ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 18, Issue 8, 2025

Online - 2455-3891
Print - 0974-2441

Research Article

COMPARATIVE HISTOLOGICAL CHANGES IN BONE AND LIVER IN RESPONSE TO EXTRACT OF *TRIFOLIUM REPENS* AND *EQUISETUM ARVENSE* IN ADULT FEMALE WISTAR RATS

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Received: 10 April 2025, Revised and Accepted: 09 June 2025

ABSTRACT

Objectives: The objective of this study is to evaluate the histological changes in bone tissue and assess potential adverse effects on liver tissue in an estrogen-deficient rat model (ovariectomized rats) treated with *Trifolium repens* (white clover) and *Equisetum arvense* (horsetail) extracts.

Methods: A total of 56 female Albino Wistar rats (7–9 weeks old, 200–220 g) were obtained from Cape Bio Laboratory and Research Centre. Animals were acclimatized under standard laboratory conditions in animal housing (temperature: 22±3°C; humidity: 50–55%; 12-h light/dark cycle). Bilateral Ovariectomy was performed to induce estrogen deficiency. One month post-surgery, Ovariectomized (OVX) rats were orally administered 60 mg/day of *T. repens* and *E. arvense* extract for 45–90 days. At the end of the treatment period, femur and liver tissues were collected for histological examination.

Results: Ovariectomy resulted in a significant increase in body weight and an increased number of osteoclasts, along with noticeable histological changes in the femur compared to the control group. Treatment with *E. arvense* and *T. repens* did not cause any adverse histological changes in the liver. Among the two treatments, *E. arvense* was more effective in mitigating bone loss induced by estrogen deficiency.

Conclusion: These findings suggest that *E. arvense* appears to be more effective than *T. repens* in preserving bone integrity in ovariectomized rats, likely by reducing bone turnover through the inhibition of bone resorption. In addition, both herbal extracts were found to be safe for liver tissue at the administered dose.

Keywords: *Trifolium repens*, *Equisetum arvense*, Osteoporosis, Bone histology, Ovariectomy, Liver toxicity.

 DOI: http://dx.doi.org/10.22159/ajpcr.2025v18i8.54537. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

To attain optimal bone health, achieving peak bone mass is necessary, thereby reducing the risk of osteoporosis in the future [1]. The peak bone mass is achieved until late adolescence or the early 20s [2], depending on the interactions between hormones and growth factors, genetics, physical activity, and nutrition, particularly calcium and Vitamin D [3,4]. Bone mineral density is influenced by various factors, including diet, age, and the use of both chemical and natural medications. Osteoporosis is a bone disease characterized by the loss of bone and its structural deterioration. The characteristics of this disease include a reduction in bone mineral content and bone matrix. Therefore, although the bone is completely normal in terms of the compounds, it is reduced in terms of its content. As part of this disease, the reduction of bone mass leads to an imbalance between bone loss and its formation and eventually results in demineralization. Osteoporosis causes pain and deformity or bone fractures as a result of demineralization of the bone.

In countries like India, where herbs are an integral part of daily life, herbal medicine is a widely trusted system of health care. Traditional medicinal plants have gained significant importance in treating diseases and promoting optimal health in both humans and animals [5,6]. These plants are favored for being cost-effective, safe, and associated with minimal or no side effects. However, since these plants are often used in their raw form, it is essential to determine their accurate dosage and validate their safety [7,8]. The findings from such studies help in determining appropriate dosages for humans and in assessing potential toxicity [9].

Clover, scientifically known as *Trifolium*, belongs to the Leguminosae family [10]. *Trifolium repens*, commonly referred to as white clover, white trefoil, Dutch clover, creeping *Trifolium*, honeysuckle clover, or ladino clover [11], is currently utilized for its therapeutic properties, a practice rooted in traditional medicine. Research on the biological effects of various *Trifolium* species has revealed the presence of flavonoids, isoflavonoids, and other antioxidant compounds [12]. Due to these beneficial compounds, certain *Trifolium* species are employed as therapeutic agents with antiseptic, analgesic, anti-inflammatory, and anti-cancer properties, as well as for promoting angiogenesis [13]. The health benefits of *Trifolium* may be attributed to the mechanistic actions of the various biologically active compounds found in these plants.

Equisetum arvense, a perennial herb from the order *Equisetales*, has been traditionally used in folk medicine to treat conditions such as diuresis, bleeding, skin infections, kidney and liver disorders, and gastric ulcers [4,14]. While its potential role in treating bone-related diseases has been suggested, it has not yet been conclusively proven. There is ongoing debate regarding the use and toxicity of *E. arvense* [15-17]. The extracts of *E. arvense* are rich in phenolic compounds, flavonoids, and phenolic acids, which have antioxidant and reducing properties [18,19]. In addition, well-known phytochemicals such as alkaloids, phytosterols, tannins, and triterpenoids have been identified in the plant [19]. *E. arvense* also has a high silica content, and studies have shown that its extract can promote bone regeneration *in vitro* and inhibit osteoclastogenesis [20]. Furthermore, horsetail extract has been reported to enhance bone mineralization and formation in ovariectomized rats [3]. A diet containing horsetail extract (120 mg/kg) also increased BMD in rats [21]. Due to its high silica content, *E. arvense* is considered a promising treatment for osteoporosis, as it supports calcium absorption and collagen formation [4].

Hence, the present study was undertaken to evaluate the histological changes of bone tissue and any adverse effect on liver tissue in association with estrogen deficiency (ovariectomy) in rats in treatment with white clover/*T. repens* and *E. arvense*/horsetail extract.

MATERIALS AND METHODS

This study was conducted in the Cape Bio Laboratory and Research Centre, Marthandam, Tamil Nadu.

Duration of the study: 6 months.

Animals to be used

Female Albino Wistar rats (n=54), 7 weeks old, weighing about 200–220 g, were obtained from Cape Bio Laboratory and Research Centre. All animals are allowed to acclimatize in animal housing standard conditions, temperature of (22±3°C), relative humidity (50–55%), and a 12 h light/dark cycle before being used for the study.

Experimental osteoporosis (ovariectomy in female rats)

After the period of adaptation (1 week), the first group of female rats was anesthetized with ketamine hydrochloride and xylazine, and their ovaries were removed bilaterally according to the method described by Waynforth 1980 and Lasota and Danowska 2004. After recovery from surgery, the extract was given to the rats in an oral dose of 60 mg/kg body weight for 45–90 days. Histological staining is performed on the section of the left femur bone and liver tissue in all groups.

Collection of plant material

The first plant, *T. repens* (white clover), was collected from the Botanical Garden, Ooty, Tamil Nadu. The second plant, *E. arvense* (field horsetail), was collected from the Senapati district, Manipur state, in India.

Preparation of powdered material

After authentication, the fresh healthy plant was dried properly in shade for 3 weeks, segregated, pulverized by a mechanical grinder, and passed through a 40 mesh sieve. The powdered plant materials were stored in an airtight container and used for further studies.

Preparation of organic extracts

About 4 kg of air-dried plant of *T. repens* (white clover) and *E. arvense* (field horsetail) was extracted in a Soxhlet assembly with ethanol. The extract was concentrated using a rotary vacuum evaporator. The extract obtained with each solvent was weighed, and the percentage yield was calculated in terms of dried weight of the plant material. The color and consistency of the extract were also noted. All the solvents used for this entire work were of analytical reagent grade (Merck, Mumbai).

Toxicological study

The successive extracts of the herbal plant will be subjected to toxicological study as per OECD guidelines 423.

Chemical/reagents/diagnostic kits

Hematoxylin and Eosin stain kit procured from Merck chemicals, Mumbai.

Name of various instruments/equipment used in the study

Lyca microtome RTS 2125.

Histological procedures

Samples were fixed in 10% neutral buffered formalin. After paraffin embedding, 3–4 µm serial sections were prepared and then stained with hematoxylin and eosin.

Hematoxylin and Eosin stain

1. Deparaffinize with xylene and hydrate the section with water
2. Nuclear stain with hematoxylin stain
3. Wash-5 mint
4. Differentiation with acid alcohol
5. Bluing with Ammonia or $LiCo_3$
6. Counterstain with Eosin
7. Wash in water
8. Dehydration
9. Clearing
10. Mounting.

Interpretation

- Nucleus-blue
- Cytoplasm-pink.

RESULTS

Histological examination of bone in treatment with *T. repens*/white clover and *E. arvense*/field horsetail extract

Histological examination of upper end of femur bone of albino Wistar rats of control group shows bony trabeculae with intervening marrow spaces showing hematopoietic elements (Fig. 1). Where 2a group – OVX control rats for 45 days – show faintly stained bony trabeculae with free floating trabeculae and few decalcified osteoid bones (Fig. 2). In the 2b group – OVX control rats kept for 90 days – show faintly stained cortical area, decalcified osteoid bone and bony trabeculae with free floating trabeculae (Fig. 3). In 3a group, i.e., OVX rats treated with trifolium repens (TR) for 45 days show surface articular cartilage with bony trabeculae appearing slightly thicker compared to group 2a. It shows a potential protection from osteoporotic changes induced by ovariectomy was observed (Fig. 4). In 3b group, i.e., OVX rats treated with TR for 90 days show surface articular cartilage with bony trabeculae appearing more thicker with lesser marrow

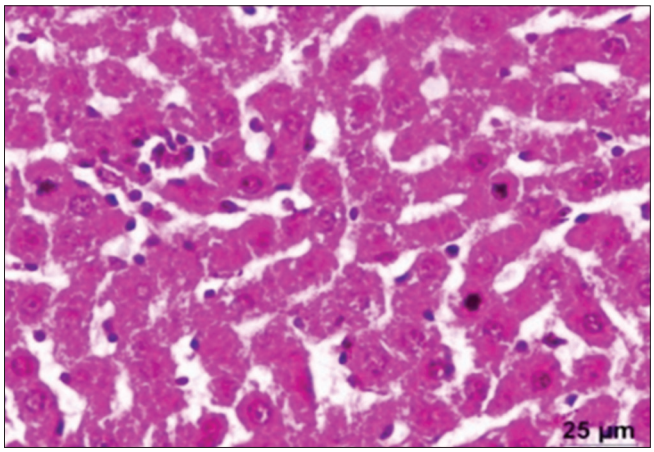

Fig. 1: Liver from a non-ovariectomized control

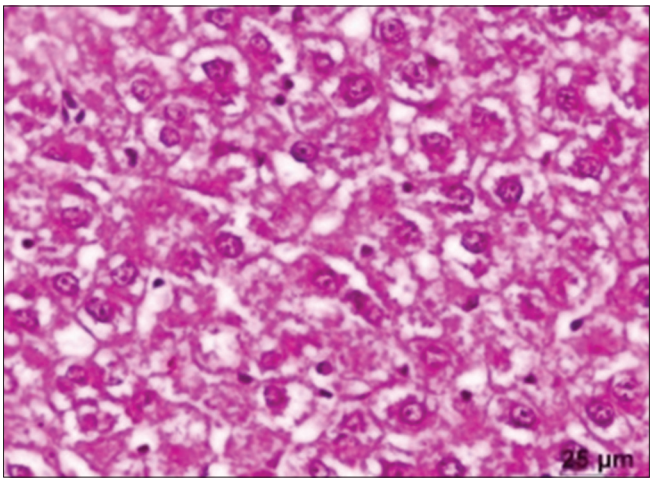

Fig. 2: Group 2a, Liver from ovariectomized control rats – 45 days

spaces compared to group 2b and decreased osteoclastic activity or characterized by negative histochemical reaction as in figures along with a thick cortex, which is a marker of slight improvement compared with OVX untreated rats (Fig. 5). In 4a group, i.e., OVX rats treated with Equisetum arvense (EA)-45 days show interconnected bony trabeculae. It is shown that the potential of protection from osteoporotic changes induced as a result of ovariectomy was observed (Fig. 6). In 4b group, i.e., OVX rats treated with Equisetum arvense (EA)-90 days show thick bony trabeculae compared to group 2b, with only occasional floating trabeculae. It was characterized by negative histochemical reaction (absence of osteoclast) along with a thick cortex, which is a marker of significant improvement compared with OVX untreated rats (Fig. 7).

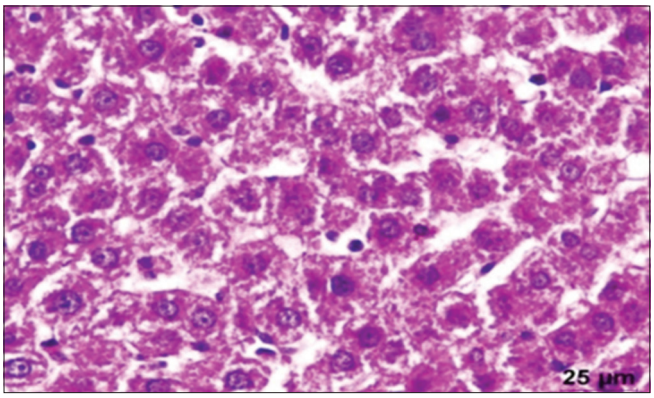

Fig. 3: Group 2b, Liver from ovariectomized control rats – 90 days

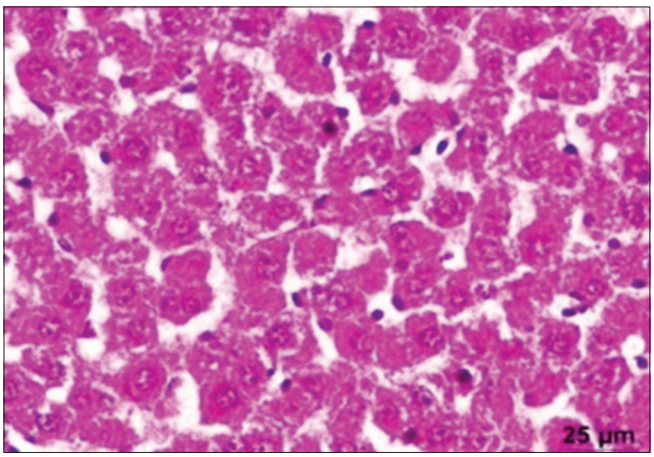

Fig. 4: Group 3a, Liver from ovariectomized rats with trifolium repens – 45 days

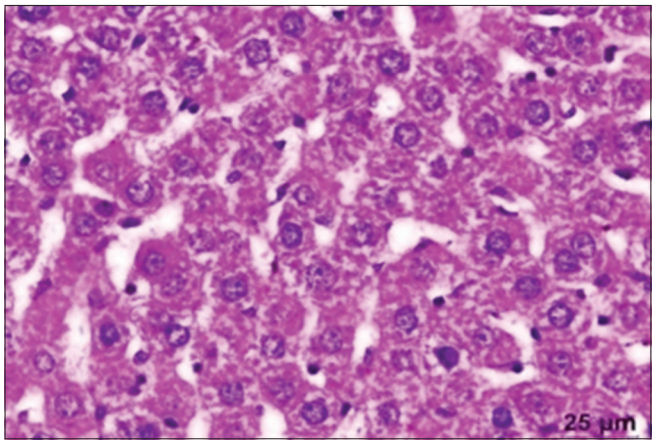

Fig. 5: Group 3b, Live from ovariectomized rats with trifolium repens – 90 days

Histological examination of liver in treatment with white clover/*T. repens* and *E. arvense*/horsetail

Histological examination of liver tissue of albino Wistar rats in the control group shows hepatocytes in trabeculae with intervening sinusoids (Fig. 8). In group 2a, i.e., OVX rats kept for 45 days, and group 2b, i.e., OVX rats kept for 90 days, sections from the liver show hepatocytes in trabeculae with scant inflammation (Figs. 9 and 10). Group 3a, i.e., OVX rats treated with TR for 45 days, and 3b, i.e., OVX rats treated with TR for 90 days, show liver parenchyma with hepatocytes in trabeculae and sinusoids. Hepatocytes appear unremarkable (Figs. 11 and 12). In group 4a, i.e., OVX rats treated with EA for 45 days, and 4b, i.e., OVX

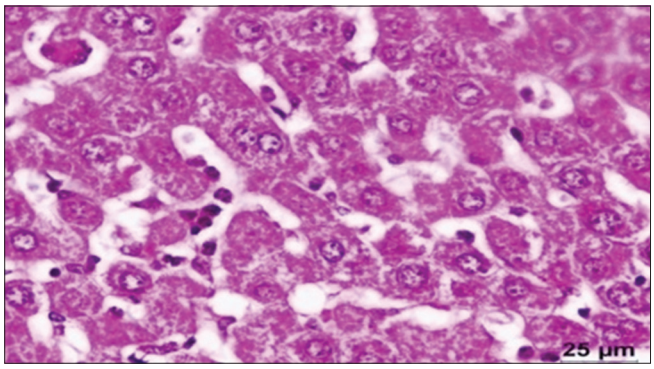

Fig. 6: Group 4a, Liver from ovariectomized rats with equisetum arvense – 45 days

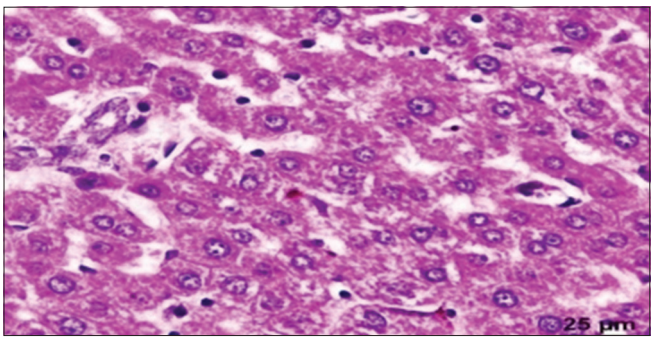

Fig. 7: Group 4b, Liver from ovariectomized rats with equisetum arvense – 90 days

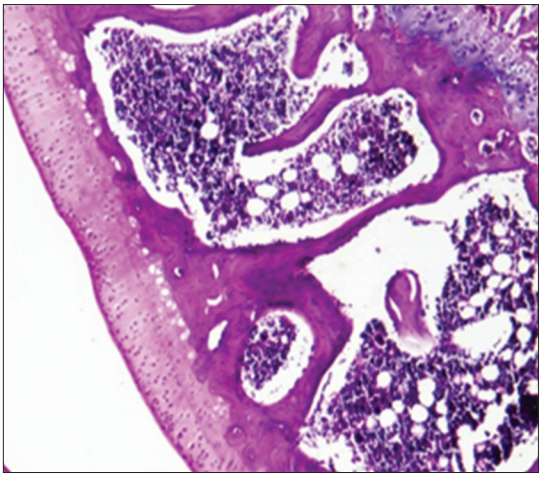

Fig. 8: Group 1, Bone from non-ovariectomized control rats

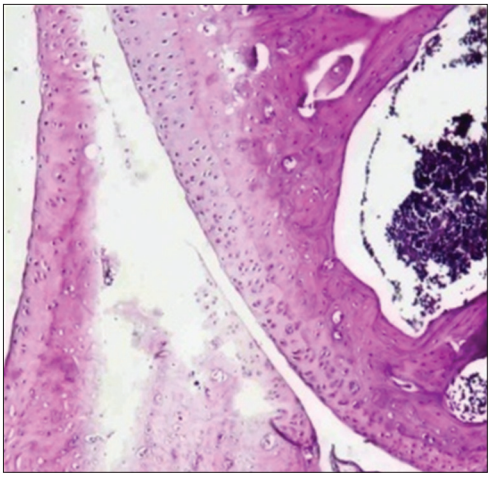

Fig. 9: Group 2a, Bone from ovariectomized control rats – 45 days

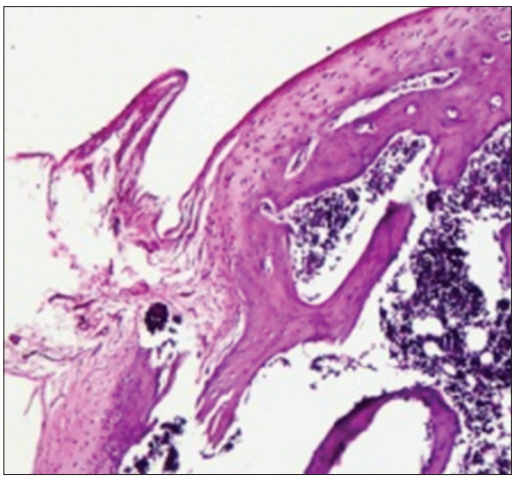

Fig. 10: Group 2b, Bone from ovariectomized control rats – 90 days

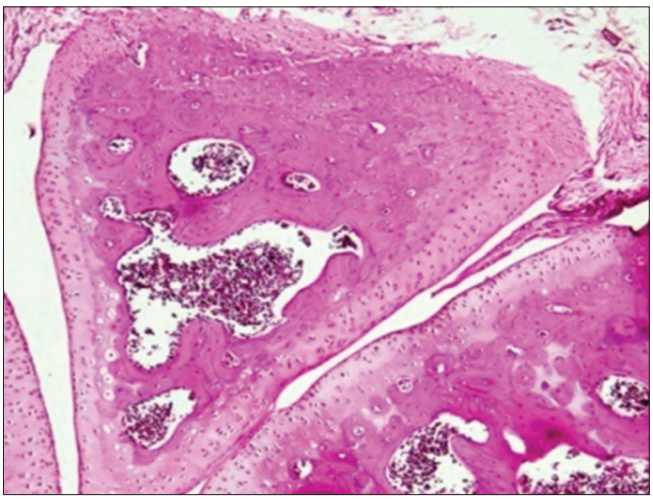

Fig. 11: Group 3a, Bone from ovariectomized rats with trifolium repens– 45 days

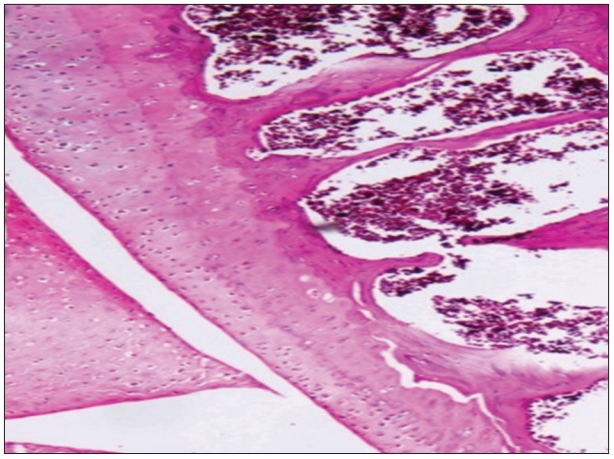

Fig. 12: Group 3b, Bone from ovariectomized rats with trifolium repens – 90 days

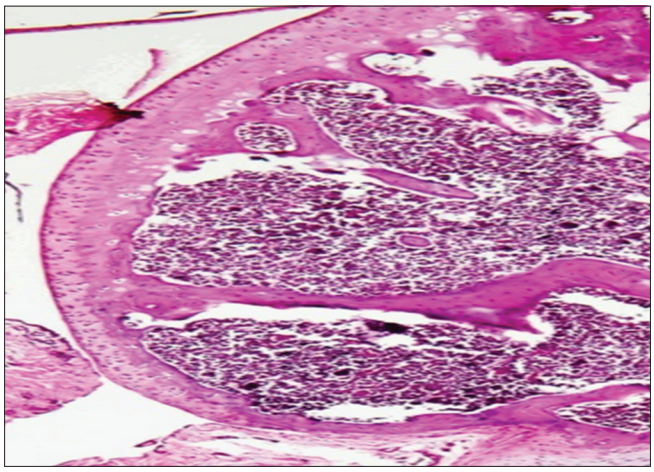

Fig. 13: Group 4a, Bone from ovariectomized rats with equisetum arvense– 45 days

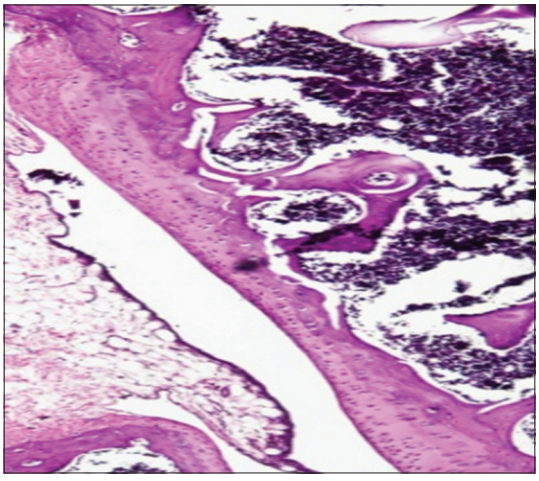

Fig. 14: Group 4b, Bone from ovariectomized with equisetum arvense – 90 days

rats treated with EA for 90 days, show liver parenchyma with normal-looking hepatocytes. Focal bland cholestasis noted (Figs. 13 and 14).

DISCUSSION

Medicinal plants have been employed for centuries in the treatment of various diseases. Before assessing their therapeutic potential, the initial step in studying these plants is to identify and evaluate their toxicity [9,22]. *E. arvense*, a perennial herb traditionally used in medicine for various health conditions, was evaluated for acute toxicity in male Wistar rats. The study administered graded doses of 30 mg/kg, 50 mg/kg, and 100 mg/kg of the plant extract over 14 days. The results revealed that these doses did not induce any significant toxic effects, as there were no notable changes in clinical signs, body weight, or organ

weight compared to the control group. Research by Oh *et al*. (2004) demonstrated that the aerial parts of *E. arvense* possess antioxidant and liver-protective properties. Conversely, Semprini *et al*. (2009) found that oral administration of *E. arvense* led to liver damage in Wistar rats. Notably, there is a lack of clinical data regarding the safe dosage range and the effects of *E. arvense* on female albino Wistar rats. In the present study, a dose of 60 mg/kg body weight did not cause any adverse effects on liver tissue.

Badole and Kotwal conducted a study to evaluate the effects of *E. arvense* extract on biochemical, hematological, and histological parameters in adult female Wistar rats. The rats were administered three different doses of the extract: Low (30 mg/kg), medium (60 mg/kg), and high (120 mg/kg) for 30 days. The study found that the low and medium doses did not exhibit any toxic effects, as there were no significant changes in clinical signs, body weight, or organ weight. However, the high dose led to a noticeable reduction in body weight, suggesting potential toxicity, although no deaths occurred. Histopathological analysis revealed that rats receiving the high dose exhibited mild hepatocellular necrosis in the liver and reduced trabecular width in the femur bone, indicating possible toxic effects at this dose. Based on these findings, the study concluded that the medium dose (60 mg/kg) could be considered a safe therapeutic dose for further research. Supplementation with calcium and Vitamin D, alongside *E. arvense*, contributed to a reduction in osteoporosis. Specifically, administration of 60 mg/kg body weight of *E. arvense* extract resulted in enhanced BMD, characterized by thicker bony trabeculae and a robust cortical bone structure, compared to ovariectomized (OVX) untreated rats. In contrast, a 2016 study by Menghini *et al*. demonstrated that a natural formulation combining lactoferrin, *E. arvense*, soy isoflavones, and Vitamin D3 effectively prevented and treated osteoporosis by influencing bone remodeling and inflammatory markers. This combination therapy showed promise in modulating bone metabolism and reducing inflammatory responses associated with bone loss. In the present study, with a dose of 60 mg/kg body weight, *E. arvense* shows thick bony trabeculae compared with only occasional floating trabeculae. It was characterized by a negative histochemical reaction (absence of osteoclast) along with a thick cortex, which is a marker of significant improvement compared with OVX untreated rats.

Research indicates that diets rich in phytoestrogenic isoflavones, such as those found in red clover (*Trifolium pratense* L.), may be associated with a reduced risk of osteoporosis and alleviate menopausal symptoms. These isoflavones, including formononetin and biochanin A, exhibit estrogen-like effects, potentially improving bone density and reducing osteoclast activity, thereby supporting bone health. Estrogen deficiency can lead to calcium loss in two main ways are decreased intestinal calcium absorption and decreased renal calcium conservation [23].

This can contribute to bone health issues, such as osteoporosis. In the present study, administration of 60 mg/kg body weight of *T. pratense* extract led to the development of thicker bony trabeculae with reduced marrow spaces and decreased osteoclastic activity in female albino Wistar rats, compared to ovariectomized untreated controls. These histological improvements suggest a positive effect on bone structure and density.

CONCLUSION

The objective of the present work was to study the effects of the extract of *T. repens*/white clover and *E. arvense*/field horsetail in female albino Wistar rats when given at a dose of 60 mg/kg of body weight. In the final part of the study, we have done the liver histology to analyse any side effects/adverse effects at a dose of 60 mg/kg of body weight of *E. arvense* and *T. repens*. The present study also helps to determine the range of therapeutic use for further studies. From the present study, it can be concluded that *T. repens* and *E. arvense* do not produce any toxic effects at a dose of 60 mg/kg body weight in female albino Wistar rats, and it can be used as a therapeutic dose for further studies. Since these two extracts show positive changes in the bone histology, I would like to conclude that these two extracts have therapeutic value and, in the future, these extracts can be used as a treatment for bone diseases. It was also noticed in the present study that the extracts of *E. arvense* are more effective than *T. repens* in the treatment of bone diseases.

ACKNOWLEDGMENT

Sukritha KM -PhD Scholar,Department ofAnatomy,Meenakshi Academyof Higher Education and Research. Dr Vishali N -Associate Professor, Meenakshi Academy of Higher Education and Research. Dr B Kavitha- Professor, Meenakshi Academy of Higher Education and Research. Dr Bhavya J Menon. Assistant Professor,Dr Moopen's Medical College.

ETHICAL APPROVAL

Ref. no-CBLRC/IAEC/15/01-2023.

AUTHOR'S CONTRIBUTIONS

Sukritha KM was responsible for collecting the plant material, preparing the plant extract, performing the OVX procedure, collecting tissue samples for histopathological analysis, interpreting the results, and analyzing the study data. Dr. Vishali N provided guidance in the design and drafting of the research work. The final manuscript was reviewed and approved by all authors involved in the study.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest. They take full responsibility for the accuracy and integrity of the content presented in this manuscript.

AUTHOR'S FUNDING

This article receives no specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Badole S, Kotwal S. Evaluation of proximate, free radical scavenging activity, and phytochemical analysis of *Equisetum arvense* L. Extracts. Indian J Nat Prod Resour. 2017;8(2):146-50.
2. Menghini L, Ferrante C, Leporini L, Recinella L, Chiavaroli A, Leone S, *et al*. A natural formula containing lactoferrin, *Equisetum arvense*, soy isoflavones and vitamin D3 modulates bone remodeling and inflammatory markers in young and aged rats. J Biol Regul Homeost Agents. 2016;30(4):985-96.
3. Kotwal SD, Badole SR. Anabolic therapy with *Equisetum arvense* along with bone mineralising nutrients in ovariectomized rat model of osteoporosis. Indian J Pharmacol. 2016;48(3):312-5. doi: 10.4103/0253-7613.182880, PMID 27298503
4. Badole S, Kotwal S. *Equisetum arvense*: Ethanopharmacological and phytochemical review with reference to osteoporosis. Int J Pharm Sci Health Care. 2014;1(4):131-41.
5. Khandelwal VK, Koneri R, Balaraman R, Kandhavelu M. Biological activities of some Indian medicinal plants. J Adv Pharm Educ Res. 2011;1:12-44.
6. Rajalakshmi A, Jayachitra A, Gopal P, Krithiga N. Toxicity analysis of different medicinal plant extracts in Swiss albino mice. Pharmacol Toxicol Res. 2014;1(1):1-6.
7. Sani D, Sanni S, Sandabe UK, Ngulde SI. Toxicological studies on *Anisopus mannii* crude aqueous stem extract: Biochemical and histopathological effects in albino rats. Int J Pharm Sci. 2010;2(1):51-9.
8. Kumar VK, Lalitha KG. Acute oral toxicity studies of *Anacyclus pyrethrum* DC root in albino rats. Int J Pharm Pharm Sci. 2013;5(4):675-8.
9. Ali R, Ali R, Jaimini A, Nishad DK, Mittal G, Chaurasia OP, *et al*. Acute and sub-acute toxicity and efficacy studies of *Hippophae rhamnoides* based herbal antioxidant supplement. Indian J Pharmacol. 2012;44(4):504-8. doi: 10.4103/0253-7613.99329, PMID 23087514
10. Zoric L, Merkulov L, Lukovic J, Boza P. Comparative analysis of qualitative anatomical characters of *Trifolium L*. (*Fabaceae*) and their taxonomic implications: Preliminary results. Plant Syst Evol. 2012;298(1):205-19. doi: 10.1007/s00606-011-0538-8
11. Corral MG. Effects of WCLMV and Root-Flooding on White Clover.

Dunedin, NZ: University of Otago; 2013.
12. Engelmann NJ, Reppert A, Yousef G, Rogers RB, Lila MA. *In vitro* production of radiolabeled red clover (*Trifolium pratense*) isoflavones. Plant Cell Tissue Organ Cult. 2009;98(2):147-56. doi: 10.1007/s11240-009-9547-5, PMID 19672329
13. Leporatti ML, Ivancheva S. Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. J Ethnopharmacol. 2003;87(2-3):123-42. doi: 10.1016/s0378-8741(03)00047-3, PMID 12860298
14. Asgarpanah J, Roohi E. Phytochemistry and pharmacological properties of *Equisetum arvense* L. J Med Plants Res. 2012;6(21):3689-93. doi: 10.5897/JMPR12.234
15. Semprini M, Prado FO, Paz K, Regalo SC, Lopes RA, Mal S. Hepatotoxicity of medicinal plants. IX. Action of *Equisetum arvense* L. infusion in rats. Braz J Pharm Sci. 2003;39:217.
16. Oh H, Kim DH, Cho JH, Kim YC. Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*. J Ethnopharmacol. 2004;95(2-3):421-4. doi: 10.1016/j.jep.2004.08.015, PMID 15507369
17. Mimica-Dukic N, Simin N, Cvejic J, Jovin E, Orcic D, Bozin B. Phenolic compounds in field horsetail (*Equisetum arvense L.*) as natural antioxidants. Molecules. 2008;13(7):1455-64. doi: 10.3390/molecules13071455, PMID 18719517
18. Pallag A, Pasca B, Jurca T, Suciu R, Nemeth S, Vicas L. Comparative histo-anatomical researches on the vegetative organs and assessment of antioxidant capacity of two species from *Equisetum* genus. Farmacia. 2016;64:372-7.
19. Cetojević-Simin DD, Canadanović-Brunet JM, Bogdanović GM, Djilas SM, Cetković GS, Tumbas VT, *et al*. Antioxidative and antiproliferative activities of different horsetail (*Equisetum arvense L.*) extracts. J Med Food. 2010;13(2):452-9. doi: 10.1089/jmf.2008.0159, PMID 20170379
20. Costa-Rodrigues J, Carmo SC, Silva JC, Fernandes MH. Inhibition of human *in vitro* osteoclastogenesis by *Equisetum arvense*. Cell Prolif. 2012;45(6):566-76. doi: 10.1111/j.1365-2184.2012.00848.x, PMID 23106302
21. Arbabzadegan N, Moghadamnia AA, Kazemi S, Nozari F, Moudi E, Haghanifar S. Effect of *Equisetum arvense* extract on bone mineral density in Wistar rats via digital radiography. Caspian J Intern Med. 2019;10(2):176-82. doi: 10.22088/cjim.10.2.176, PMID 31363396
22. De Smet PA. Health risks of herbal remedies. Drug Saf. 1995;13(2):81-93. doi: 10.2165/00002018-199513020-00003, PMID 7576267
23. Uebelhart D, Schlemmer A, Johansen JS, Gineyts E, Christiansen C, Delmas PD. Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium cross-links. J Clin Endocrinol Metab. 1991;72(2):367-73. doi: 10.1210/jcem-72-2-367, PMID 1991806