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In vivo and *in vitro* Study to Evaluate the Anti-osteoporotic Activity of *Punica granatum* Seed, *Bambusa arundinaceae* Leaves and *Trichosanthes diocio* Fruit Ethanolic Extract

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ABSTRACT

Background: In the present study, it was aimed at evaluating the efficacy of ethanolic extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit on their anti-osteoporotic activity in ovariectomized (OVX) rats and mcf-7 cell line to identify their osteoprotective role.

Methods: Thirty six female albino wistar OVX rats were treated with the ethanolic extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit for 45 days. The ethanolic extracts were given in different concentrations for 24 h and a cytotoxicity study was carried out by trypan blue dye exclusion assay.

Result: The ethanolic extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit treated notably averted an OVX-induced increase in body weight. Moreover, exposure to ethanolic extract of *Bambusa arundinacea* significantly decreased lipid peroxidation and protein carbonyl formation. Treated mcf-7 cell line viability was also increased. All these finding suggest that the ethanolic extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit have an important role in reducing osteoporosis.

Key words: Ethanolic extract, *In vitro* study, Mcf-7 cell line, Ovariectomized rats.

INTRODUCTION

Osteoporosis is a bone disease that causes a decrease in bone density and makes the bones fragile with an increased susceptibility to fracture (Sinaki, 2021). This is most commonly seen in women, which relates to estrogen deficiency, which causes osteoclast formation, leading to an imbalance between osteoblasts and osteoclasts (Zhang *et al.,* 2020). Women are more prone to have osteoporosis than men. For the treatment of osteoporosis, calcium selective estrogen receptor modulators such as raloxifene and droloxifene, estrogen, bis-phosphonates, fluoride, and calcitonin are useful. But, their use is now limited due to many side effects (Eichner *et al.,* 2003; Migliaccio *et al.,* 2007). So, more new studies focused on the use of natural remedies for osteoporosis management for obvious reasons, like minimal side effects (Tu *et al.,* 2018). Ayurveda has been the pioneer of medical treatment in India for ages. Notably, it suggests various herbs and medicines for healing fractures. Phyto-pharmacotherapy for bone and fracture healing is expected to be safer when compared with synthetic drugs, keeping in mind the side effects (Tu *et al.,* 2018). The burnt form of the *Bambusa arundinacea* root is potentially effective against ringworm, bleeding gums, and painful joints (Singh and Jawaid, 2012). The seeds are known to be acrid, laxative and effective in urinary discharge (Zihad *et al*., 2018). The bark of this tree possesses many dermatological properties (Pandey *et al*., 2018). *Trichosanthes diocio* possesses antidiabetic (Rai *et al*., 2008, 2013), hepatoprotective (Yesmin *et al.,* 2017) and anti-inflammatory properties (Shahana and Nikalje, 2019). It has also shown a significant effect on lowering cholesterol (Shrivastava *et al.,* 2021), skin disorders (Kumar *et al.,* 2012), fungal and bacterial infections. The plant also possesses antioxidant properties (Kumar *et al.,* 2012). *Punica granatum* has antioxidant, anti-carcinogenic, and anti-inflammatory properties (Yusefi *et al.,* 2020). It is also effective in the prevention and treatment of many chronic and infectious diseases (Wu and Tian, 2017).

This study was conducted to explore the effect of ethanolic extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit against osteoporosis as well as in mcf-7 cell lines.

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MATERIALS AND METHODS

Study area

This experiment was conducted at Vivek College of Technical Education, Bijnor, Uttar Pradesh during June 2015 to December 2016.

Ethical clearance

The experiment protocol was approved by the Institutional Animal Ethical Committee (IAEC) according to the regulations of the committee for the purpose of control and supervision of experiments on animals (CPCSEA; Ref. No. VCTE/07/2016 CPCSEA).

Plant collection and authentication

In the present study, the matured leaves of *Bambusa arundinaceae* were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. The leaves were authenticated from Council of Scientific and Industrial Research-National Botanical Research Institute (CSIR–NBRI), Lucknow (Ref. No.: NBRI/CIF/276/2012 and Specification No. : NBRI-SOP-202). Mean while, *Punica granatum* (*seed*) and *Ttrichosanthes diocio* (*fruit*) were collected from Council of Scientific and Industrial Research-National Botanical Research Institute (CSIR-NBRI), Lucknow (Ref. No.: NBRI/CIF/539/2017, Dated: 06.02.2017 and Specification No. : NBRI-SOP-202).

Experimental animals, housing and feeding conditions

Thirty six female albino wistar rats were procured from the Animal House of Bilwal Medchem and Research Laboratory, Jaipur (Reg. No.-2005/PO/RcBT/S/18/CPCSEA). Rats were kept in a laboratory with an unlimited su pply of drinking water and a temperature of 22°C (±3°C) and a relative humidity of atleast 30%. The exposure to light was 12 hours light, 12 hours dark; the room was lit for 12 hours per day. All the animals were grouped into six experimental group like Group A - (2% CMC (Carboxy methyl cellulose) solution 5 ml/kg), Group B - (2% CMC) solution 5 ml/kg (ovariectomized rats), Group C - (Raloxifene - 5.4 mg/kg i.p.), Group D - (Ethanolic extract of *Bambusa arundinaceae* leaves at a dose of 200 mg/kg), Group E - (Ethanolic extract of *Trichosanthes diocio* fruit at a dose of 200 mg/kg) and Group F - (Ethanolic extract of *Punica granatum* seed at a dose of 200 mg/kg). Rats were ovariectomized after following the method of Høegh-Andersen *et al.* (2004).

Osteoporosis induction

The osteoporosis induction was done in thirty six female wistar rats (six animals in each group) through the intramuscular administration of dexamethasone disodium phosphate (Decadron ® 4 mg/ml) at the dose level of 7 mg/kg of body weight, once a week for five weeks in all groups.

Blood sample collection, processing and analysis

After 30 days of the treatment period, all the rats were sacrificed and blood sample was collected from the carotid artery. The serum calcium level, serum phosphorus levels were done by the standard methods (Rathi *et al*., 2020).

Weight of femoral bone (gm)

The length was measured from the proximal tip of the femur head to the distal tip of the medial candyle using a digital caliper (Partadiredja *et al*., 2019).

Estimation of bone calcium level

The bone mineral content was estimated by preparing left femur bone ash in a muffle furnace (700°C for 6 h) and dissolving it in a 0.1 mol/L HCl solution. Bone mineral (calcium) was measured by a UV-visible spectrophotometer (Cherni *et al.,* 2020).

Histopathological study

All the animals were sacrificed and the femur was dissected for histopathology study. The bones were collected and immediately fixed in 10% formalin and allowed to remain in it till they were taken up for processing.

In vitro study

The MCF-7 cell line was obtained from the National Center for Cell Science (NCCS), Pune, India. The cell lines were grouped into three groups, like Group I (Control), Group II (ethanolic extract of *Bambusa arundinacea* (leaves), *Ttrichosanthes diocio* (fruit) and *Punica granatum* (seed) @ 100 mg/kg) and Group III (ethanolic extract of *Bambusa arundinacea* (leaves), *Ttrichosanthes diocio* (fruit) and *Punica granatum* (seed) @ 200 mg/kg).

Determination of cell viability

Cells were fixed *in situ* by the gentle addition of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. Drug concentration resulting in total growth inhibition (TGI) was calculated as described by (Skehan *et al.,* 1990; Vichai and Kirtikara, 2006).

RESULTS AND DISCUSSION

The effect of ethanolic extracts of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit on serum ALP, calcium and phosphorous is shown in Fig 1A, 1B and 1C, respectively. Groups treated with *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit ($P<0.01$) and raloxifene ($P<0.05$) significantly suppressed the rise in serum ALP levels.

The effect of *Bambusa arundinacea* on cell viability was an osteoprotective effect when treated with trypan blue dye exclusion. The exposure of ethanolic extracts of *Bambusa arundinacea* 100 mg/kg on the MCF-7 cell line showed 59% cell viability (Fig 4A). On treatment with ethanolic extract 200 mg/kg, the cell viability was 71% of live cells. At a higher dose of *Bambusa arundinacea,* the cell mortality was decreased by up to 12%. Thus, a promising osteoprotection over MCF-7 cell line cell line was displayed by *Bambusa arundinacea* (Fig 4A, 5A, 6A).

The effect of *Trichosanthes diocio* on cell viability was significant and showed osteoprotective when treated with trypan blue dye exclusion. The exposure of ethanolic extract of *Trichosanthes diocio* 100 mg/kg on the MCF-7 cell line

showed 59% cell viability (Fig 5A). On treatment with ethanolic extract 200 mg/kg, the cell viability was 71% of live cells. At a higher dose of *Trichosanthes diocio,* the cell mortality was decreased by up to 12%. Thus, a promising osteo-protection over MCF-7 cell line cell line was displayed by *Trichosanthes diocio* (Fig 5A).

The effect of *Punica granatum* on cell viability was significant. The exposure of ethanolic extracts of *Punica granatum* 100 mg/kg on the MCF-7 cell line showed 78.2% cell viability (Fig 6A). *Punica granatum* showed that total protein increased significantly at low and higher doses (P<0.05 and P<0.01, respectively), whereas non-significant

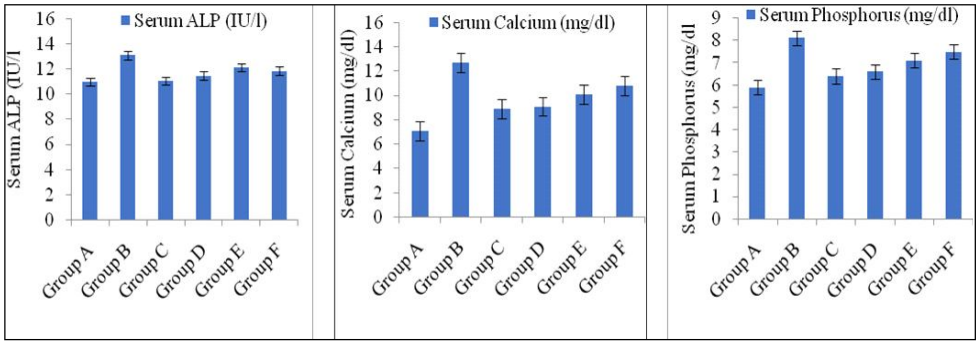


Fig 1A **Fig 1B** **Fig 1C**

Fig 1A-1C: Effect of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit on serum ALP, calcium and serum phosphorus level.

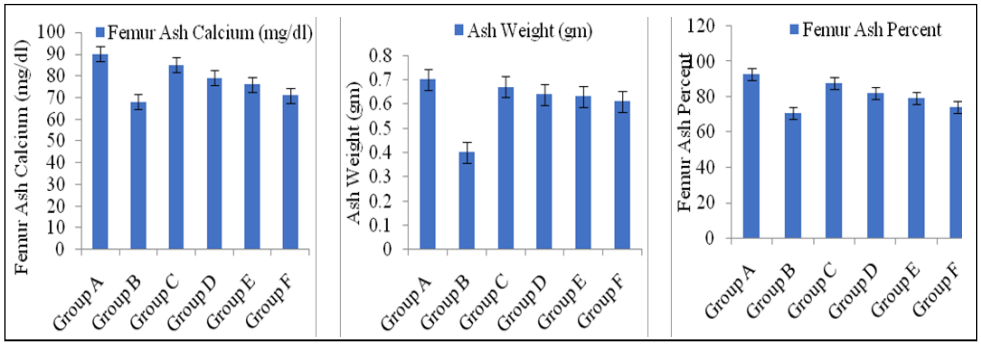
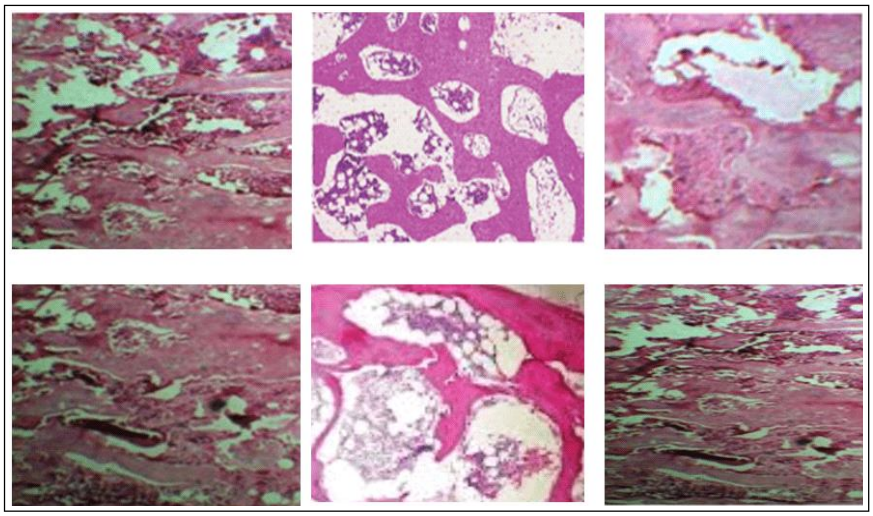


Fig 2A **Fig 2B** **Fig 2C**

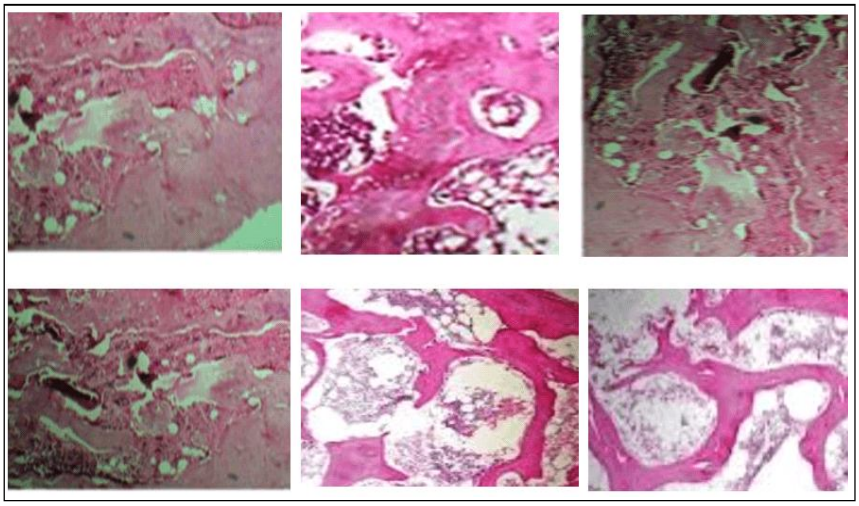
Fig 2A-2C: Effect of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit on femur ash calcium, ash weight and femur ash per cent.



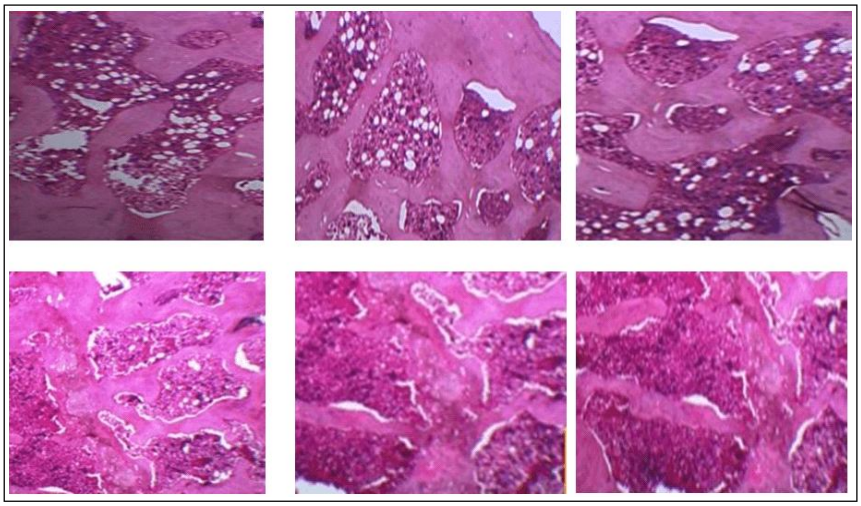
Group A - Epiphyseal region showing sparse, thinning of trabeculae, loss of connectivity and widening of inter trabecular space in group A (Negative Control Group).

Figure 3: Continue.....

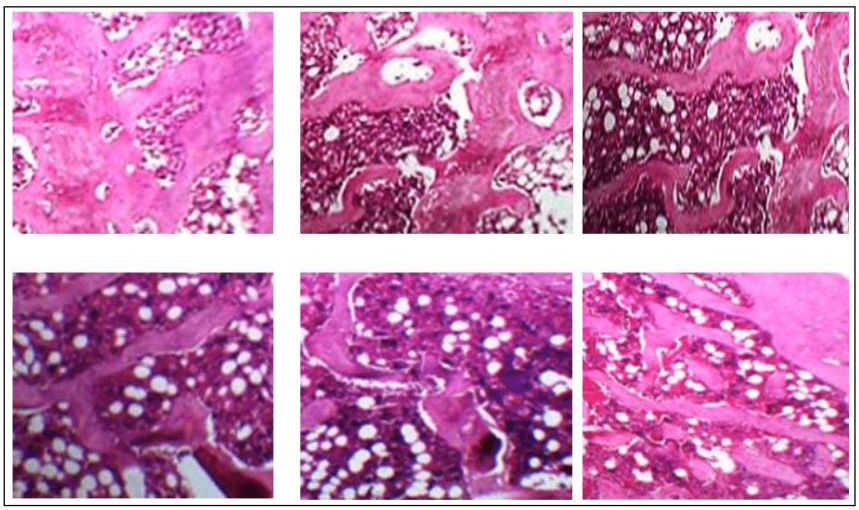
Figure 3: Continue.....



Group B - Epiphyseal region showing moderately thick elongated trabeculae and narrow inter trabecular space in Test Group B.



Group C - Epiphyseal region showing moderately thick elongated trabeculae and narrow inter trabecular space in Test Group C.



Group D - Epiphyseal region showing slightly thick elongated trabeculae and narrow inter trabecular space in Test Group D.

Fig 3: Histopathology of Epiphyseal region of different groups.

alteration was observed at low dose treatment as compared to control (Fig 4B). At a higher dose of *Punica granatum* treatment, the results were near to normal (P<0.001) as there was least breakage of protein (Fig 4B). *Punica granatum* treatment showed a decrease (P<0.001) in LPO as compared to control (Fig 4D). The SOD activity was not prominent in any groups (P<0.001) in comparison to control (Fig 4E).

Treatment of *Trichosanthes diocio* showed that the value of total protein increased significantly at mid and higher doses (P<0.05 and P<0.01 respectively), whereas non-significant alteration was observed at low dose treatment as compared to control (Fig 5B). A protein carbonyl can be formed inside the cell due to the breakage of the protein backbone by generation of ROS or direct oxidation of amino acids and it showed no significant change at a low dose of *Trichosanthes diocio* treatment. At a higher dose of *Trichosanthes diocio,* the results were somewhere close to normal (P<0.001) as there was no breakage of protein (Fig 5C). On the other hand, high doses of *Trichosanthes diocio*

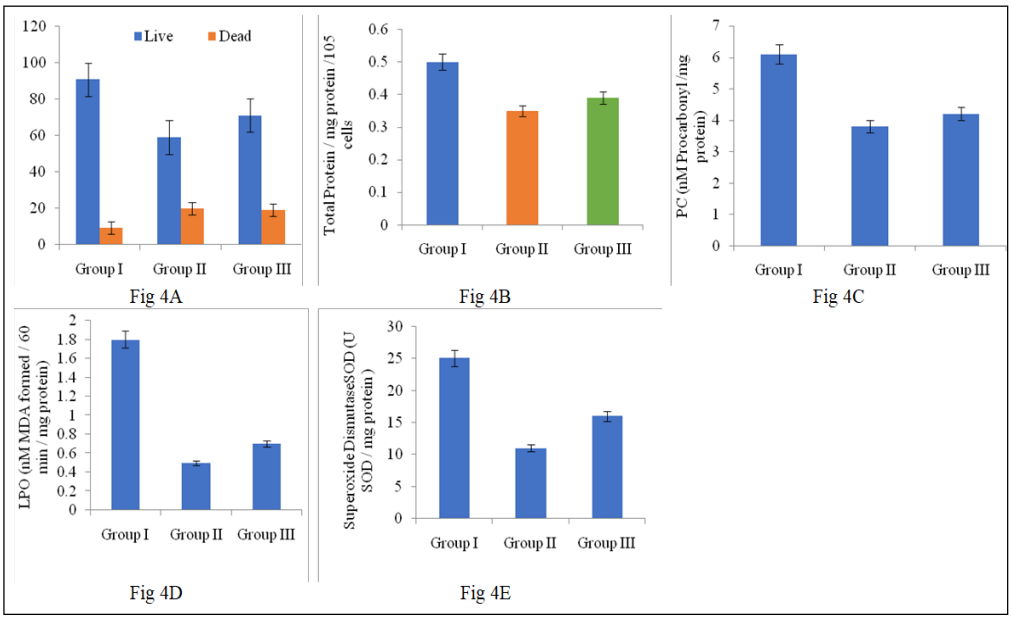


Fig 4A-4E: Percentage of live and dead cells after trypan blue dye exclusion assay, total protein, protein carbonyl, lipid peroxidation and SOD in control and all *Bambusa arundinacea* treated groups, respectively.

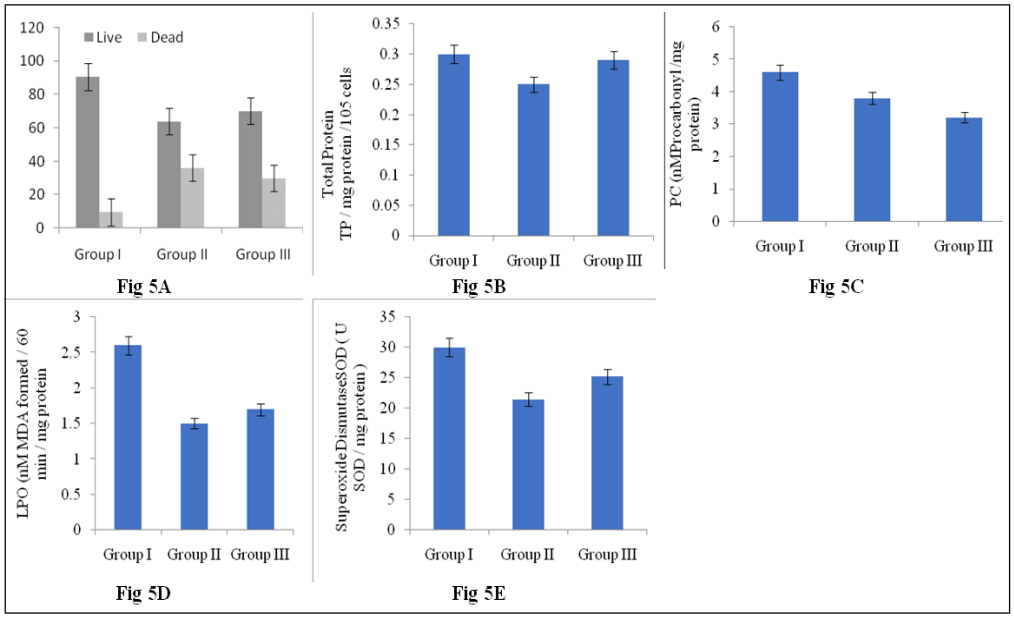


Fig 5A-5E: Percentage of live and dead cells after trypan blue dye exclusion assay, total protein, protein carbonyl, lipid peroxidation and SOD in all control and treated *Trichosanthes diocio* groups, respectively.

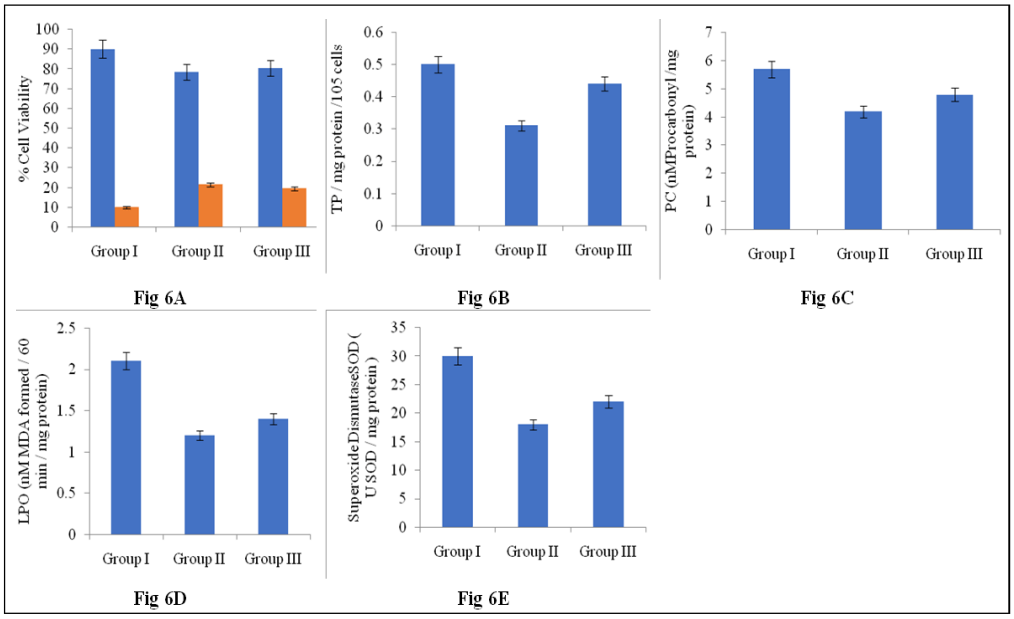


Fig 6A-6E: Percentage of live and dead cells after trypan blue dye exclusion assay, total protein, protein carbonyl, lipid peroxidation and SOD in all control and treated *Punica granatum*, respectively.

treatment (Group 2 and 3) showed a promising decrease (P<0.001) in LPO as compared to control (Fig 5D). These parameters showed an excellent linear dose response relationship in cultured MCF-7 cells after 24 h of Trichosanthes diocio exposure. The SOD activity was not prominent in any groups (P<0.001) in comparison to control (Fig 5E). Treatment of Punica granatum showed that the values of total protein, protein carbonyl, LPO and SOD increased significantly (P<0.05) at higher doses (Ethanolic extract 200 mg/kg) whereas non-significant alteration was observed at low dose treatment as compared to control (Fig 6B-6E).

The present study evaluated the effect of ethanolic extract of *Bambusa arundinacae, Punica granatum* and *Trichosanthes diocio* on corticosteroid induced osteoporosis induced by corticosteroid as they alter skeletal integrity by affecting bone metabolism, reducing the life span of osteoblasts and inhibiting osteoblastogenesis in female rats. Corticosteroid creates an imbalance in the rhythm between bone formation and bone reabsorption (Sato *et al*., 2019). Osteoporosis was induced by intraperetional administration of dexamethasone for 5 weeks and test samples for 30 days. The hardness and rigidity of a bone is due to the presence of mineral salts in the osteoid matrix, which is the crystalline complex of calcium and phosphate (Suarez-Bregua *et al.,* 2018). A significant increase in BMD on treatment with extract of *Punica granatum* seed, *Bambusa arundinaceae* leaves and *Trichosanthes diocio* fruit, on the other hand, confirmed the remodelling of bones and the prevention of osteoporosis. The histology of osteoporosis rats' bones found that the epiphyseal region showed sparse, thinning of trabeculae, loss of connectivity and widening of inter trabecular space found in group A (Negative Control) and after treatment with test samples, thickening of trabeculae in the epiphyseal region in each test group. But test group D found significant cellular changes in bone microscopy. Test Group D was found to be more therapeutically active than other test samples. The ovariectomy model in female rats simulates many common characteristics of postmenopausal osteoporosis occurring in humans, such as increased bone turnover, bone resorption exceeding bone remodelling, resulting in micro-architectural deterioration of bone mass (Medina-Contreras *et al*., 2020). Thus, result suggested that plant's extract has the potential to stop bone restoration by promoting bone mineralization in ovariectomized rats.

The protein carbonyl can be formed inside the cell due to the breakage of the protein backbone by generation of ROS or direct oxidation of amino acids (Sharma *et al*., 2019) and it showed no significant change at a low dose of *Bambusa arundinacea* treatment. At a higher dose of folic acid treatment, the results were somewhere close to normal (P<0.001) as there was no breakage of protein (Fig 2). In order to determine the level of malondialdehyde, the level of lipid peroxidation (LPO) is measured. The *Bambusa arundinacea* treatment (Group II and III) showed a promising decrease (P<0.001) in LPO as compared to control (Fig 4). Superoxide dismutase is an enzyme that catalyzes the disputation of O_2^- into oxygen and H_2O_2, whereas catalase converts H_2O_2 into non-toxic water molecules (Mannaa, 2017). The SOD activity was not prominent in any groups (P<0.001) in comparison to control (Fig 4).

CONCLUSION

In this study, it could be concluded that all the ethanolic extracts of the plants *Bambusa arundinacea* (leaves),

Ttrichosanthes diocio (fruit) and *Punica granatum* (seed) have shown osteoprotective on MCF 7 cell lines as well as in overectomized rats. On comparing all the three plants, the most significant effect was seen in *Bambusa arundinacea* (leaves), *Ttrichosanthes diocio* (fruit) and *Punica granatum* (seed) also showed non-significant alteration were observed at low dose treatment as compared to control. Total protein protein carbonyl, LPO and SOD activity were less after treatment of *Ttrichosanthes diocio* (fruit) and *Punica granatum* (seed) when compared to *Bambusa arundinacea* (leaves). Thus, this study concluded the ethanolic extract of these three plants has potential in preventing osteoporosis in overectomized rats and in the MCF-7 cell line.

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Conflict of interest

All authors declared that there is no“conflict of interest.

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