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Original Article

ANTI-OBESITY EFFECT OF THE POMEGRANATE LEAVES ETHANOL EXTRACT (*PUNICAGRANATUM*L.) IN HIGH-FAT DIET INDUCED MICE

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ABSTRACT

Objective: The aim of the study was to evaluate *in vivo* and *in vitro* assay from the pomegranate leaves ethanol extract (PLEE)as the anti-obesity.

Methods: The anti-obesity effect was evaluated to *in vivo* model high-fat diet induced obese mice and used two doses level 50 mg/kg and 100 mg/kg b.w. from the extract. *In vitro* assay was measured through inhibitory effect of pancreatic lipase enzyme activity by pomegranate leaves ethanol extract with various concentrations.

Results: The pomegranate leaves ethanol extractat a dose 50 mg/kg and 100 mg/kgb.w. showed asignificant decrease of body weight, faeces index, total fat index, food index, and lee's index compared to control mice. Furthermore, significantly inhibitory activity also showed from *in vitro* assay.

Conclusion: The pomegranate leaves ethanol extract may be a potentially therapeutic alternative in the treatment of obesity caused by a high-fat diet.

Keywords: Pomegranate leaves ethanol extract, Anti-obesity, Pancreatic lipase enzyme, High fat diet

INTRODUCTION

Obesity is most common health problems become epidemic on a global scale, especially in the developed countries in the world including Europe, United States of America (USA), and Japan, presenting increase in the risk of morbidity and mortality. Obesity according to *The World Health Organization* (WHO) defines as an over fat accumulation which influence to human health. Obesity has also been defined as an increased of adipose tissue mass **[1]**. Furthermore, obesity has an associated with many diseases like diabetes mellitus type-2, atherosclerotic, cardiovascular diseases, osteoarthritis, hypertension, and some cancers**[2].**

Obesity is an exceedly complex group of diseases and probably should be characterized as a syndrome. Its results from an imbalance between energy intake and expenditure. Has been reported various of dietary composition are important role in the regulation of metabolic process. So, dietary fat could promotes body fat storage more effective than dietary carbohydrate. Thus, inhibition of digestion and absorption of dietary fat is a first key to treating obesity. This inhibition involve pancreatic lipase enzyme, the principlelipolytic enzyme synthesized and secreted by the pancreas.Pancreatic lipase is important enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerol to monoacylglycerol and fatty acid. Substrates for lipase enzyme are long-chain triacylglycerol, which are separated from the aqueous medium by the surface phase. Thus, lipase enzyme must be adsorbed on the substrate lipid surface and the nature of the surface of the substrate is an key role for lipase activity **[3].**

Theraupeutic regimens were given for the long-term to obesity reduction of body weight are largely ineffective causes several adverse effects**[4]**. Recent, phytotherapy more than preference and considered to synthetic drugs for obesity treatment, as a complementary approach for preventing and treating disease.

Pomegranate (*Punicagranatum* L.) is plant can found in Asia, including Himalayas, India, Mediterran region,South East Asia, and the drier regions of USA. Pomegranate tree grows until over 12 feet, spiny branches, and the leaves are glossy. In India, the pomegranate used as *Ayurvedic* medicine and is used as treatment of parasite infections, blood tonic, and some gastrointestinal disorders like diarrhea, and peptic ulcer. In traditional chinese, pomegranate have been used to treat diarrhea, metabolic acidosis, and microbes infections**[5, 6]**. The parts of plants including barks, roots, flowers, and leaves have medicinal benefit as well **[11-14].**

According to Lei *et al*.(2007), the pomegranate leaves extract also exhibited best potency as the anti-obesity after oral administration treatment through appetite suppresant. Based on the above information, the present study has been undertaken to evaluate *in vivo*and *in vitro* assay from pomegranate leaves ethanol extract in high-fat diet induced obese mice as the pancreatic lipase inhibitor.

MATERIALS AND METHODS

Plant collection and identification

The fresh leaves of pomegranate were collected from the garden in Lembang, Bandung, West Java. The leaves was identified in *Herbarium Bandungense***[7-9],**School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

Chemicals

All the chemicals used in this study were of analytical grade. Ethanol, chloroform, n-heptane, sesame oil, NaCl, $CaCl_2$, phosphate buffer solution, copper reagent, diethyldithiocarbamate sodium, oleic acid, porcinepancreatic lipase, bovine albumin serum (BSA) were obtained by Sigma-Aldrich and orlistat (Xenical) ® were purchased from Kimia Farma pharmacy.

Preparation of extract

Leaves were collected, cutting the leaves to small, and extracted to ethanol through maceration for 24 hours. After 24 hours, filtrate were separated from the reciduewith filter paper. Furthermore, the filtrate were concentrated using *rotary evaporator* and stored at the room temperatureprotected from sunlight. The percentage of yield of ethanol extract were found to be 13.7 % w/w. The extract were used for the pharmacological studies by dissolving in 1.0% w/v carboxymethylcellulose sodium (CMC-Na).

Phytochemical screening of leaves extract

The pomegranate leaves ethanol extract (PLEE) were subjected to preliminary phytochemical screening for plant constituents **[10]**.

***In vivo* anti-obesity evaluation**

High-fat diet induced obese mice

Animal study was conducted in the Laboratory of Pharmacology and Toxicology, School of Pharmacy, Bandung Institute of Technology, Indonesia. Fourty fives Swiss-Webster mice of 2-3 month age, weighed about 20 and 30g, were acclimatized under room temperature (28+2° C) with a regular light/dark cycle and were given access to food and drink for a week before treatment.

Following acclimatization, animals were divided into five groups of five animals in each group. The control mice group, PLEE at dose 50 mg/kg and 100 mg/kgb.w. group was fed high-fat diet and the normal group was fed standard Animal Laboratory, School of Pharmacy, Bandung Institute of Technology, for 1 month. The compositions of the experimental diets are shown in Table 1. The food was cut into smaller pieces before being given to the animals. Water and dietary were given *ad libitum*. By the end of 1 month period, the body weight of the obese group was approximately 20% higher than the normal group. The food index, urine index, faeces index, and lee's index was recorded at early period, after induced, and after treated. The body weight of each mouse was recorded at everyday. At the end of experiment, the mice was sacrified with chloroform and the organs were collected and isolated including liver, spleen, perirenal fat, perianal fat, testicle, and kidney. The experimental protocol and handling throughout the study were in accordance with guidelines approved by the institution ethics committee where the study was conducted.

Table 1: Compositions of the experimental diets

Component	Standard Diet (SD) g/Kg	High-Fat Diet (HFD) g/Kg
Cornstarch	250	250
Fish flour	160	160
	140	140
Bean flour		
Wheat flour	410	130
Fat	-	320
Vegetable oil	40	-

***In vitro* pancreatic lipase enzyme assay**

Pancreatic lipase activity was determined by measuring the release rate of oleic acid from emulsified sesame oil. This method was performed by the method of Han *et al.***[19]**, with slight modification. The substrate emulsion (5 ml in a 10-ml centrifuge tube) was prepared by sonification for 5 min of 15 mmol/l sesame oil in a solution containing 1 mmol/l NaCl, 1 mmol/l $CaCl_2$, 10 mg of bovine serum albumin/ml, and phosphate buffer solution (pH 8.0). After sonification, substrate emulsions was incubated with 50µl of *porcine*pancreatic lipase and various concentrations of the pomegranate leaves ethanol extract for 30 min at 37°C. Finish of incubation, for 30 min at 37°C, were added to 3 ml of a 1: 1 (v/v) mixture of chloroform and n-heptane, extracted by shaking the centrifuge tube for 10 min in a shaker. The mixture was centrifuged at 2000 rpm for 10 min. The upper aqueous phase was removed and the lower phase was added with copper reagent (0.5 ml). The tube was shaken again for 10 min, was centrifuged at 2000 rpm, and 0.5 ml of the upper phase (organic phase) was added with 0.5 ml diethyldithiocarbamate-sodium solution. The absorbance was then measured at 480 nm in a spectrophotometer UV-Vis.

Statistical Analysis

The data obtained were analyzed using the Statistical Package for the Social Sciences (SPSS) version 15. Analysis of variance (ANOVA) with the post-hoc tukey HSD was used to analyze the data, and a value of $p<0.05$ was used to statistical significance. The results are expressed as mean + standard error of the mean (SEM).

The percentage inhibition was calculated as

$$\% \text{ Inhibition} = \frac{Control-Test}{Control} \text{ X } 100$$

RESULTS AND DISCUSSIONS

Phytochemical screening of leaves extract

Preliminary phytochemical screening of pomegranate leaves ethanol extract (PLEE)showed the presence of flavonoid, saponin, tannin, quinone, and steroid/triterpenoid.The biological activities of plant constituents are complex. Hence the anti-obesity effect may be attributed to the complex pharmacological action of phytoconstituents present in the extract. Iswantini*et al.* **[17, 18]** reported that the phytoconstituents such as flavonoids, saponin, tannin, and steroid/triterpenoid have a ability to inhibited pancreatic lipase activity.

Effects of PLEE in high-fat diet induced obese mice

In this study, obese mice was induced by feeding a high fat diet for 28 days. Obese mice compared with mice fed a standard diet. Mice fed a high-fat diet increased body weight until 20.0% after 28 days induced of high fat diet. Increased of the body weight in obese mice was followed higher of the visceral adipose include perirenal fat, perianal fat, and liver than in normal group at the end of induced period. As shown in Figure 1, the PLEE group at the dose 50 mg/kg and 100 mg/kg b.w. significantly decreased the body weight compared with control group.

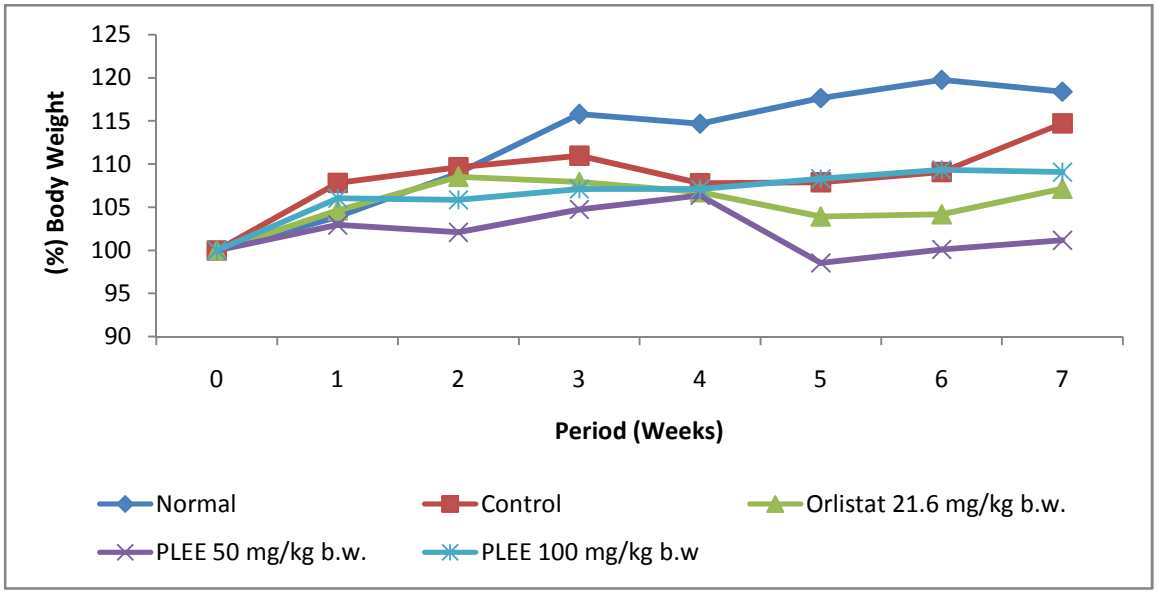

Fig. 1: Effect of the pomegranate leaves ethanol extract administration on decrease the daily body weight

The administration of obese mice with PLEE at dose 50 mg/kg and 100 mg/kg b.w. exhibited a remarkable reduction of body weight compared to the control group. The findings demonstrated that PLEE at 50 mg/kg and 100 mg/kg b.w can reduce body weight, especially PLEE at dose 50 mg/kg. Similar results had been reported Lei *et al.* **[4],**whereby mice supplemented with PLE at dose 400 mg/kg and 800 mg/kgb.w.showed a significant decreased in mice body weight after 5 weeks of treatment.

In addition, increased of organ body which is expressed as organ index has also occur in obese mice involvevisceral adipose region such as perianal and perirenal fat compared with normal group. Fat accumulation not only occurs on the visceral adipose but also on the liver. Liver enlargement shows that pathologic development on the body. It was found that mice fed with high-fat diet can develop increased of liver weight. Furthermore, there are reports that both adipocyte size and number are increased in animals with obesity caused by high fat diet **[22].**

Treatment of both PLEE at the dose 50 mg/kg and 100 mg/kg b.w. also decreased organ index involves total fat(periranalfat and perirenal fat)compared to control group.However, the PLEE administration did not effective on decreased the liver enlargement(Fig 2). Others organ include kidney, testicle, and spleen also determine to know undesirable effect on the body and shows that were not significantly different for all groups. Kidney, testicle, and spleen weight was also under normal conditions for all groups.

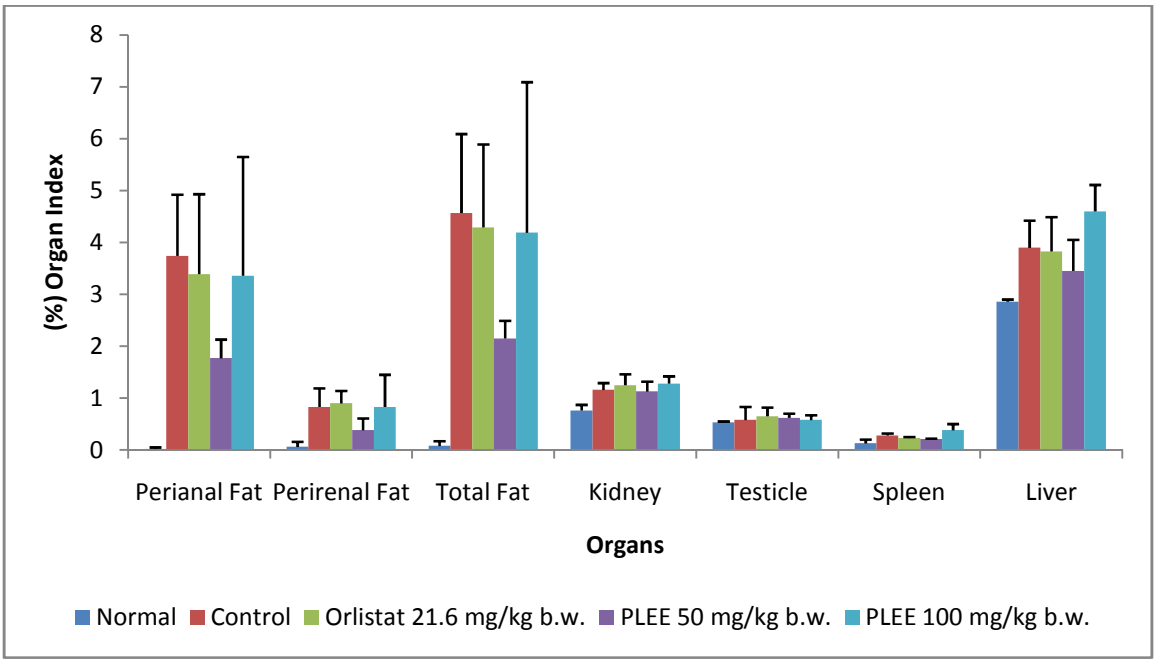

Fig. 2: Effect of the pomegranate leaves ethanol extract administrationon decrease the organ index after treated

Group treated with PLEE at 50 mg/kg and 100 mg/kgb.w. showed a significantly lower perianal fat, perirenal fat weight than that of the control group ($P < 0.05$).Decreased of total fat was shown at PLEE 50 mg/kgb.w. which is significant difference compared PLEE 100 mg/kgb.w and orlistat-treated group(Fig 3).

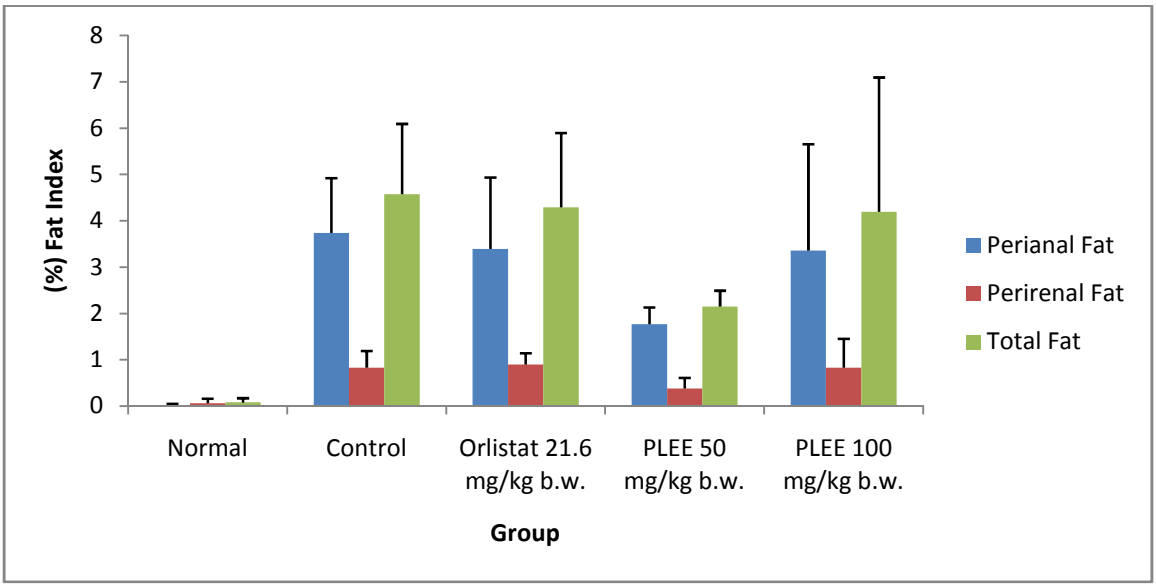

Fig. 3: Effect of the pomegranate leaves ethanol extract administrationon decrease of total fat (perianal fat and perirenal fat) after treated

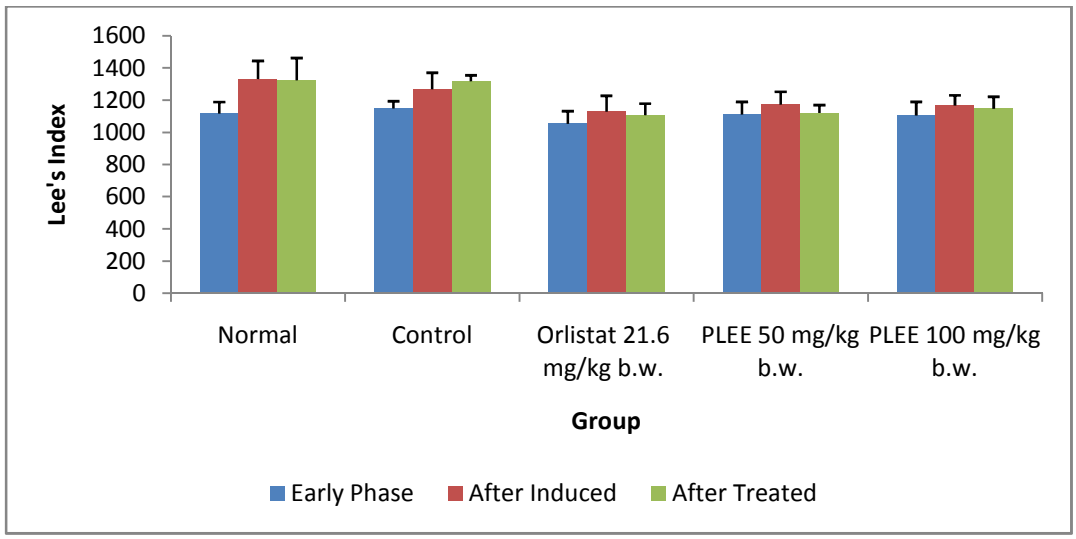

Fig. 4: Effect of the pomegranate leaves ethanol extract administrationon Lee's index

As shown in Figure 4, the lee's index in both PLEE dose 50 mg/kg and 100 mg/kg b.w, and orlistat-treated group was significantly decreased compared with control group after treatment. The aim to lee's index measurement is to known obesity level on the rodent models. Lee's index measurement was performed according to Lei *et al.* (2007).

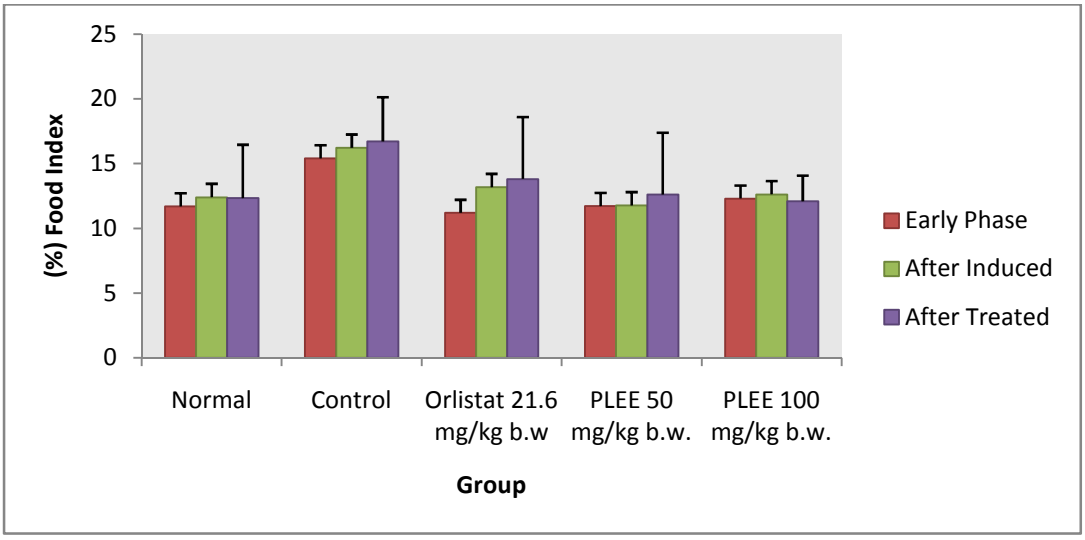

Fig. 5: Effect of the pomegranate leaves ethanol extract administration on food consumption (food index) after treated

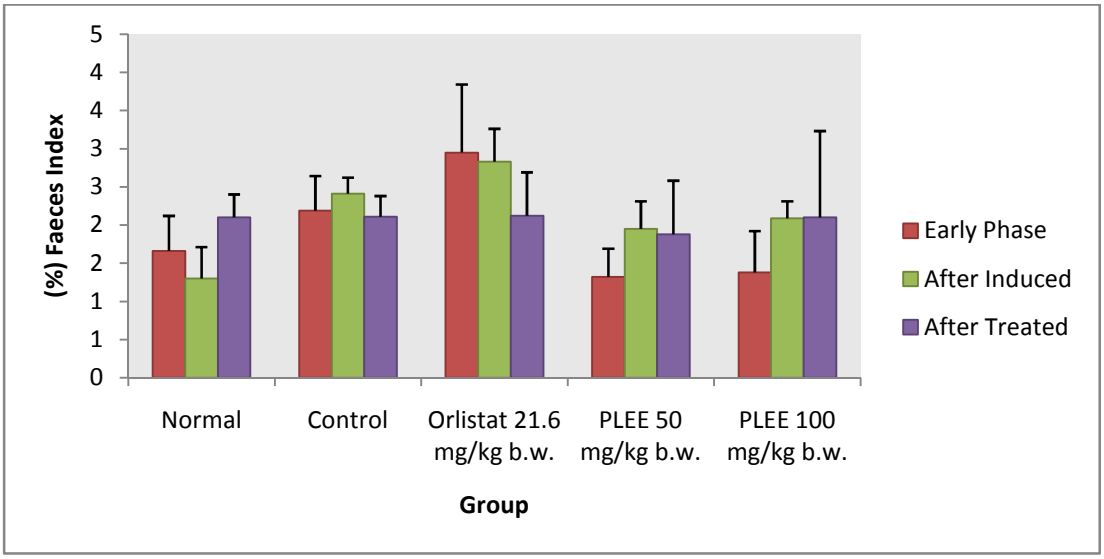

Fig. 6: Effect of the pomegranate leaves ethanol extract administration on faeces index

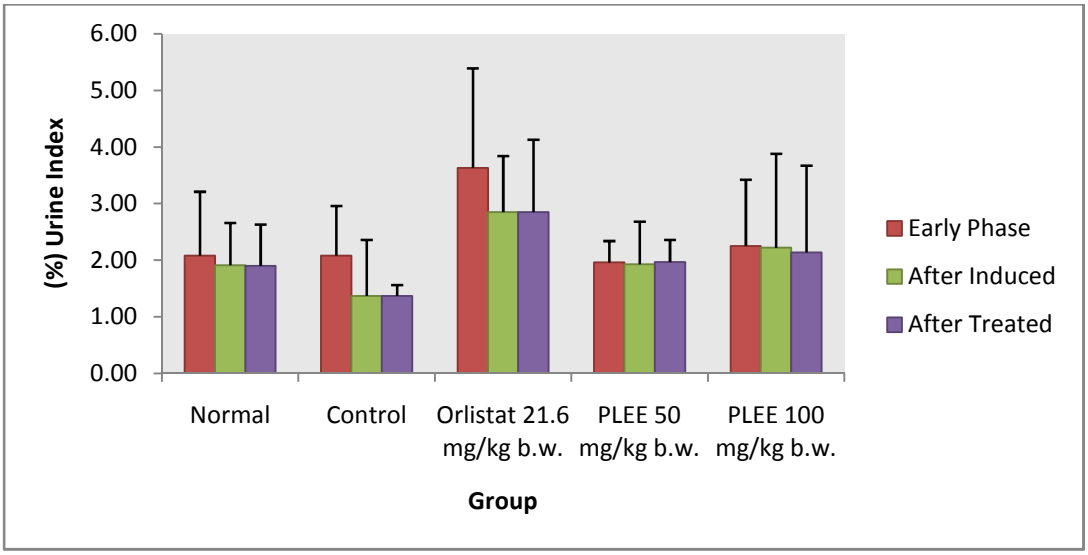

Fig. 7: Effect of the pomegranate leaves ethanol extract administration on urine index

The food index (Fig. 5) and urine index (Fig. 7) were not significantly different between treated group and the control group. Its mean, PLEE and orlistat as treated group did not influence the mice's appetite and did not have diuretic activity for 3 weeks period.

As the Figure 6 shows that both PLEE dose 50 mg/kg and 100 mg/kg b.w. significantly different increased faeces index with control group.Inhibition of pancreatic lipase activity makes lipid did not absorbed to the intestine, its result occurs increased of lipid excretion to the faeces. Based on some studies reported that most common of pomegranate leaves compound is hydrolysable tannin. It is known that hydrolysable tannin which is found on the pomegranate leaves have a high affinity to proteins, forming insoluble complexes with protein and other macromolecules.

Effects of PLEE on pancreatic lipase inhibition activity

As shown in Table 2, PLEE inhibited pancreatic lipase activity as concentration-dependently on the *in vitro* assay. PLEE significantly inhibitedpancreatic lipase with 50% inhibition (IC_{50}) of enzyme activity at the concentration 20.64 µg/ml compared about orlistat as standard drug with various concentrations.

Based on the *in vitro* assay, PLEE exhibited maximum inhibition with increased of concentration. Its mean inhibition effect of PLEE dependent on concentration and how the substrate was presented to the lipases. The lipolytic process occurs at the substrate surface (lipid – water interface) and is dependent on surface adsorption of the lipase. Then, the PLEE compounds could be considered efficiently inhibits pancreatic lipase activity by interaction with the emulsified sesame oil – BSA, adsorbing to the substrate surface and retarding the lipolytic process. This mechanism isdifferent with orlistat. Orlistat (tetrahydrolipstatin), is a derivative of the naturally produced from *Streptomyces toxitricinii*, which strongly inhibits pancreatic lipase. The mechanism of lipase inhibition is through a covalent bond to the active site serine of the pancreatic lipase and did not worked to the substrate **[20, 21]**.

Table 2: *In vitro* pancreatic lipase enzyme activity assay

Group	Concentration (µg/ml)	Inhibitory effect (%)
PLEE	0.1	44.26 + 9.49
	1	63.92 + 4.43
	10	49.65 + 10.52
	100	65.78 + 5.69
	1000	74.19 + 8.66
Orlistat	0.1	94.52 + 1.06
	1	96.49 + 0.18
	10	97.19 + 0.67
	100	97.93 + 0.18
	1000	98.32 + 0.41

Value are expressed as %, are mean + SEM from 3 replicate.

CONCLUSION

The present study suggested that pomegranate leaves ethanol extract (PLEE) showed a promising role in therapy to high fat diet induced obesity. Our present documented findings may suggest the use of pomegranate leaves ethanol extract to treat the obesity patients.

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