Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

Document heading 10.1016/S2305-0500(13)60109-8

Estrogenic activity of *Punica granatum* L. peel extract

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ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form 28 February 2013
Accepted 1 March 2013
Available online 20 March 2013

Keywords: Postmenopausal syndrome
Biochemical parameters
Biomechanical parameters
Histopathological studies
Punica granatum

ABSTRACT

Objective: To evaluate the estrogenic effect of alcoholic extract of *Punica granatum* peel (ALPG) in ovariectomized (OVX) rats. **Methods:** Female wistar rats were divided into sham control and ovariectomized (OVX), OVX rats receiving standard drug, raloxifene (5.4 mg/kg) and groups treated with 500 mg/kg and 750 mg/kg of ALPG daily for 90 days. The vaginal cornification, uterine weight, bone loss, biomechanical, biochemical and histopathological observation were carried out to ascertain the effect of test drug in post menopausal syndrome. **Results:** The experimental animals treated with the ALPG showed dose dependent activity. The significant increase in uterine weight, femur BMD, femur hardness was observed. In addition, increased levels of calcium and phosphorus in serum and significant decrease in urine, were observed as compared to control OVX group. The histopathological results also confirm the protective effect of ALPG. **Conclusions:** The present findings strongly suggest that the peel of *P. granatum* possess potent estrogenic activity in ovariectomized rats and substantiate the ethnic use in treatment of postmenopausal osteoporosis.

1. Introduction

Menopause is the time of life when menstrual cycle ceases, and is caused by reduced secretion of the ovarian hormones oestrogen and progesterone which is characterized by low bone mass and microarchitectural deterioration of bone tissues, leading to enhanced bone fragility [1]. Many symptoms have been attributed to menopause, but only vasomotor dysfunction and vaginal dryness are consistently associated with this time of life in epidemiological studies. Other common symptoms such as mood changes, sleep disturbances, urinary incontinence, cognitive changes, somatic complaints, sexual dysfunction, and reduced quality of life may be secondary to other symptoms, or related to other causes [2]. Menopause brings about increased bone turnover, an imbalance between bone formation and bone resorption [3]. Estrogen deficiency is considered as the main determinant for bone loss in postmenopausal women [4]. Osteoporosis is caused by an imbalance in the normal bone remodelling process, in which there is excessive osteoclast resorption and adequate new bone formation by osteoblasts reduction. Hormone replacement therapy (HRT) has proven to be efficacious in preventing bone loss and reducing the incidence of skeletal fractures in postmenopausal women. However, long-term HRT increases the high risk of breast cancer, endometrial cancer, thromboembolic events and vaginal bleeding [5]. Traditional Indian medicines have been used from long days in prevention and treatment of postmenopausal osteoporosis. Since these medicines are prepared from medicinal plants they have fewer side effects and are suitable for long-term use. Punica granatum (Punicaceae) is a large shrub, known for estrogenic activity [6]. It has potent antioxidant activity also [7]. Tendency of pomegranate to reverse ovariectomy-related urothelial changes was determined by investigating different parameters in ovariectomized rats viz. the thickness of urogenital epithelium, tail skin temperature, serum levels of sex hormones, lipid profile, homocysteine, and thiobarbituric acid reactive substances [8]. It is conceivable that pomegranate juice is clinically effevtive on a depressive state and bone loss in post menopausal syndrome in women [9]. Pomegranate components possess an ability to inhibit the estrogenic action of 17-β-estradiol, an activity best explained through competitive binding to estrogen receptors by a number of non-steroidal estrogenic flavonoids such as kaempferol, quercetin, naringenin, and luteolin [10]. A methanolic eluate of pomegranate juice competed with 17-β-estradiol for estrogen receptors, stimulated estrogen receptor

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positive (ER+), MCF-7 breast cancer cells and increased uterine weight in ovariectomized mice [11], though elsewhere, pomegranate fermented juice inhibited MCF-7 growth through a range of concentrations [10]. Further, both oral and intramuscularly injected pomegranate seed oil increased uterine weight and vaginal cornification in ovariectomized mice and immature female rabbits, respectively [12-13]. The present study is designed to investigate the estrogenic property of the fruit peel of pomegranate in ovariectomized rats.

2. Materials and methods

2.1. Chemicals and reagents

Calcium, phosphorous and alkaline phosphatase (ALP) kits were obtained from Pathozyme Diagnostics, India. Raloxifene obtained from Dr. Reddy Limited and ketamine obtained from Bharat Serum and Vaccines Limited, India. All other chemicals and reagents were of analytical grade.

2.2. Plant material and preparation of extract

Punica granatum (P.granatum) peels were obtained from the Local Market, Pune, India, dried under shade, powdered and preserved in air tight container. The coarsely powdered materials (150 g) were extracted with 400 mL of 95% ethanol using soxhlet apparatus. The extract was evaporated by rotary vacuum evaporator to dryness and preserved until further use. The yield of the extract was found to be 34.5%.

2.3. Preliminary phytochemical screening

Therapeutic potential of vegetable drugs depends upon the type of constituents present in them. The above extract was screened for the presence of various groups of phytoconstituents viz. alkaloids, carbohydrates, glycosides, saponins, steroids, proteins, amino acids, tannins, flavonoids and organic acids using different chemical tests [14-16].

2.4. Animals

Female Wistar rats weighing 220-250 g were used for study. The animals were housed in polypropylene cages and maintained under the environmental conditions of temperature of (25±1)℃, relative humidity of 45%-55% and 12h light: 12h dark cycle. The animals had free access to food pellet and water *ad libitium*. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA).

2.5. Acute oral toxicity study

Acute toxicity was measured by administering a dose 2 000, 3 000 and 5 000mg/kg b.w. orally of ALPG. The mice were observed by housing them individually in the polypropylene metabolic cages continuously for 14 days. At this dosage, neither mortality nor any signs of clinical abnormality were observed in the treated group. The dosage of 500 and 750 mg/kg were selected for the pharmacological screening.

2.6. Estrogenic activity of test drug

2.6.1. Experimental protocol

The female Wistar rats were divided into 5 groups of 6 rats in each and treated as follows:

Group-I: Sham operated receive vehicle (Saline water)

Group-II: Ovariectomized control receives vehicle (Saline water)

Group-III: Raloxifene (5.4 mg/kg) in ovariectomized rats

Group-IV: ALPG (500 mg/kg) in ovariectomized rats

Group-V: ALPG (750 mg/kg) in ovariectomized rats

After seven days of acclimation, the rats were ovariectomized and sham operated. The rats were anesthetized with Ketamine HCl (80 mg/kg, *i.p*), and ovaries were removed bilaterally. Sham operated animals were performed in same manner, but only exposing the ovaries [17]. The treatment of test drugs by oral gavages administration continued for 90 days. Body weight of all animals was measured at the beginning and end of the dosage schedule. At the end of 90 days, all the rats were deprived of food for whole night. On the next day, urine (0-24 h) was collected, then the animals were anesthetized by anaesthetic ether and blood samples were withdrawn by retro orbital puncture. The blood samples were centrifuged (Remi R4C, India) at 2 500 rpm for 15 min to separate the serum and preserved (-20 ℃) for analysis of calcium, phosphorus and alkaline phosphatase (ALP) [18]. Immediately, after collecting the urine and blood samples, uterus was carefully removed and weighed. The Femur bones were isolated and stored at -70 ℃ until biomechanical and histopathological studies were performed.

2.6.2. Effect on vaginal cornification

The various stages of estrus cycle can be identified by preparing the vaginal smears and observing under microscope. An adult (2-3 month old) female rat has an estrus cycle of 5 days. The drugs were administered twice daily on two following days at 10.00 AM and 5.00 PM, continued at 5.00 PM of the third day and at 10.00 AM on the fourth day. On fourth day vaginal fluids were collected by inserting the tip of dropper filled with 1-2 mL of normal saline [sodium chloride (NaCl) 0.9%] into the rat vagina. A drop of vaginal fluid smeared on the slide and observed under light microscope, with 10 and 40× objective lenses. The smears were examined microscopically and scored according to the following guidelines [19]:

0: Diestrus phase- mainly leukocytes with few epithelial cells

1: Presence of mixture of leukocytes and epithelial cells

2: Proestrus phase- Presence of nucleated or nucleated plus corniified cells

3: Estrus phase- Presence of corniified cells only.

Only animals showing score of 2 or 3 were considered to be positive for estrogenic activity.

2.6.3. Determination of biochemical parameters

The calcium level of serum and urine was estimated by Calcium Modified Arsenazo method, level of phosphorus by Phosphorus Molybdate UV method and serum alkaline phosphatase activity were estimated by alkaline phosphatase DEA-PNPP method using commercially available test kits [20-21].

2.6.4. Determination of biomechanical parameters

After removing the blood for biochemical analysis the animals were sacrificed by cervical dislocation and the femurs were collected. The bone was dried overnight in the oven and bone marrow was carefully removed. The thickness of femur was measured with a vernier calliper. Bone was also weighed. Bone volume was measured by using plethysmometer (UGO Basile, Model. No. 7140, Italy) and bone density was calculated (mass/volume). The breaking strength of right femur was also measured using hardness tester (Pharma Test PTB, Incorp., India) [17, 21].

2.6.5. Determination of body weight and organ weight

Body weight of each animal was measured on day 1 and again on the last day of the dosage regimen (90th day), increase in body weight was determined for all the groups; and organ weight (uterus) of each rat was recorded at the end of the experiment [21-22].

2.6.6. Histopathology study

The right femur was fixed in 10% formalin for 12 h at 4 ℃, decalcified in 5% ethyenediamine tetracetic acid (EDTA) for 7 days and embedded in paraffin wax and cut into sagittal plane section of 5 μm thickness of the femur. The sections were stained with hematoxylin and eosin (HE), and examined for histopathological changes under a light microscope [23].

2.7. HPTLC finger printing profile of P. granatum extract and analysis of ellagic acid

One milligram per millilitre ellagic acid standard stock solution was prepared in methanol. Standard working solutions were prepared by diluting 100 μL stock solution into 1 mL with methanol.

To determine the presence of ellagic acid in the extract, 100 mg of pomegranate extract was transferred into 50 mL methanol, sonicated for 10 min. Filtered on a Whatman no. 1 filter paper, an aliquot of sample 10 μL was applied on the HPTLC plate. The samples were spotted in the form of bands with a Camag microlitre syringe on a precoated silica gel aluminium plates 60F-254 (10 cm × 10 cm width, 250 mm thickness, E.merck, Darmstadt, Germany) using a Camag Linomat IV (Muttenz, Switzerland) applicator. The plates were prewashed by methanol and activated at 60 ℃ for 5 min prior to chromatography.

Linear ascending development was carried out in 10 cm ×10 cm twin trough glass chamber (Camag Muttenz, Switzerland) using mobile phase consists of Toluene : Ethyle acetate : Formic acid : Methanol (3.0:3.0:0.8:0.2). Subsequent to scanning, TLC plates were air dried and scanning was performed on a Camag TLC scanner III in absorbance mode at 280 nm and operated by Cats software 4.03 version.

2.8. Statistical analysis

Data are expressed as the mean values ± S.E.M. (n=6). Inter-group differences were analyzed by one-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison Test as post-test after ANOVA was performed to compare the group means by the statistical software (GraphPad Prism5). P<0.05 was considered statistically significant.

3. Results

3.1. Preliminary phytochemical screening

Preliminary phytochemical screening revealed presence of alkaloids, proteins, amino acids, steroids, flavonoids, organic acids, tannins and phenolic compounds in the alcoholic extract of *P. granatum* peel (ALPG) (Table 1).

Table 1
Preliminary phytochemical investigation.

Group of phytoconstituent	Alcoholic extract of *P. granatum* peel
Alkaloids	+
Carbohydrates	-
Proteins	+
Amino acids	+
Steroids	+
Cardiac glycosides	-
Anthraquinone glycosides	-
Saponin glycosides	-
Flavonoids	+
Tannins and phenolic compounds	+
Organic acids	+

+ indicates present and - indicates absent.

3.2. Acute oral toxicity

The test drug did not show any signs of toxicity or mortality even at 5 000 mg/kg of body weight dose during the observation period. Therefore, 500 mg/kg of body weight and 750 mg/kg of body weight of test drug were used for pharmacological studies.

3.3. Estrogenic activity of test drug

3.3.1. Effect on vaginal cornification

The vaginal epithelium cells observed under the microscope and representative cell type was determined by choosing the majority of cells. The appearance of cornified cells was used as an indicator of estrogenic activity. It was observed that treatment with raloxifene showed only cornified cells in the vaginal smear (score-3). Treatment with test drug (both 500 and 750 mg/kg) exhibited the presence of nucleated as well as cornified cells (score-2), but the majority of cells are cornified in the group administered with the higher dose of the test drug. The

vaginal smear of ovariectomized control did not show any vaginal cornification (score–0).

3.3.2. Biochemical parameters

The serum alkaline phosphatase level was significantly increased in the OVX group when compared with the sham control, but significant (P<0.05) differences were found in the level of serum alkaline phosphatase in ovariectomized rats to treated ovariectomized rats. Test drug at the higher dose (750 mg/kg) showed better effect than the standard drug (Raloxifene) also (Table 2). The decrease in serum calcium and phosphorus was significantly (P<0.05) increased by the standard drug and the test drug (750 mg/kg). Lower dose of the test drug did not show any significant change in the serum calcium and phosphorus level (Table 2). Loss of calcium and phosphorus ion in urine was significantly (P<0.05) decreased when rats were treated with raloxifene and test drug when compared to ovariectomized rats (Table 2).

3.3.3. Biomechanical parameters

As shown in Table 3, the femoral weight, volume, density, thickness and hardness were significantly changed in ovariectomized rats as compared to sham operated animals. In groups treated with raloxifene (standard drug) and test drug (750 mg/kg b.w.) significantly (P<0.05) changed femoral weight, volume, density, thickness and breaking strength when compared with those in ovariectomized control (Table 3). The low dose of the test drug (500 mg/kg b.w.) exhibited significant change in body weight only, when compared with OVX rats.

3.3.4. Body weight and organ weight

The mean values of body weight and uterus weight of sham control, ovariectomized and treated animals are shown in Table 4.

The mean body weight gain was (39.00±2.46) g in OVX group which was significantly greater than sham control group, (25.17±2.33) (P<0.05). However, this ovariectomy–induced increase in body weight was abolished by the administration of the test drug (750 mg/kg b.w.). Low dose of the test drug did not show any significant decrease in body weight when compared with OVX group.

The mean uterus weight was dramatically decreased to a level of one–fourth by ovariectomy: 121.0±5.45 mg for sham control versus 31.83±0.70 mg for OVX group (Table 4). However, administration of raloxifene (standard drug) and test drug (750 mg/kg b.w.) after ovariectomy prevented the loss of uterus weight showing a significant difference (P < 0.05) when compared with OVX group.

3.3.5. Histopathology study

The ovariectomy resulted in a significant reduction in

Table 2

Effect of test drug on biochemical markers in ovariectomized rats.

Group/treatment	Alkaline phosphatase (IU/L)	Serum calcium (mg/dL)	Serum phosphrous (mg/dL)	Urine calcium (mg/dL)	Urine phosphrous (mg/dL)
Sham control	228.00±7.86	8.30±0.30	7.10±0.29	2.10±0.09	5.10±0.32
Ovariectomized control (OVX)	337.70±9.10*	6.30±0.16*	2.30±0.36*	4.70±0.22*	8.30±0.47*
OVX + Raloxifene (RL)	224.90±11.60**	8.00±0.23**	5.20±0.31**	2.80±0.14**	7.10±0.44**
OVX + ALPG (500 mg/kg b.w)	265.20±14.10**	6.90±0.14	3.60±0.36	3.40±0.20**	7.70±0.26
OVX + ALPG (750 mg/kg b.w)	203.40±11.50**	7.80±0.25**	4.30±0.22**	2.20±0.21**	6.70±0.37**

Values are represented as mean ± SEM (n=6); *P<0.05 *vs.* sham control; **P<0.05 *vs.* OVX.

Table 3

Effect of test drug on biomechanical parameters in ovariectomized rats.

Group/Treatment	Bone weight (g)	Bone volume (mL)	Bone density (g/mL	Bone thickness (cm)	Bone breaking strength (N)
Sham control	0.660±0.012	0.570±0.012	1.160±0.008	0.230±0.011	60.50±1.96
Ovariectomized control (OVX)	0.440±0.007*	0.430±0.012*	1.020±0.027*	0.090±0.015*	42.00±2.28*
OVX + Raloxifene (RL)	0.640±0.008**	0.550±0.010**	1.150±0.006**	0.240±0.015**	62.83±3.40**
OVX + ALPG (500 mg/kg b.w)	0.520±0.015**	0.430±0.008	1.040±0.006	0.150±0.019	52.33±3.68
OVX + ALPG (750 mg/kg b.w)	0.610±0.008**	0.530±0.011**	1.150±0.005**	0.260±0.02**	65.00±4.64**

Values are represented as mean ± SEM (n=6); *P<0.05 *vs.* Sham control; **P<0.05 *vs.* OVX.

Table 4

Effect of test drug on body weight and uterus weight in ovariectomized rats.

Group/treatment	Body weight (g)			Uterus weight (mg)
	Initial	Final	Gain	
Sham control	224.50±5.65	251.30±6.15	25.17±2.33	121.00±5.45
Ovariectomized control (OVX)	220.80±5.23	259.80±7.06	39.00±2.46*	31.83±0.70*
OVX + Raloxifene(RL)	221.60±5.86	248.10±7.39	26.50±2.57**	58.50±7.54**
OVX + ALPG (500 mg/kg b.w)	224.00±5.70	256.30±6.28	32.33±1.99	46.17±6.12
OVX + ALPG (750 mg/kg b.w)	221.30±5.09	247.00±5.67	25.67±2.37**	59.17±2.65**

Values are represented as mean ± SEM (n=6); *P<0.05 *vs.* sham control; **P<0.05 *vs.* OVX.

trabecular thickness compared to the sham control groups. Treatment with raloxifene and the test drug significantly increased the trabecular thickness compared to the OVX group (Table 5).

Table 5
Effect of test drug on trabecular width of femur.

Group/treatment	Trabecular width (μ m)
Sham control	193.20
Ovariectomized control (OVX)	96.60*
OVX + Raloxifene(RL)	165.60**
OVX + ALPG (500 mg/kg b.w)	124.20**
OVX + ALPG (750 mg/kg b.w)	151.80**

Values are represented as mean±SEM (n=6); *P<0.05 *vs.* sham control; **P<0.05 *vs.* OVX.

3.4.HPTLC finger printing profile of P. granatum extract

The presence of ellagic acid in the pomegranate extract was confirmed from the chromatograms (Figure 1 & 2) and the concentration of ellagic acid in the pomegranate extract was found to be 11.8% *w/w*.

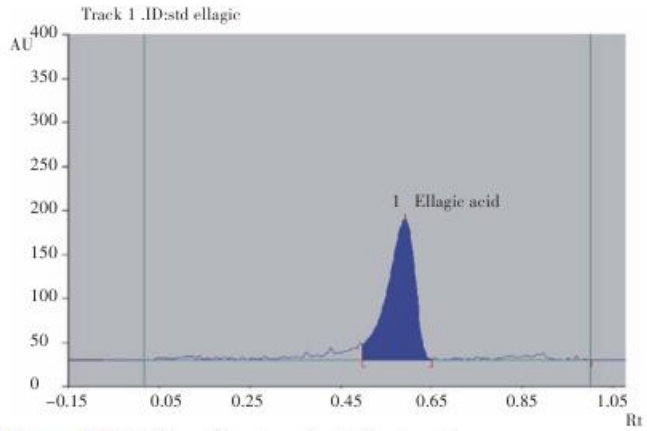

Figure 1. HPTLC profile of standard ellagic acid.

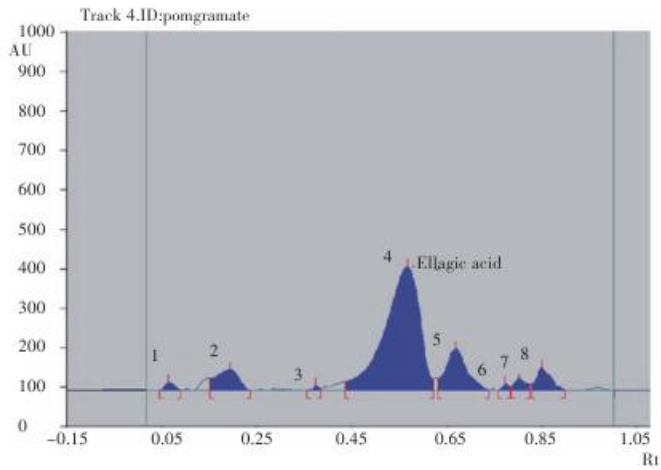

Figure 2. HPTLC profile of pomegranate extract.

4. Discussion

Vaginal cytology assay is particularly used to determine the estrogenic activity of the synthetic estrogens. It is a sensitive, simple and inexpensive method to predict the estrogenic activity. The assay can be performed in either immature or ovariectomized rodents [24]. Our results confirm the estrogenic activity of the test drug which may be due to the phytoestrogens present in the drug.

The most common type of osteoporosis is the bone loss associated with ovarian hormone deficiency at menopause. Especially, in womens the estrogen deficiency, results to augment plasma calcium levels as a result of increased bone resorption [25]. The ovariectomized rat exhibited most of the characteristics of human postmenopausal osteoporosis [26]. Increase in loss of calcium and phosphorous through excretion in urine are supporting factor for bone loss in the ovariectomized rats [27]. Similarly, the increase in level of ALP was observed with respect to decreased serum calcium and phosphorus in control OVX rats. Significant fall in serum calcium and phosphorus was also observed in OVX animals. Significant decrease in serum ALP of Test drug treated OVX rats which may be a suggestive factor for enhanced bone formation may account for bone disorders [28]. The decrease in urine calcium and phosphorus, and increase in serum calcium and phosphorus by the test drug as compared to OVX group indicates the antiosteoporetic activity of the drug.

Estrogen deficiency is a well-known risk factor in the pathogenesis of osteoporosis. Our present study clearly demonstrated the usefulness and beneficial effects of the test drug in the treatment of osteoporosis induced by ovariectomy. Estrogen influences bone loss, either directly by binding to the receptor on the bone or indirectly by influencing calcium regulatory hormones (PTH and vitamin D) and cytokines IL-1 and IL-6. Biomechanical data suggest that bone becomes stronger after treatment with the test drug, which may be due to enhanced mineralization of the bone [20]. Interestingly, the test drug (750 mg/kg b.w.) shows better activity to that of a standard antiosteoporotic drug, raloxifene. It is well known that ovariectomy induces the increase of body weight [29]. Also in the present study, the body weight of the animals increased after ovariectomy, and the increase was inhibited by administration of test drug. Regarding the role of estrogens in lipid metabolism, estrogen insufficiency is thought to be largely responsible for an increase in adiposity during menopause because postmenopausal women under estrogen replacement therapy do not display the characteristic pattern of abdominal weight gain usually associating with menopause [30]. Thus, the test drug is able to regulate the lipid metabolism.

The mean uterus weight was dramatically decreased in ovariectomized control and administration of test drug after ovariectomy prevented the ovariectomy-induced loss of uterus weight. Hence, it may be said that the test drug is effective on the development and function of the uterus as well.

The activity of the test drug on the thickness of trabecular bone is indicative of the antiosteoporotic activity of the drug. The observed osteoprotective role may be attributed to its phytogenic, steroid-like components present in the extracts. The anti-osteoporotic activity of the test drug may be justifiably attributed to its steroid components, which probably act as phytoestrogens to prevent bone loss [31].

Reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anions, which are produced as by-

products in aerobic organisms, are implicated in oxidative damage to various cellular macromolecules. Antioxidant enzymes including super-oxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) *etc.*, constitute an important defense system in clearing up the detrimental ROS *in vivo*. Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage. Against this background, evaluation of the antioxidant properties of specific chemical scavengers is of particular importance for their potential use in preventing or limiting the damage induced by free radicals. A number of classes of drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity. Hence, ellagic acid which is present in pomegranate extract and a known antioxidant agent might be somehow responsible for the various activities of the plant.

Basing on the findings of the present study it can be concluded that the test drug could be used to treat the complications of menopause. Further, the test drug at higher dose exhibited better response than the standard drug (raloxifene). However, further studies are required to investigate the molecular mechanism of action of the drug and to confirm the specific phytoconstituent(s) responsible for the estrogenic activity.

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