy of glomerular tufts, in addition to sever dilation and congestion of interstitial blood vessels with perivascular lymphatic cuffing, figure (1,2,3,4) which confirmed that saffron high doses given for one month had led to renal toxicity.

3.3.3. Liver Injury Enzyme Aspartate Aminotrasferase (AST) and Alanine Aminotrasferase (ALT)

There was a significant increase $P<0.05$ in the serum activity of AST of mice of both treated groups with two doses 4000 and 5000 mg/Kg.BW of ethanolic extract of saffron stigma in comparison with the mice of the controlled one which was dosed with distilled water, but there were no significant changes between them. The serum activity of ALT of both dosed groups showed no significant changes in comparison with control group, but we can observe there is no significant increase in it, table(6). The changes in the serum activity of these enzymes may be considered due to harmful effect of ethanolic extract of saffron stigma in the liver tissue of both dosed groups, which included dilation of central veins with vacuolar degeneration of hepatocytes accompanied with

Table1. The outcomes of the range finding study of ethanolic extract of saffron stigma in male Balb-C mice orally.

Dose mg/Kg.Bw	Number of animals	after 7 days of treatment with plant extract	
		No of dead animals	No of a live animals
2000	5	0	5
3000	5	0	5
4000	5	0	5
5000	5	0	5

Table 2. The results of pilot study of Nikathamide dosed orally to male Balb-C mice for producing convulsion.

Dose ml/Kg.Bw	Number of animals	No of animals without signs	No Of animals showed convulsion	No of dead animals
0. 1	2	2	0	0
0.12	2	2	0	0
0.14	2	2	0	0
0.16	2	2	0	0
0.18	2	2	0	0
0.2	2	0	2	0
0.22	2	0	2	2

Table 3. The result of Up and Dow method in male Balb-C mice for estimation ED_{50} of ethanolic extract of saffron stigma orally**.**

Initial dose Mg/Kg.BW	Last dose Mg/Kg.BW	No of animals	Result after plant extract dosing	D_{50} mg/Kg.BW.
500	500	5	XOXOX	570

X = no relieve of convulsion.
O = relieve of convulsion.
ED_{50} = Xf+ Kd
Xf = last dose
K = constant(0.70).
d = difference between doses = 100 mg.

Table 4. Total WBC, RBC count and Hmoglobine of male Balb-C mice Exposed to two different doses of ethanolic extract of saffron stigma orally for 30 five weeks**.**

Group	WBC cell/Cumm M ± SE	RBC cell/Cumm M ± SE	Hb mg/dL M ± SE
T1 4000mg/Kg.BW	8360 ± 573.20 A	$5.660X10^6 \pm 2.418X10^6$ B	9.02 ± 0.52 B
T2 5000mg/Kg.BW	9108 ± 440.98 A	$4.572X10^6 \pm 2.279X10^6$ B	7.58 ± 0.26 C
Control (C) DW	5800 ±1276.30 B	$10.852X10^6 \pm 2.454X10^6$ A	15.44 ± 032 A

LSD of WBC=2134.46 LSD of RBC= $1.822X10^6$ LSD of Hb=0.97
The different capital letters denote significance differences P <0.05 between groups.

Table 5. Serum creatinine ,Blood Urea Nitrogen (BUN) and serum uric acid of male Balb-C mice exposed to two different doses of ethanolic extract of saffron stigma orally for five weeks.

Group	Serum creatinine mg/dl M ± SE	BUN mg/dL M ± SE	Serum uric acid mg/dL M ± SE
T1 4000mg/Kg.BW	*1.40 ± 0.17* B	59.20 ± 1.15 B	4.99 ± 0.58 B
T2 5000 mg/Kg.BW	2.42 ± 0.20 A	94.80 ± 4.49 A	9.00 ± 0.32 A
Control (C) DW	0.52 ± 0.02 B	53.40 ± 2.22 B	3.38 ± 0.44 C

LSD of creatinine=0.96 LSD of BUN=8.19 LSD of uric acid=0.80
-The different capital letters denote significance differences P <0.05 between groups.

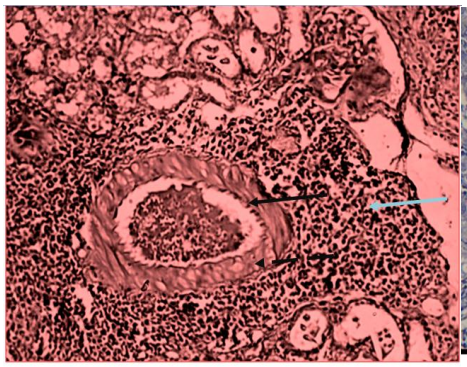

Figure 1: Kidney of mouse dosed orally 4000mg/Kg .BW ethanolic extract of saffron stigma for one month. Severe dilation and congestion of interstitial blood vessels ( ) with perivascular lymphocytic cuffing ( ) (H&E 10 X).

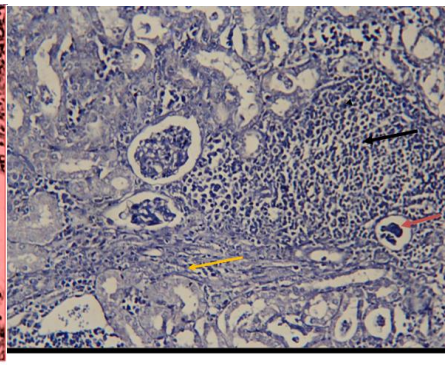

Figure 2: Kidney of mouse dosed orally 5000mg/Kg .BW ethanolic extract of saffron stigma for one month. Sever interstitial mononuclear cells infiltration ( ) with atrophy of glomerular tuft ( ) and slight interstitial fibrosis ( ) (H&E 10 X).

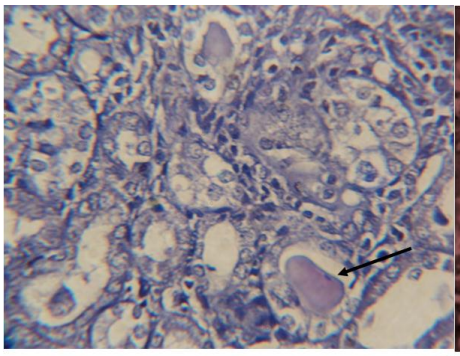

Figure 3: Kidney of mouse dosed orally 5000mg/Kg .BW ethanolic extract of saffron stigma for one month. Formation of eosinophilic hyaline cast ( ) (H&E40X)

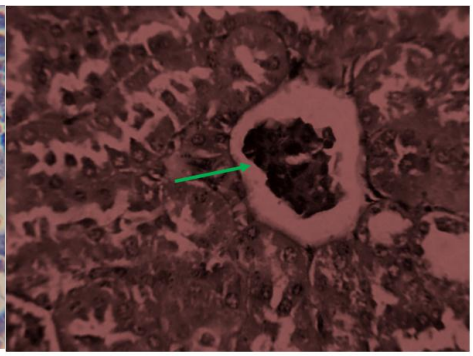

Figure 4 : Kidney of mouse dosed orally 4000mg/Kg .BW ethanolic extract of saffron stigma for one month. Atrophy of glomerular tuft ( ) (H&E 40 X).

Table 6. Liver injury enzymes (AST) U/L and (ALT) U/L of male Balb-C mice exposed to two different doses of ethanolic extract of saffron stigma orally for five weeks.

Group	AST U/L	ALT U/L
T1 4000mg/Kg.BW	176.40±6.70 A	125.29±38.02 A
T2 5000 mg/Kg.BW	184.00±5.64 A	139.87±49.94 A
Control (C) DW	100.60±5.60 B	66.00±21.57 A

LSD of AST=15.14 LSD of ALT=78.95
-The different capital letters denote significance differences P <0.05 between groups.

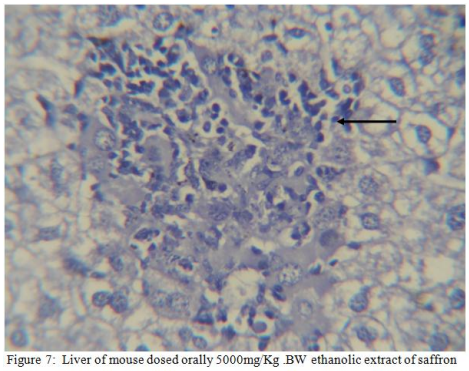

Figure 7: Liver of mouse dosed orally 5000mg/Kg .BW ethanolic extract of saffron stigma for one month. Formation of early granuloma (⟶) (H&E40).

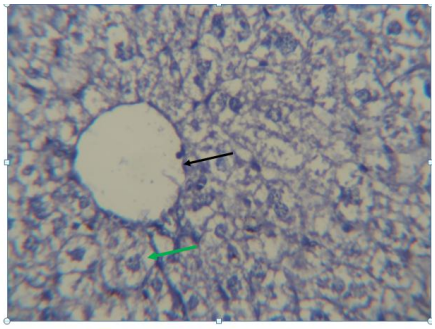

Figure 5: Liver of mouse dosed orally 4000mg/Kg .BW ethanolic extract of saffron stigma for one month. Dilation of central vein (⟶) with vacuolar degeneration of hepatocytes (⟶) (H&E40X)

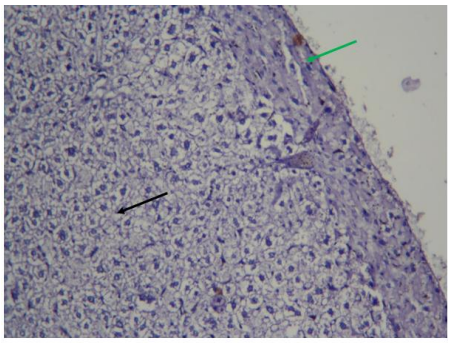

Figure 6: Liver of mouse dosed orally 5000mg/Kg .BW ethanolic extract of saffron stigma for one month. Vacuolar degeneration of hepatocytes (⟷) with moderate fibrous thickening of Glasson's capsule (⟶) (H&E40).

Table 7. Weekly body weight (gm)of male Balb-C mice exposed orally to two different doses of ethanolic extract of saffron stigma for five weeks.

Group	Before treatment (day zero) M ± SE	During treatment 1 week M ± SE	2 weeks M ± SE	3 weeks M ± SE	4 weeks M ± SE	5 weeks M ± SE
T1 4000 mg/Kg.BW	28.20±1.24 A a	27.99±1.67 A ab	26.00±1.09 A ab	26.00±1.18 AB ab	25.00±1.43 AB ab	24.00±0.94 BC b
T25000 mg/Kg.BW	28.00±1.39 A a	27.40±1.20 A ab	25.40±0.87 A ab	24.20±0.66 B b	23.00±0.63 B cb	22.20±0.37 C dcb
Control (C) DW	26.60±1.69 A a	26.60±1.69 A a	27.00±1.37 A a	28.80±1.85 A a	28.40±2.01 A a	29.60±2.04 A a

LSD= 3.55
-The different Capital letters denote significance differences P <0.05 between groups.
-The different small letters denote significance differences P <0.05 within groups.

proliferation of Kupffers cells (figure 5), fibrous thickening of Glasson's capsule with formation of multiple parenchematous granuloma (figure 6) and granuloma (figure 7)

3.4. The Effect of Ethanolic Extract of Saffron Stigma in Body Weight of Male Balb-C Mice

The mice treated with 5000mg/Kg.BW of ethanolic extract of saffron stigma showed significant decrease P <0.05 in body weight after 3,4 and 5 weeks of treatment incomparison with its pretreatment (day zero) body weight and control mice body weight, while the mice that were treated with 4000 mg/Kg.BW of ethanolic extract of saffron stigma showed significant decrease P <0.05 in body weight only after five weeks of treatment in comparison with its pretreatment (day zero) body weight and control group mice body weight, table (7).

Saffron (***Crocus Sativus L***.) is considered a source of polyphenols and carotenoids which have antioxidant ability (28). Natural extracts from plants such as polyphenols and carotenoids reported to enhance body weight loss (29,30). Carotenoids and polyphenols are the antioxidants which have the ability to reduce blood glucose, triglyceride, blood LDL cholesterol, increase energy expenditure and fat oxidation consequently reducing body weight (31,32), and they are capable of inhibition of pancreatic lipase, Lipoprotein lipase and glycerophosphate dehydrogenase (33,34).

Capsule 176.5 mg/day of ethanolic saffron extract given for two months reported a decrease in snacking and body weight in overweight women (28). Crocin, a saffron carotene is a reversible lipase inhibitor in comparison with a commercially

available anti–obesity Orlista which is irreversible (35). Ethanolic extract of saffron stigma was found to significantly reducing body weight in rats through decreasing appetite as a side effect (36, 37). We thought the possible reduction in body weight of both dose 4000 and 5000mg/Kg.BW of ethanolic extract of saffron stigma for Blab-C mice in our present study is dose dependent, and may have contributed to decreasing fat metabolism through inhibition of pancreatic lipase which resulted in decreasing calorie intake and also satiety effect which suppressed appetite that noticed in mice of both treated groups.

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