International Journal of Herbal Medicine
Available online at www.florajournal.com

IJHM
International Journal of Herbal Medicine

ISSN 2321-2187
IJHM 2014; 2 (1): 65-70
Received: 26-03-2014
Accepted: 07-04-2014

Anton Bahtiar
(a) Graduates School of Herbal Medicine, Faculty of Pharmacy, University of Indonesia.
(b) Department of Pharmacology, Faculty of Pharmacy, University of Indonesia.

Sunarmi Arifin
Graduates School of Herbal Medicine, Faculty of Pharmacy, University of Indonesia.

Amalia Razalifha
Graduates School of Herbal Medicine, Faculty of Pharmacy, University of Indonesia.

Nurul Qomariah
Graduates School of Herbal Medicine, Faculty of Pharmacy, University of Indonesia.

Puspita E. Wuyung
Faculty of Medicine University of Indonesia.

Ade Arsianti
Faculty of Medicine University of Indonesia.

Correspondence:
Anton Bahtiar
(a)Graduates School of Herbal Medicine, Faculty of Pharmacy, University of Indonesia.
(b)Department of Pharmacology, Faculty of Pharmacy, University of Indonesia.
Email: anton.bahtiar@ui.ac.id

Polar Fraction of *Punica granatum* L. peel extract increased osteoblast number on ovariectomized rat bone

Anton Bahtiar, Sunarmi Arifin, Amalia Razalifha, Nurul Qomariah, Puspita E. Wuyung, Ade Arsianti

ABSTRACT
The effects of Polar fraction of *Punica granatum* (L) peel extract on bone loss in ovariectomized (ovx) rats model of osteoporosis were investigated. Forty two 6 weeks old female Sprague–Dawley rats were randomly assigned to seven groups as followed, sham-operated, OVX, OVX-Estradiol (0.5 mg/kgBW), OVX-Tamoxifen (50 mg/kg BW), OVX-Punica fraction (PF) 50 mg/kg BW, OVX-PF 100 mg/kg BW and OVX-PF 200 mg/kg BW for 4 weeks. The administration of Punica fraction was given orally using a stomach tube. The results demonstrated that the administration Punica fraction 50, 100, and 200 mg/kg BW significantly prevented bone loss in OVX rats which these effect equivalent to Tamoxifen. These effects were described in increased mineral content of calcium. On histology data shown that fraction could increased osteoblast number. This result indicated that polar fraction of ethanolic extract of pomegranate have potential as a drug for osteoporosis.

Keywords: Pomegranate, Peel, Polar Fraction, Osteoblast, Ovariectomy.

1. Introduction
Osteoporosis is one of the major health problems, and expected to increase dramatically in the recent decades and the majority of these patients are women. Recent epidemiological studies have suggested that the incidence of osteoporosis is a complex interaction due to many factors such as variety of genetic, geographic, and ethnic factors [1]. Estrogen deficiency is generally not one of the major of the main risk factors for osteoporosis, but it is indirect and strongly related with the many recognized osteoporosis risk factors especially in women such as thin, advanced age, postmenopausal, amenorrhea, and more drinking alcohol [2].
Several line of evidence reported the importance of estrogen in bone remodeling and metabolism. Furthermore, the evident from the clinical used that the administration of hormone replacement therapy (HRT) in a dose dependent manner effectively prevents bone loss in postmenopausal women [3] and reduces the incidence of osteoporosis [4]. Unfortunately, the use of HRT for long term caused several unwanted side effect associated with these powerful steroids and increased risk for breast and endometrial cancers [5, 6]. Therefore, further exploration of alternatives and/or adjunctive approaches that can produce clinically relevant prevent bone loss like in osteoporosis would be interest. Non-hormonal therapy or natural product therapy may more acceptable for the treatment and prevent osteoporosis. Recently, much attention has been focused on phytoestrogens, especially isoflavone, as a potential safe alternative for pharmaceutical HRT [7, 8]. Phytoestrogen is one of the natural alternatives that appear to offer the most potential for the prevent bone loss. Phytoestrogen is non-steroid plant-derived compounds which structurally similar to estrogen and possesses both weak estrogenic and antiestrogenic effects [9, 10]. Previous study in animals showed that phytoestrogen had a protective effect against bone loss due to estrogen deficiency. The consumption of natural phytoestrogen from soybean instead of a casein-based diet had been demonstrated to prevent bone loss in ovariectomized (OVX) rats [11]. Similarly, genistein, a phytoestrogen found predominantly in soybean, prevented bone loss in OVX rats [12].
Both in vitro and in vivo studies have shown that daidzein, genistein, and their glycosides exert a weak estrogenic effect [13]. In addition, raloxifene showed the positive effects of selective estrogen receptor modulators in animals and humans. Because of their similarity to raloxifene in conformational binding to estrogen receptors, genistin have selective actions in bone. Other studies demonstrated that human dietary studies shown the effects of isoflavone-rich soy protein diets on markers of bone turnover and preventing bone loss as measured from bone mineral density (BMD) and content.

Recent studies indicate that oral administration of daidzin, genistin, genistein and their succinyl derivatives significantly prevents bone loss in an ovx model of osteoporosis [13].

Punica granatum (L) is one of the natural plant contain some phytoestrogens. Many years, Punica juice known and use for traditional medicine such as dried pericarp and the juice of the fruits are employed as orally medication in the treatment of colic, colitis, leucorrhea, menorrhagia, oxyuriasis, paralysis, and external application to caked breast and to the nape of the neck in mumps and headache. Pomegranate juice is rich in antioxidants which general possess numerous important biological properties against cholesterol oxidation, protection against atherogenesis, anti-inflammatory, anti-aging, and protection against Alzheimer's disease and diabetes [14]. However, the estrogenic effect of phytoestrogen as Selective Estrogen Receptors Modulator from *Punica granatum* (L) have not been investigated especially the tannin compound of pomegranates. Therefore, there is a great interest to investigate the effect and action of Tannins-contain fractions of pomegranate on bone and reproductive tissue on osteoporosis model rat.

2. Materials and Methods

2.1 Plant and Chemical Materials

Punica granatum (L) used in the present study were collected from "Kampoeng Djamoe Organic" PT Martha Tilaar Ethanol 96%, methanol p.a, ethyl acetate p.a and petroleum ether p.a. were obtained from Sigma (St. Louis, MO, USA). Estradiol were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2 Preparation of Polar fraction of *Punica granatum*

The dried powders of peel of *Punica granatum* were extracted by soxhlet using methanol. Further, the residue that obtained was fractionated using etil acetat and hexane. All the fraction was evaporated to obtain concentrated fraction. The extractive value of methanol from dried powders was calculated as % w/w yield and was found to be 3.71%.

2.3 Animals

Female Sprague–Dawley rats, aged 42 days, were purchased from Litbangkes. The animals were grouped and housed in polyacrylic cages with one animal per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet and water ad libitum.

2.4 Administration Procedure

Rats were acclimatized to laboratory condition for 1 week before commencement of experiment. All procedures described conducted in accordance with Guideline for Care and Use of Faculty of Medicine, University of Indonesia. At 50 days of age, bilateral ovariectomy was performed via a dorsal midline incision under ketamine injection. Upon recovery from anesthesia, animals were assigned to experimental groups, normal (sham-operated), OVX, OVX-estradiol, OVX-Tamoxifen, OVX-FR 50, OVX-FR 100, and OVX-FR 200, with six animals per group, per experiment. Twenty days after ovariectomy, all the rats were allowed controlled access to a commercial standard pellet and free access to deionized water for 20 days. Normal (sham-operated) and OVX rats were sacrified under light anesthesia to determine the baseline at 70 days. From 70 days, estradiol (0, 1 mg/kg BW), FR (50, 100 and 200 mg/kg BW/day) was given orally using a stomach tube for 4 weeks. The food intake of all rats was measured everyday.

On the day after the last dose, the rats were blood collected from orbital plexus after and sacrified under light anesthesia. The uterus was removed and the wet weight, was determined. The femurs and tibiae were also removed immediately for bone analyses.

2.5 Mineral content

The femurs were also removed immediately after sacrified for bone analyses. The right and left femurs were freed of soft tissue. The removed right femurs were freed of soft tissue using small scissors, tweezers and cotton gauze. Then the bones were dehydrated and defatted in acetone and anhydrous ether, dried for 12 h at 110 °C and reweighed to obtain the dry bone weights. Bone calcium content in ash bone were determined by atomic absorption spectrophotometry (AAS).

2.6 Histological Analysis

The uterus and mammary glands were fixed in 10% buffered formalin for 48 hr. Uterine were cut for three cross section per area. Mammary glands were cut to obtain sections from the nipple through the fat pad toward the abdominal muscles. All samples were embedded in paraffin and 3-µm thick sections were cut, mounted, and stained with hematoxylin and eosin (H&E) for microscopic analysis

2.7 Statistical Analysis

Data from the animal experiments were expressed as the mean ± S.E.M. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni or LSD post-hoc test analysis using software SPSS. p values of less than 0.05 were considered to indicate significant differences.

3. Results

3.1 Body and Uterine Weights

The effect of Pomegranate fraction (PF) on average body weight gain and uterus are presented in table 1. As described in table 1, ovariectomized caused atrophy of uterus. This effect was prevented by the administration of estradiol and tamoxifen but not by PF 50, 100 and 200 mg/kg BW. In other hand, ovariectomy increased average daily body weight gain. This effect also was prevented by the administration of estradiol and tamoxifen and also by PF 50, 100 and 200 mg/Kg BW. The effect of PF 200 mg/kg BW was greater than estradiol and tamoxifen. This result indicated that PF work on body fat but not on uterine cell, this also indicated that PF works specifically on each type estrogen receptor.

3.2 Calcium Content of the Bone

The activities of Pomegranate Fraction on bone were demonstrated in table 2. The result demonstrated that OVX caused bone loss which determined by decreased bone density, content of calcium bone. The results shown that the administration of Pomegranate Fraction(PF) 50, 100 and 200 mg/kg BW for 28 days capable to increase calcium content in bone as well as the density of bone. The effect of PF on dose 100 mg/kg BW were same effect with tamoxifen and estradiol for calcium content.

Table 1: Body and uterine weight Values are means± S.E.M., n = 6 rats. Within a column, values with a superscript are significantly different: # p, 0.05 compared with sham rats; *p<0.05 compared with OVX rats.

Groups	Initial Body weight (g)	Final Body weight (g)	Average body weight gain (g/day)	Uterine Weight (g)
Sham	178.60 ± 8.98	198.27 ± 8.71	0,54	319.35 ± 76.68
OVX	196.85 ± 19.31	212.47 ± 24.28	1,04*	112.9 ± 57.73*
EST	193.22 ± 17.02	203.62 ± 17.72	0,14#	194.2 ± 135.65#
TAM	197.63 ± 16.43	180.47 ± 20.68	-0,34#	143,1 ± 20.27#
PF 50 mg/kgBW	196.77 ± 22.71	201.28 ± 17.38	0,32#	111.1 ± 5.22#
PF 100 mg/kgBW	203.38 ± 16.94	205.53 ± 21.52	0,02#	103.08 ±11.64#
PF 200 mg/kgBW	179.17 ± 7.52	177.00 ± 19.38	-0,24#	81.56 ± 18.56#

Table 2: Bone mineral content Values are means± S.E.M., n = 6 rats. Within a column, values with a superscript are significantly different: # p, 0.05 compared with sham rats; *p<0.05 compared with OVX rats.

Groups	Bone ash content of calcium
Sham	27.32 ± 3.69
OVX	16.52 ± 1.53*
EST	23.02 ± 1.53#
TAM	24.24 ± 4.51#
PF 50 mg/kg BW	20.96 ± 2.83#
PF 100 mg/kg BW	21.27 ± 2.91#
PF 200 mg/kg BW	19.94 ± 3.89

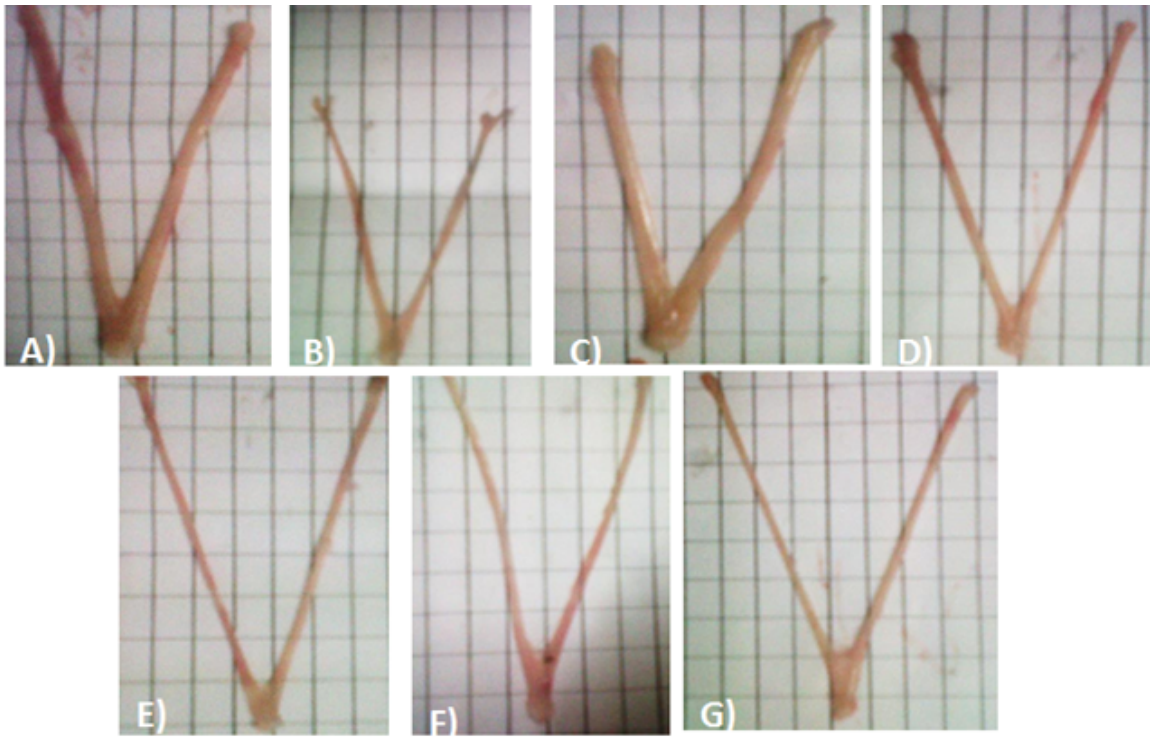

Fig 1: Uterine morphology. A). Sham-operated rat, B). Ovariectomized rat, C). Estradiol, D). Tamoxifen, E) FD 50 mg/Kg BW, F). FD 100 mg/kg BW, G). FD 200 mg/kg BW.

3.3. Effects of Pomegranate Fraction on Histomorphometry of Mammary Gland and Uterine wall.

Figure 1 shows morphology of uterus taken from one animal per treatment group. In ovariectomized rats the uterus showed decrease in size and become atrophy. The administration of estradiol could reverse the uterine, But the administration of Tamoxifen and

Punica Fraction only reverse the size of uterus without any effect on uterine wall as shown in Figure 2 and Table 4

3.4. Effects of Pomegranate Fraction on bone

Table 5 shows that Pomegranate fraction could increase osteoblast number of ovx rat, this finding seem could be the reason prevention of pomegranate fraction in bone.

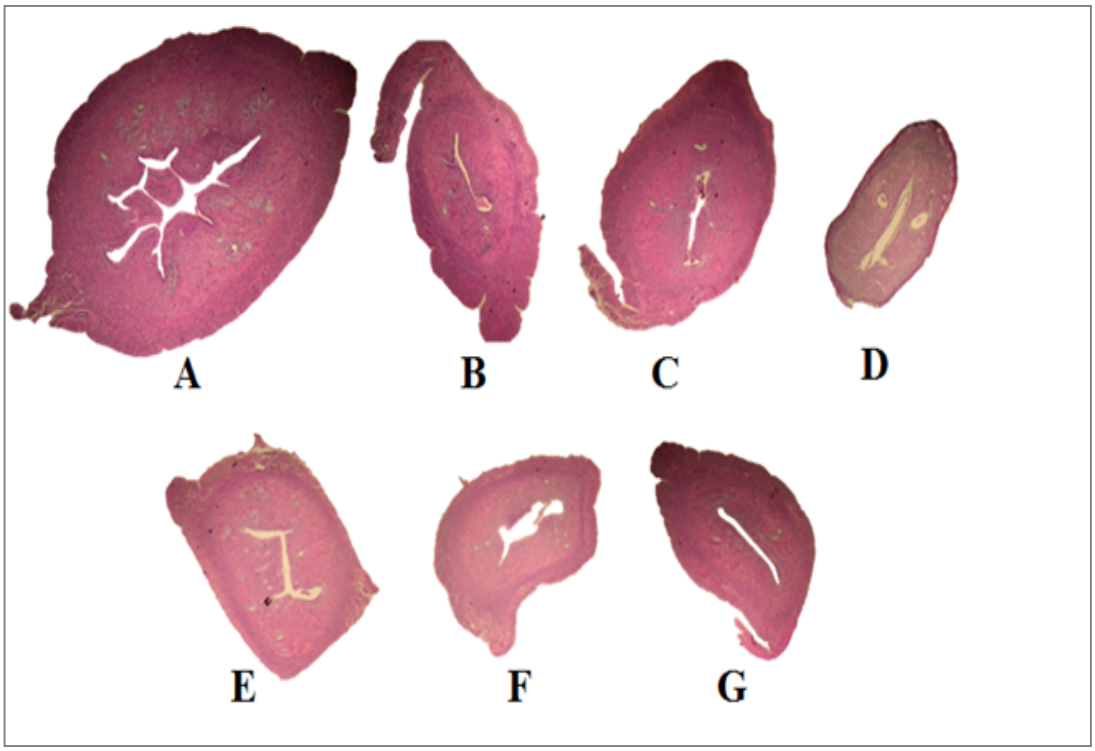

Fig 2: Photomicrographs of representative uterus walls sections A). Sham-operated rat, B).Ovariectomized rat, C). Estradiol, D). Tamoxifen, E) FD 50 mg/Kg BW, F). FD 100 mg/kg BW, G). FD 200 mg/kg BW.

Table 3: Quantification of Endometrium wall thickness and Uterine wall thickness Values are means± S.E.M., n = 6 rats. Within a column, values with a superscript are significantly different: # p, 0.05 compared with sham rats; *p<0.05 compared with OVX rats

Groups	Endometrium wall thickness (µm)	Uterine wall thickness (µm)
Sham	22,01 ± 5,37	34,01 ± 7,87
OVX	11,27 ± 3,96	18,40 ± 7,05
EST	16,00 ± 4,34	22,44 ± 5,56
TAM	9,57 ± 1,10	14,40 ± 1,42
PF 50 mg/kg BW	10,97 ± 1,49	15,42 ± 2,24
PF 100 mg/kg BW	10,09 ± 2,16	16,55 ± 4,11
PF 200 mg/kg BW	7,93 ± 1,18	12,90 ± 2,58

Table 4: Quantification of Endometrium wall thickness and Uterine wall thickness Values are means± S.E.M., n = 6 rats. Within a column, values with a superscript are significantly different: # p, 0.05 compared with sham rats; *p<0.05 compared with OVX rats

Groups	Osteoblast number	Osteoclast number
Sham	38,55 ± 3,87	5,2 ± 1,15
OVX	32,97 ± 4,21*	7,4 ± 1,20
EST	41,33 ± 2,91#	9,2 ± 5,09
TAM	37,33 ± 1,28#	7,5 ± 0,64
PF 50 mg/kg BW	36,4 ± 0,28#	6,4 ± 0,1
PF 100 mg/kg BW	39,3 ± 0,42#	9,0 ± 2,54
PF 200 mg/kg BW	38,8 ± 0,1#	7,2 ± 0,1

4. Discussion

The aims of this work were to investigate the effects of the pomegranate fraction on bone protection and effects on reproductive organs.

Ovariectomized rats are classically used as an animal model for studying the effect of postmenopausal bone loss. Furthermore, they may provide a useful model for investigating the biological effect of PF on bone loss in ovariectomized rats. Pomegranate Fraction from polar part of *Punica granatum* (L) extract contain tannins especially Ellagic acid [115, 16].

Previous study shown that ellagic acid prevent bone loss by increasing mineralization of bone through osteoblast. And others studies shows ellagic acid has no effect on epithelial cell of uterus and antiestrogenic on MCF7 cell lines. The present study was investigated the potential preventive effects of tannin-content pomegranate fraction of *Punica granatum* L. which contain ellagic acid to prevent bone loss in animal model of osteoporosis. The administration of PF prevented OVX-induced increase average body weight gain in rats. This results also support by previous study that compound that prevented OVX-induced uterine atrophy and increases in body weight gain, abdominal fat, serum total cholesterol and triglyceride. In addition, other study also reported that soybeans-rich isoflavones dietary interventions effectively reduce cholesterol serum in OVX-induced increased cholesterol serum in rats.

According with previous report, rats in the OVX group had lower densities of the right femur and tibiae because of reducing the ovariectomy-induced increase in bone resorption. The administration of Pomegranate fraction 50, 100 and 200 mg/kg BB effectively prevented OVX-induced lowering bone density. These observations are supported by previous study that ellagic acid significantly prevented bone loss in OVX rats by increasing mineralization of bone.

Further studies are needed to investigate the efficacy of that ellagic acid in humans.

5. Acknowledgements

This work was supported by Ministry of Education and Culture Grant

6. References

1. Lau EMC, Cooper C. The epidemiology of osteoporosis the oriental perspective in a world context. Clin Orthop 1996; 323:65–74.
2. Maggi S, Kelsey JL, Litvak J, Heyse SP. Incidence of hip fractures in the elderly a cross-national analysis Osteoporos Int 1991; 1:232–41
3. Lindsay R, Hart DM, Aitken JM, MacDonald ED, Anderson JB, Clarke AC. Long-term prevention of postmenopausal osteoporosis by oestrogen Lancet 1976; 1:1038–1041.
4. Hutchinson TA, Polansky SM, Feinstein AP. Postmenopausal oestrogens protect against fractures of hip and distal radius Lancet 2:706–709.
5. Lacey JV, Mink JR, Lubin PJ, Sherman JH, Troisi ME, Hartge P, *et al*. Menopausal hormone replacement therapy and the risk of ovarian cancer. JAMA 2002; 288:334–341.
6. Lindsay R, Hart DM, Clark DM The minimum effective dose of estrogen for prevention of postmenopausal bone loss. Obstet Gynecol. 1984; 63:759–763.
7. Anderson JJB, Garner SC. The effects of phytoestrogens on bone. Nutr Res 2007; 20:220–224.
8. Hertrampf T, Gruca MJ, Seibel J, Laudenbach U, Fritzemeier KH, Diel P. The bone-protective effect of the phytoestrogen genistein is mediated via ERα-dependent mechanisms and strongly enhanced by physical activity Bone 2007; 40:1529–1535.
9. Ishida H, Uesugi T, Hirai K, Toda T, Nukaya H, Yokotsuka K, Tsuji K. Preventive effects of the plant isoflavones daidzin and genistin, on bone loss in ovariectomized rats fed a calcium-deficient diet Biol Pharm Bull 1998; 21,62–66.
10. Michaëlsson K, Baron JA, Farahmand BY, Johnell O, Magnusson C, Persson PG, *et al*. Hormone replacement therapy and risk of hip fracture population based case-control study. The Swedish Hip Fracture Study Group. BMJ 1998; 316:1858–1863.
11. Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, *et al*. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis J Nutr 1998; 126:161–167.

12. Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, *et al*. The phytoestrogen genistein reduces bone loss in shortterm ovariectomized rats. Osteoporos Int 1998; 8:274–281
13. Glazier MG, Bowman MA. A review of the evidence for use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch Internal Med 2001; 161: 1161–1172
14. Sreekumar S, Thankayyan R, Santhosh K, Baddireddi SL, Sreeharshan S. Pomegranate extract demonstrate a selective estrogen receptor modulator profile in human tumor cell lines and in vivo models of estrogen deprivation. Journal of Nutritional Biochemistry 2012; 23:725–732
15. Sepúlveda L, Ascacio A, Rodríguez-Herrera R, Aguilera-Carbó A, Aguilar CN. Ellagic acid Biological properties and biotechnologicaldevelopment for production processes. African Journal of Biotechnology 2011; 10(22):4518-4523.
16. Mori-Okamoto J, Otawara-Hama moto Y, Yamato H, Yoshimura H. Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice. Journal of Ethnopharmacology 2004; 92:93–101.