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Original Article

Estrogenic Effect of *Asparagus racemosus, Cissus quadrangularis, Punica granatum* and *Pueraria tuberosa* in Post-menopausal Syndrome

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ABSTRACT

Background: *Asparagus racemosus, Cissus quadrangularis*, *Punica granatum* and *Pueraria tuberosa* are employed for their supposed properties in treatment in alleviating symptoms of menopause traditionally from ages. The aim of the present study is to evaluate the estrogenic effect of *Asparagus racemosus, Cissus quadrangularis*, *Punica granatum* and *Pueraria tuberosa* in ovariectomized (OVX) rats. **Materials and 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 700 mg/kg of test drug (containing equal quantity of *Asparagus racemosus, Pueraria tuberose, Cissus quadrangularis* and *Punica granatum* extracts) 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 mixture of extract of four drugs 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 decreased in urine were observed as compared to control OVX group. The histopathological results also confirm the protective effect of extracts. **Conclusion:** The present findings strongly suggest that *Asparagus racemosus, Cissus quadrangularis*, *Punica granatum* and *Pueraria tuberosa* possess the potent estrogenic activity in ovariectomized rats and substantiate the ethnic use in treatment of postmenopausal osteoporosis.

Key words: Menopause, Estrogenic, Post menopausal syndrome, Ovariectomized (OVX) rats, Biochemical, Histopathological.

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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 microarchictectural 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 post-menopausal osteoporosis. Since these medicines are prepared from medicinal plants they have fewer side effects and are suitable for long-term use. *Asparagus racemosus* (Asparagaceae), known as 'Shatavari' is a spine scent ethinomedicine, traditionally known for menopausal disorders and osteoporosis. *Asparagus racemosus* has been reported for its potential galactogogue and nervine tonic activity.[5] *Cissus quadrangularis* (Vitaceae), known as 'Hadjod' is a cactus like herb, known for osteoporosis.[6] *Cissus quadrangularis* has antiulcer and cytoprotective activity.[7] *Punica granatum* (Punicaceae) is a large shrub, known for estrogenic activity.[8] It has potent

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antioxidant activity also.[9] *Pueraria tuberosa* (Fabaceae), known as 'Vidarikand' is a climber, known for its hypolipidemic activity.[9]

MATERIALS AND METHODS

Procurement and authentification of raw materials

Powered drugs of *Asparagus racemosus* roots and tubers and *Pueraria tuberosa* tubers with specified physicochemical characteristics were purchased from, NIEC, lucknow, India. *Punica granatum* peels were obtained from the Local Market, Lucknow, India. Fresh *Cissus quadrangularis* stem was procured from NBRI, Lucknow, India and identified by Dr. A K S Rawat, NBRI, Lucknow, India. *Punica granatum* peels and *Cissus quadrangularis* stems were dried under shade, powdered and preserved in air tight container.

Extraction and preliminary phytochemical screening of the test drugs

Extraction of *Asparagus racemosus* roots and tubers

Defatted air-dried plant powders were extracted with methanol in soxhlet apparatus set at 60°C for 24 hr. The solvent was evaporated at 50°C using rotary vacuum evaporator to obtain a semisolid extract and stored in a deep freezer.[10]

Extraction of *Cissus quadrangularis* stem

The coarsely powdered plant material was exhaustively extracted with 95% ethanol using a soxhlet apparatus. The total ethanol extract was concentrated in a vacuum and preserved until further use.[11]

Extraction of *Punica granatum* peel

Dried pomegranate peels weighing 1kg was extracted with 2lt of 95% ethanol. The extract was concentrated using vacuum evaporator and stored until further use.[12]

Extraction of *Pueraria tuberosa* tubers

The tubers powder was extracted with 95% ethanol in a soxhlet apparatus set at 60-80°C for 18hrs.The alcoholic extract was subjected to evaporation in a beaker in a water bath maintained at 50°C till a thick paste of extract remained in the beaker and was kept in refrigerator below 4°C till the experimental study.[13]

Preliminary phytochemical screening of different extracts

Therapeutic potential of vegetable drugs depends upon the type of constituents present in them. The above extracts of the plants were 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]

Preparation of drug solution (test drug)

Equal amount of the extracts of different plant material were mixed; using the powder mixture, fresh solution was prepared by dissolving in distilled water every day for animal studies.

Acute oral toxicity study

Animals

Either sex immature and Swiss albino mice (18-23gm) were purchased from Yash Pharm, Pune, India. They were maintained at a temperature of 25±1°C and relative humidity of 45 to 55% under 12h light: 12h dark cycle. The animals had free access to food pellets (NIEC, Lucknow, India) and water was available *ad libitum*. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Northern India Engineering College, Lucknow. Constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA).The protocol approval number is Registration no. 319/01/ab/CPCSEA.

Treatment schedule

Acute toxicity was measured by administering a dose 2000, 3000 and 5000mg/kg b.w. orally of the test drug (mixture of all four extracts in equal amount). The mice were observed by housing them individually in the polypropylene metabolic cages continuously for 2h for behavioral, neurological and autonomic profiles and for any lethality during next 48h.

Estrogenic activity of test drug

The most common type of osteoporosis is the bone loss associated with ovarian hormone deficiency at menopause. It is well known that in human females estrogen deficiency caused by ovariectomy (OVX) as a well as menopause leads to acceleration of bone resorption and rapid bone loss resulting in osteoporosis. The ovariectomized rat is the most appropriate model for studying the mechanism as well as potential treatments of post-menopausal osteoporosis in humans and is useful model to study the efficacy of various pharmaceutical candidates for their prevention and or reversal of bone loss. Rodents do not experience a natural menopause but OVX has become a time honored method used to produce an artificial menopause.

Animals

Female Wistar rats weighing 220-250 gm were purchased from Yash Pharm., Pune, India. The animals were housed in polypropylene cages and maintained under the environmental conditions of temperature of 25±1°C, relative humidity of 45-55% and 12h light: 12h dark cycle. The animals had free access to food pellet (NIEC, Lucknow) and water *ad libitum*. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of NIEC, Lucknow constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). The protocol approval number is Registration no. 319/01/ab/CPCSEA.

Drugs and chemicals

The drugs and chemicals used were Ketamine Hydrochloride Injection (Ketavan, Bharat Serum and Vaccines Limited), Raloxifene Hydrochloride Tablets (Fiona tablet, Dr. REDDY'S Limited).

Ovariectomy

The procedure was carried out by the method described.[17] The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (80mg/kg body weight). A single transversion was made on the dorsolateral side of the back. The incision was shifted readily from one side to the other side so as to lie over each ovary in turn. A small nick was then made over the site of the ovary. The ovary was seen through the abdominal wall, embedded in pad fat. The top pair of a force was introduced and fat around the ovary was grasped. Care was taken so that capsule around ovary was not ruptured.

The tip of uterine horn was ligated and ovary, together with the fallopian tube was removed with a single cut by a pair of scissors. The ovary of the other side was also removed in the same way. Skin wound was closed with cotton sutures. The whole procedure is depicted in Figure. The animals were housed individually in polypropylene cages during recovery period of 21 days.

Treatment schedule

Animals were divided into various groups and treated with vehicle / standard drug / test drug as given below:

Group I: Sham control group (n = 6) underwent sham surgery, in which the ovaries were exposed but not removed and treated with normal saline.

Group II: The ovariecomized (OVX) group (n = 6) received an ovariectomy and treatment with normal saline for 90 days commencing from 22nd post ovariectomy day.

Group III: The ovariectomized + raloxifene (OVX + RL) group (n = 6) received an

ovariectomy and were treated with raloxifene (5.4 mg/kg/day, p.o.) for 90 days commencing from 22nd post ovariectomy day.

Group IV: The ovariectomy + test drug (OVX + T1) group (n=6) received an ovariectomy, and were treated with the test drug (500 mg/kg body weight daily, p.o.) for 90 days commencing from 22nd post ovariectomy day.

Group V: The ovariectomy + test drug (OVX + T2) group (n=6) received an ovariectomy, and were treated with the test drug (750 mg/kg body weight daily, p.o.) for 90 days commencing from 22nd post ovariectomy day.

Effect on vaginal cornification

The method described by the Vogel and Vogel, 2002 was used. 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 estrus cycle can be divided into different stages:

1. Diestrus phase characterized by the presence of leukocytes in the vaginal smear.
2. Proestrus phase characterized by the presence of large number of nucleated epithelial cells.
3. Estrus phase characterized by the presence of corniified epithelial cells and leukocytes (Meta or late estrus).

The drugs were administered twice daily on two following days at 10:00 AM and 5:00 PM and continued 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 1-2ml of normal saline [sodium chloride (NaCl) 0.9%] into the rat vagina. A drop of vaginal fluid smeared on the slide. Unstained vaginal smear was observed under light microscope, with 10 and 40 x objective lenses. The smears were examined microscopically and scored according to the following guidelines.[18]

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.

Determination of biochemical parameters

Animals were anaesthetized with anesthetic ether. After 90 days of drug treatment blood was withdrawn by retro orbital puncture and was collected separately in EDTA tubes. The blood in the EDTA tubes was centrifuged to separate the serum. Serum from EDTA tube was used for estimation of calcium (by calcium modified arsenazo method), phosphorus (by phosphorus molybdate uv method) and alkaline phosphatase (by alkaline phosphatase DEA-PNPP method).[19,20] For the estimation of alkaline phosphatase, calcium and phosphorus the kits were purchased from Pathozyme Diagnostics, India.

12h of urine collection was initiated one day before termination of study. Urine was collected in acid-washed tubes and total volume was measured and acidified with HCl. Urine calcium and phosphorous were estimated by Calcium Modified Arsenazo Method and Phosphorus Molybdate UV Method as discussed above.

Determination of biomechanical parameters

After removing the blood for biochemical analysis the animals were sacrificed by cervical dislocation and the femurs were collected. The right femur was removed and was freed of soft tissue using small scissors, tweezers and cotton gauge. 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 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).[19,17]

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.[21,19]

Histopathology study

The animals of the respective groups were then systematically necropsied and left femur were collected, fixed in 10% neutral buffered formalin (NBF) for 12h at 4°C, decalcified in 5% ethylenediamine tetra acetic acid (EDTA, p^{H} 7.4) for 7 days, embedded in paraffin, and cut into longitudinal sections of 5µ thickness. The sections were stained with hematoxylin and eosin (H&E). Then the slides were prepared and the thickness of individual trabeculae was measured by using a calibrated ocular micrometer in the microscope.[22]

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.

RESULTS

Preliminary phytochemical screening

The extracts of all the four plants were screened for various groups of phytoconstituents and the observations were furnished in Table 1.

Acute oral toxicity

The test drug did not show any signs of toxicity or mortality even at 5000 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.

Estrogenic activity of test drug
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

Table 1: Preliminary phytochemical investigation of different plant materials.

Group of phytoconstituent	Alcoholic extract of			
	P. granatum peel	*A. racemosus* root	*P. tuberosa* tuber	*C. quadrangularis* stem
Alkaloids	+	-	-	+
Carbohydrates	-	+	+	+
Proteins	+	-	-	-
Amino acids	+	-	-	-
Steroids	+	+	+	+
Cardiac glycosides	-	-	-	-
Anthraquinone glycosides	-	-	-	-
Saponin glycosides	-	+	-	+
Flavonoids	+	+	+	+
Tannins and phenolic compounds	+	-	+	+
Organic acids	+	+	+	+

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).

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).

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

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	228.0 ± 7.86	8.3±0.30	7.1±0.29	2.1±0.09	5.1±0.32
Ovariectomized control (OVX)	337.7 ± 9.1*	6.3±0.16*	2.3±0.36*	4.7±0.22*	8.3±0.47*
OVX + Raloxifene (RL)	224.9 ± 11.6**	8.0±0.23**	5.2±0.31**	2.8±0.14**	7.1±0.44**
OVX + T1 (500 mg/kg b.w)	265.2 ± 14.1**	6.9±0.14	3.6±0.36	3.4±0.2**	7.7±0.26
OVX + T2 (750 mg/kg b.w)	203.4 ± 11.5**	7.8±0.25**	4.3±0.22**	2.2±0.21**	6.7±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 (gm)	Bone Volume (ml)	Bone Density (gm/ml)	Bone Thickness (cm)	Bone breaking strength (N)
Sham control	0.66±0.012	0.57±0.012	1.16±0.008	0.23±0.011	60.50±1.96
Ovariectomized control (OVX)	0.44±0.007*	0.43±0.012*	1.02±0.027*	0.09±0.015*	42.00±2.28*
OVX + Raloxifene (RL)	0.64±0.008**	0.55±0.010**	1.15±0.006**	0.24±0.015**	62.83±3.4**
OVX + T1 (500 mg/kg b.w)	0.52±0.015**	0.43±0.008	1.04±0.006	0.15±0.019	52.33±3.68
OVX + T2 (750 mg/kg b.w)	0.61±0.008**	0.53±0.011**	1.15±0.005**	0.26±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.

S.No.	Group/treatment	Body weight (gm)			Uterus weight (mg)
		Initial	Final	Gain	
1.	Sham control	224.5±5.65	251.3±6.15	25.17±2.33	121.0±5.45
2.	Ovariectomized control (OVX)	220.8±5.23	259.8±7.06	39.00±2.46*	31.83±0.70*
3.	OVX + Raloxifene(RL)	221.6±5.86	248.1±7.39	26.50±2.57**	58.50±7.54**
4.	OVX + T1 (500 mg/kg b.w)	224.0±5.7	256.3±6.28	32.33±1.99	46.17±6.12
5.	OVX + T2 (750 mg/kg b.w)	221.3±5.09	247.0±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

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

S.No.	Group/treatment	Trabecular width (µm)
1.	Sham control	193.20
2.	Ovariectomized control (OVX)	96.60*
3.	OVX + Raloxifene(RL)	165.60**
4.	OVX + T1 (500 mg/kg b.w)	124.20**
5.	OVX + T2 (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

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.

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. As shown in Table 4 the mean body weight gain was 39.00±2.46 gm 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.

Histopathology Study

The ovariectomy resulted in a significant reduction in 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 and Figures 1a-e).

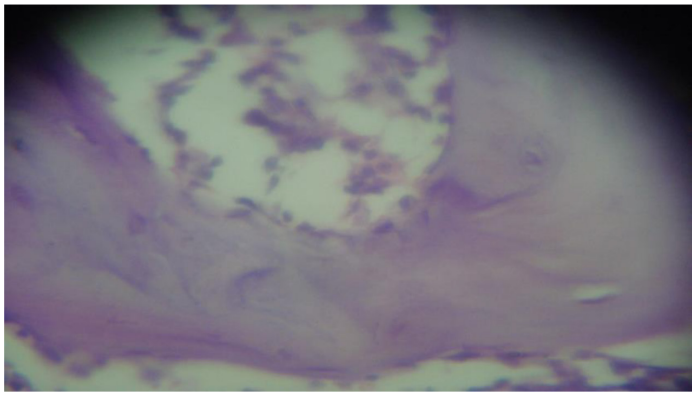

Figure 1a: Photomicrography of left femur of sham control group. No abnormality detected, almost normal width of trabeculae.

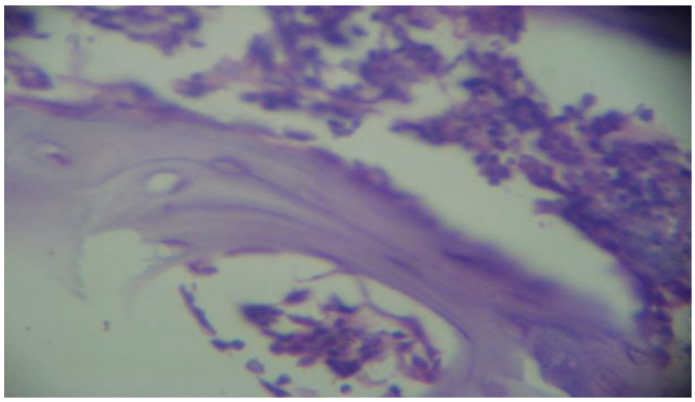

Figure 1b: Photomicrography of left femur of ovariectomized group (OVX). Significant decrease in trabecular width (a classical feature of osteoporosis).

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.[23] 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 women's the estrogen deficiency, results to augments plasma calcium levels as a result of increased bone resorption.[24] The ovariectomized rat exhibited most of the characteristics of human postmenopausal osteoporosis.[25] Increase in loss of calcium and phosphorous through excretion in urine are supporting factor for bone loss in the ovariectomized rats.[26,27] Similarly, the increase in level of ALP was observed with respect to decreased serum calcium and phosphorus in control OVX rats. Significant fall

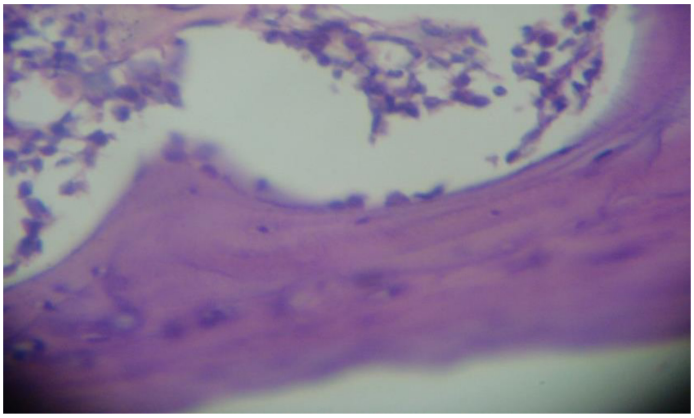

Figure 1c: Photomicrography of left femur of OVX+RL group. Showing almost normal trabecular width.

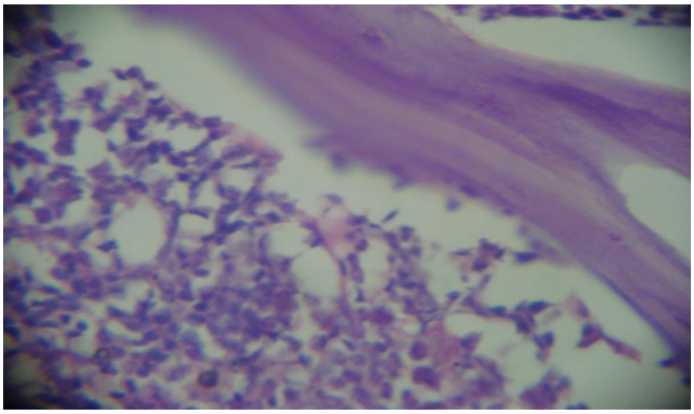

Figure 1d: Photomicrography of left femur of OVX+Test1 group. Significant increase in trabecular width when compared with OVX.

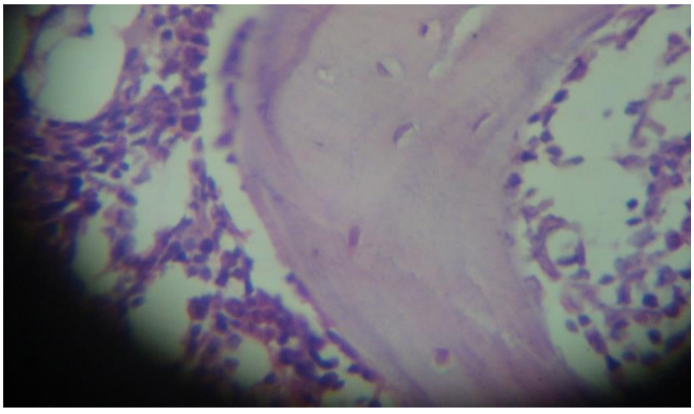

Figure 1e: Photomicrography of left femur of OVX+Test2 group. Significant increase in trabecular width compared to OVX, and also more than Test 1.

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 that may account for bone disorders.[27,28] 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.[29] 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.[30] 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.[31,32] 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.[33,34]

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 phytoconstituents responsible for the estrogenic activity in individual drugs and their potency/efficacy in combination form.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

OVX: Ovariectomy; **HRT:**Hormone Replacement Therapy; **ERα:** Estrogen Receptor alpha; **ERβ:** Estrogen Receptor beta; **ERE:** Estrogen Response Elements; **O-DMA:** O-desmethylangiolensin; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiment on Animal; **PUD:** Peptic Ulcer Disease; **ER+:** Estrogen Receptor Positive; **MCF-7:** Michigan Cancer Foundation -7; **PPD:** Purified Protein Derivative; **ABTS:** 2,2'-azino-bis (3-ethylbenzothiazolim-6-sulphonic acid; **NBT:** Nitro Blue Tetrazolium; **EPM:** Evaluated Plus Maze.

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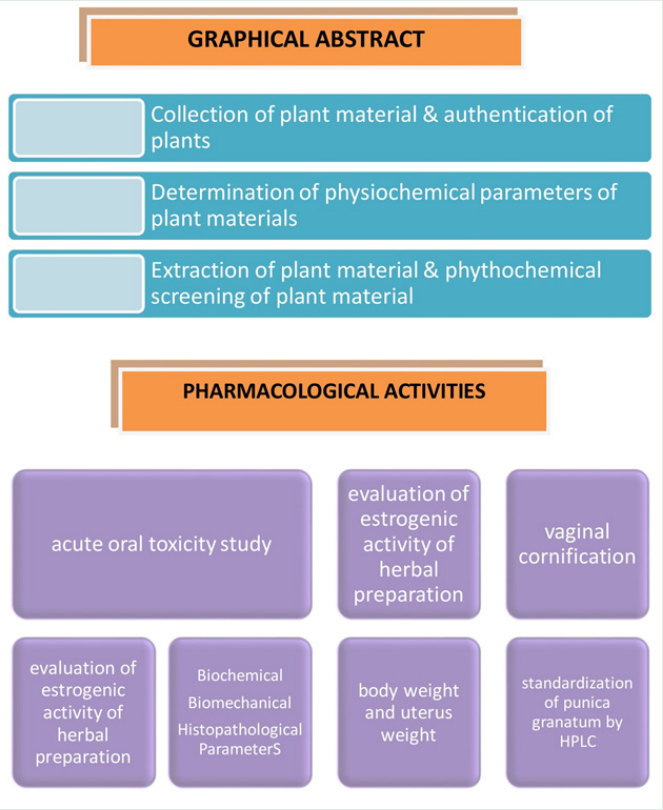
GRAPHICAL ABSTRACT



SUMMARY

The estrogenic effect of test drug (mixture of equal amount of Asparagus, Cissus, Pueraria and Pomegranate extract) was studied by determining biochemical markers (serum ALP, calcium & Phosphorus and urine calcium & Phosphorus), biomechanical parameters (bone weight, bone volume, bone density, bone thickness & bone breaking strength), change in body weight and uterus weight, and histological changes of femur by measuring the thickness of trabeculae. 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 phytoconstituents responsible for the estrogenic activity in individual drugs and their potency/efficacy in combination form.

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