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Research Article
Protocatechuic Acid as a Potential Phytomedicine in a TCM Herbal Extract Mitigates Alcohol-Induced Osteoporosis

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Abstract

Background and Objective: Alcohol consumption exerts a multifaceted influence on skeletal health, with persistent gaps in understanding its contribution to bone loss. While light to moderate alcohol intake generally benefits or remains neutral for bone health in older adults, chronic excessive drinking leads to alcoholic osteoporosis and alcohol-induced osteonecrosis, hindering bone fracture recovery. Osteoporosis, a widespread bone ailment, presents a formidable global public health concern. For centuries, Traditional Chinese Medicine (TCM) has provided remedies for diverse medical conditions, including osteoporosis and alcoholism. This study aims to investigate whether protocatechuic acid (PCA) is a potential phytomedicine in a TCM herbal extract to effectively mitigate alcohol-induced osteoporosis in mice. **Materials and Methods:** To establish murine models of alcohol-induced bone loss, oral alcohol administration is employed via gavage in Balb/c mice and Lieber-DeCarli alcohol diet in C57BL/6 mice. The impacts of the herbal extract and PCA on alcohol-induced osteoporosis were investigated by bone morphological analysis using Micro-Computed Tomography (μ-CT). **Results:** Current study revealed that there is a preventive effect of the TCM herbal extract and PCA on alcohol-induced bone loss in Balb/c mice and the supplementation of the Lieber-DeCarli alcohol liquid diet with 50 mg kg^{-1} PCA significantly attenuated alcohol-induced trabecular bone loss in C57BL/6J mice. These findings highlight PCA as one of the potential phytomedicines within the herbal extract. **Conclusion:** Natural products derived from TCM herbs have made substantial contributions to pharmacotherapies and remain an invaluable resource for drug discovery. This study demonstrates the potential of therapeutic natural products in preventing alcohol-induced osteoporosis faced by long-term drinkers.

Key words: Traditional Chinese Medicine, herbal extracts, protocatechuic acid, alcohol, osteoporosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The relationship between alcohol consumption and human health is intricate. While the benefits of moderate alcohol consumption remain a topic of debate, epidemiological studies indicate potential advantages of light to moderate alcohol consumption, such as supporting heart health, safeguarding against diabetes and correlating with reduced risks of mortality in older adults[1-4]. Chronic excessive drinking poses serious risks to various organs and tissues, being a significant factor in stroke, heart failure and mortality[5-7]. Osteoporosis stands as a prevalent global public health concern, being the most common bone disease and a leading cause of fractures and disability[8-12]. Despite considerable advancements in comprehending postmenopausal and senile osteoporosis, the relationship between alcohol consumption and its impact on bone health remains intricate, resulting in notable gaps in our understanding of how alcoholism contributes to bone loss. The complex effects of alcohol on skeleton depend on age, drinking pattern, hormonal status and the type of alcohol consumed. Light to moderate alcohol consumption is generally reported to be beneficial or have a neutral impact on bone health in older adults, while chronic excessive drinking can lead to alcoholic osteoporosis, which is a disease that compromises normal bone metabolism and increases fracture risk, diminishes bone repair and alcohol-induced osteonecrosis[13-19].

Traditional Chinese Medicine (TCM) has been practiced over thousands of years for the treatment and symptom management of a wide range of medical conditions, including osteoporosis and alcoholism. Natural products, in particularly extracted from TCM, have contributed significantly to pharmacotherapy and remain unique sources for drug discovery[20,21].

Preclinical and clinical studies demonstrated that an herbal extract (Jing extract) utilized a composite herbal formula consisting of nine Chinese herbal medicines, including *Astragalus*, *Cistanche deserticola*, *Dioscorea polystachya*, *Lycium barbarum*, *Epimedium*, *Cinnamomum cassia*, *Syzygium aromaticum*, *Angelica sinensis* and *Curculigo orchioides*, which have the anti-fatigue and immunity-enhancing properties in humans and animals[22-24]. Qian *et al*.[19] suggested that the herbal extract prevents chronic excessive alcohol consumption-induced osteopenia in male mice. This study investigates whether Jing extract holds therapeutic potential for combating alcohol-induced bone loss in female mice.

The HPLC, LC/MS and NMR analyses[22-24] revealed that Jing extract comprises a variety of bioactive components with antiosteoporotic properties, some of which also exhibit potential in managing alcohol use disorder, including protocatechuic acid, acteoside, calycosin, daidzin, echinacoside, epimedin, eugenol, ferulic acid, hyperoside, icariside, icariin, oleanolic acid, puerarin, sagittatoside, scopolin and ligustilide. Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA) is a natural phenolic compound in various plant-based foods and traditional herbal medicines and has a wide array of health benefits, including antioxidant, anti-inflammatory, anti-hyperglycemic, antibacterial, antiviral, anticancer, antiosteoporotic, anti-aging and neuroprotective properties[25-28]. This study delves into the potential of PCA, a representative bioactive component within Jing extract, to mitigate alcohol-induced osteopenia in mice. Current study not only underscores the preventive effects of TCM Jing extract against alcohol-induced bone loss in female mice, but also highlights PCA as one of the promising phytomedicines present in this herbal extract. Further investigations are warranted to explore whether other natural products identified in the extract share similar therapeutic potential in the mouse model of osteopenia.

MATERIALS AND METHODS

Study area: This study was conducted in Brigham and Women's Hospital, Harvard Medical School, United States during the period from January, 2019 to December, 2022.

Materials: In this study, a composite herbal formula consisting of nine Chinese herbal medicines was utilized. These herbs include *Astragalus*, *Cistanche deserticola*, *Dioscorea polystachya*, *Lycium barbarum*, *Epimedium*, *Cinnamomum cassia*, *Syzygium aromaticum*, *Angelica sinensis* and *Curculigo orchioides* (Fig. 1). These herbs were prepared in accordance with our description[19]. The collection of these Traditional Chinese Medicine herbs was carried out by researchers at the Jing Brand Research Institute. Voucher specimens, as outlined in a report by researchers[22-24], have been deposited at the Herbarium of Jing Brand Research Institute, located in Daye, Hubei, China. The raw Chinese herbal medicines underwent a meticulous process, which included washing, drying and slicing into pieces, followed by superfine pulverization, in accordance with the protocols outlined in the Chinese Pharmacopoeia. The traditional Chinese medicine herbal extracts, referred to as Jing extract, were prepared using a percolation extraction method,

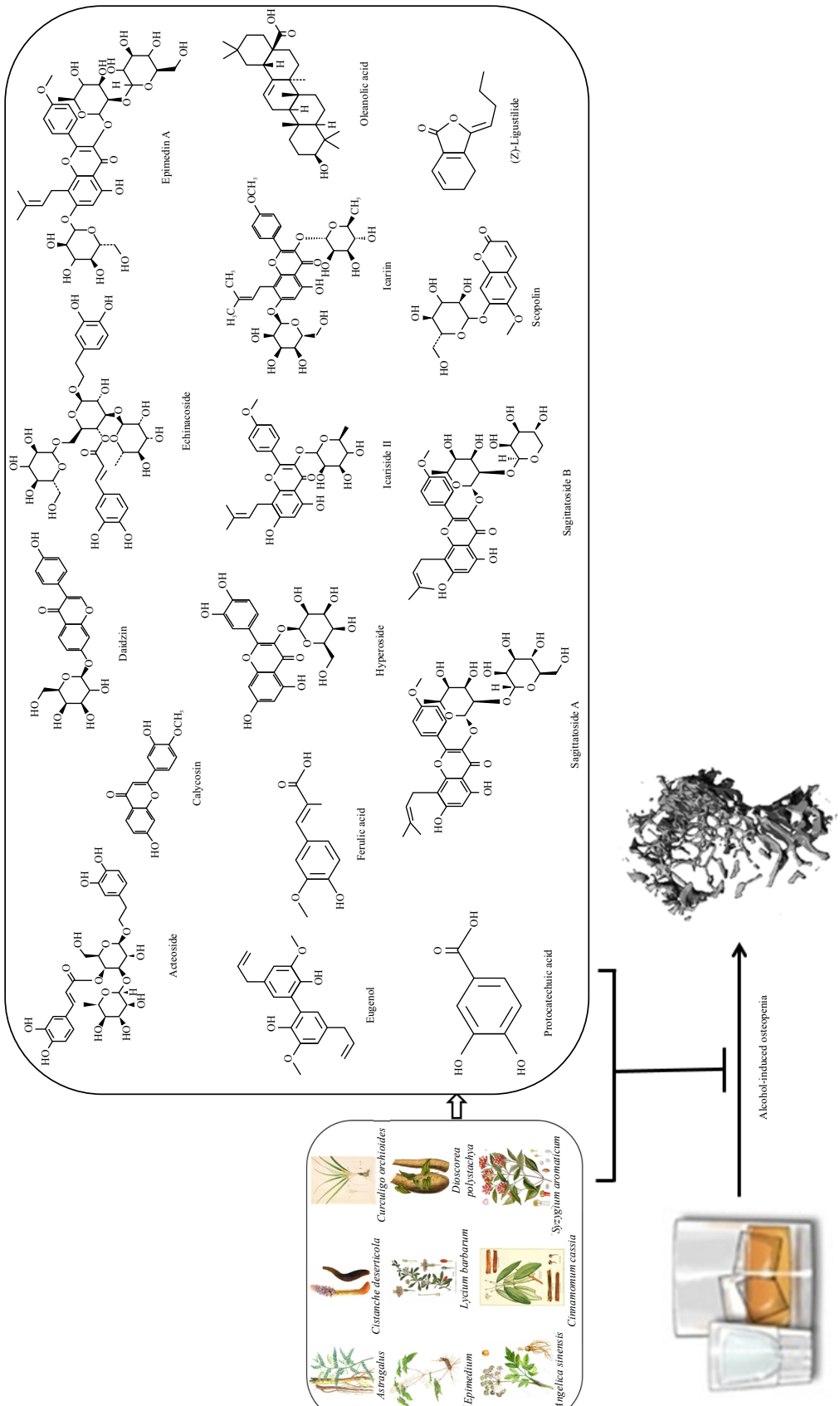

Fig. 1: Experimental design
A concentrated TCM herbal extract, referred to as Jing extract, from nine Traditional Chinese Medicine herbs and bioactive compounds in the herbal extract were investigated in animal models of alcohol-induced bone loss

as detailed by Qian *et al.*[19]. The extracts of these Chinese herbal medicines (Jing extract) have been subjected to extensive characterization through chemical constituent analysis with LC/MS and NMR[22,23] and high-performance liquid chromatogram-gas chromatography fingerprint analysis[24]. Protocatechuic acid (PCA) (3,4-dihydroxybenzoic acid) and vitamin D_3 were procured from MilliporeSigma (Burlington, Massachusetts). Xian-Ling-Gu-Bao (XLGB) capsule was obtained as an over-the-counter Traditional Chinese Medicine (TCM) product from Sinopharm Group Tongjitang Pharmaceuticals Co. Ltd., based in Guizhou, China.

Animals: The Balb/c and C57BL6 mice from Jackson Laboratory (Bar Harbor, Maine, USA) were procured, two mating pairs per mouse strain, to establish breeding colonies within the animal facilities of Brigham and Women's Hospital (BWH). This study used 70 Balb/c mice and 20 C57BL6 mice (2 months old). The animal facilities at BWH, fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care, were employed for housing the mice. The mice were maintained under controlled conditions, including a temperature of 22±2/-2.5°C with alarm setpoints at+4/-3°C and a humidity level of 40% with alarm setpoints at +15/-10%. Lighting was provided on a 12 hrs light/12 hrs dark cycle. Mice were given unrestricted access to standard chow and water.

Ethical consideration: All aspects of animal maintenance and experimental procedures were conducted in strict adherence to ethical guidelines for animal research, which were established and approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee (#2016N000361).

Oral alcohol administration models of alcohol-induced bone loss: To establish murine models of alcohol-induced osteoporosis and investigate the impacts of the herbal extract and PCA, oral alcohol administration via gavage in female Balb/c mice was employed. This approach follows the procedures outlined in previous studies[19,29]. In this study, vitamin D_3, a recognized preventive agent for alcohol-induced osteopenia[30], was employed as a control for the potential small molecule phytomedicine, PCA. Xian-Ling-Gu-Bao (XLGB) capsule is a potent traditional Chinese medicine prescription widely utilized for the prevention and treatment of osteoporosis in China, as supported by studies[31-33]. The XLGB is composed of several key ingredients, including *Epimedium brevicornum*, *Dipsacus fullonum*, *Salvia miltiorrhiza*, *Anemarrhena asphodeloides*, *Psoralea corylifolia* and *Rehmannia glutinosa*[32-33]. This study employed XLGB as the control for Traditional Chinese Medicine (TCM) anti-osteoporotic treatment. The 0.4 g kg^{-1} dosage administered to the mice corresponds to the recommended human dosage (taken 3 capsules twice per day, each capsule containing 0.3 g of XLGB) based on mouse-human dose conversion[34,35].

Chronic alcohol-induced bone loss model using the Lieber-DeCarli liquid diet: The C57BL/6 is a strain of mice known for its preference for alcohol and is frequently utilized in alcohol-related experiments[29]. For current study, C57BL/6J male mice were randomly divided into age-matched groups at 8 weeks old. They were then subjected to dietary conditions, including the Lieber-DeCarli liquid control diet (Bio-Serv) or the Lieber-DeCarli alcohol diet (Bio-Serv), which contained 5% (v/v) alcohol and maltose dextrin. These dietary conditions were administered according to previously established protocols[29,30,36,37]. A third experimental group received the Lieber-DeCarli alcohol diet containing 5% (v/v) alcohol along with the phytomedicine candidate PCA (at a dose of 50 mg kg^{-1}). The C57BL/6J male mice receiving the Lieber-DeCarli alcohol liquid diet were provided with food *ad libitum*, while calorically matched control and Lieber-DeCarli alcohol plus PCA (50 mg kg^{-1}) diets were adjusted based on the previous day's consumption by the alcohol-exposed groups. To ensure consistent experimental dosages over the 6-week study period, the quantity of PCA added to the Lieber-DeCarli alcohol liquid diet was adjusted daily in accordance with body weight and the previous day's consumption.

Bone morphological analysis

Micro-Computed Tomography (μ-CT): Bone morphological analysis of the proximal tibia was conducted using Micro-Computed Tomography System (μ-CT 35, Scanco Medical, Switzerland), following the procedures as described by Qian *et al.*[19]. For trabecular bone analysis, selected the scanned region proximal to the growth plate, extending 1.4 mm, as indicated by the outlined box in Fig. 2a. The 3-dimensional microstructural characteristics of the bone, encompassing Trabecular Bone Mineral Density (Tb. BMD), tibia Trabecular relative Bone Volume (Tb. BV/TV), trabecular number (Tb. N) and cortical bone mineral density (C. BMD), were evaluated using software provided by the manufacturer, Scanco Medical, Switzerland. Furthermore, a second segment, measuring 0.6 mm in length and positioned at the midpoint of the tibia as indicated by the enclosed box in Fig. 2c, was employed to compute diaphyseal parameters.

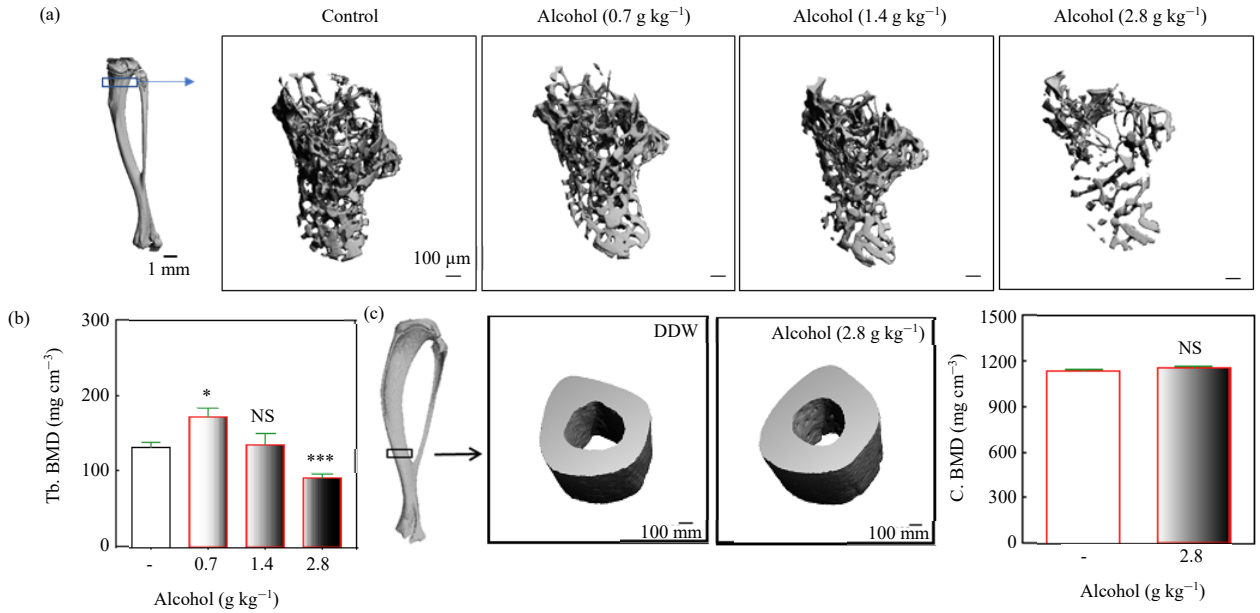

Fig. 2(a-c): Oral alcohol administration models of alcohol-induced bone loss in Balb/c female mice. The Balb/c female mice (2 months old) were administrated with different doses of alcohol, 0.7, 1.4 and 2.8 g kg^{-1} (alcohol/body weight) for 40 days, (a) Representative µ-CT 3-D microstructures of trabecular bone were obtained from female mice with alcohol gavage (alcohol) or water gavage (control) for 40 days, (b) Quantitative analysis of Trabecular Bone Mineral Density (Tb. BMD) and (c) Representative µ-CT 3-D microstructures of cortical bone were obtained from female mice with 2.8 g kg^{-1} alcohol gavage (alcohol) or water gavage (control) for 40 days

(a) Bars represent 100 µm. The 3-D microstructural properties of the tibia were calculated using software supplied by the manufacturer, (b) (Alcohol of 0.7 g kg^{-1}, n = 3, 1.4 g kg^{-1}, n = 7, 2.8 g kg^{-1}, n = 14, vs control, n = 18; *p<0.05, ***p<0.001, t-test, NS: Not significant and (c) Bars represent 100 mm. The quantitative analysis of cortical bone mineral density (C. BMD), 2.8 g kg^{-1} alcohol did not induce cortical bone loss (alcohol, n = 9, vs control, n = 14, NS: Not significant and t-test)

Statistical analysis: All experiments were conducted with a minimum of three replicates. Quantitative data were treated as continuous variables and analyzed using standard statistical tests. The Kolmogorov-Smirnov test was employed to assess the normal distribution of data sets. Group data is presented as Mean±SEM (Standard Error of the Mean). Unless specified otherwise, quantitative data were subjected to non-parametric Mann-Whitney tests for group comparisons. If the data met the assumptions of parametric analysis, t-tests were employed for two-group comparisons, while one-way ANOVA was utilized for multiple group comparisons, all conducted using GraphPad Instat (GraphPad Software, La Jolla, California). Statistical significance was defined as a p-value less than 0.05.

RESULTS

Optimal dosages for alcohol-induced bone loss in Balb/c female mice: To determine the optimal dosages for inducing alcohol-induced osteopenia in female Balb/c mice, a study was conducted using 2 months old mice and administered oral alcohol at doses of 0.7, 1.4 and 2.8 g kg^{-1} of body weight. This administration took place once per day between 3-4 PM, five days a week, over a 40-day period.

Current study showed that the dosage of 0.7 g kg^{-1} significantly increased Bone Mineral Density (BMD) in Balb/c female mice that received alcohol via oral gavage, while the 1.4 g kg^{-1} alcohol dosage did not induce significant bone loss after the 40 days administration (Fig. 2a-b). However, following 40 days of oral alcohol administration, the 2.8 g kg^{-1} dosage of alcohol did lead to significant bone loss in Balb/c female mice (Fig. 2a-b). Consequently, the 2.8 g kg^{-1} alcohol dosage was selected and maintained a 40 days duration for further investigations into the effects of the herbal extract and PCA on alcohol-induced osteopenia. It is noteworthy that, under these conditions, using the same 2.8 g kg^{-1} dosage and 40 days duration of alcohol administration did not result in bone loss in cortical bone (Fig. 2c). Therefore, current report primarily focuses on the impact of alcohol on trabecular bone.

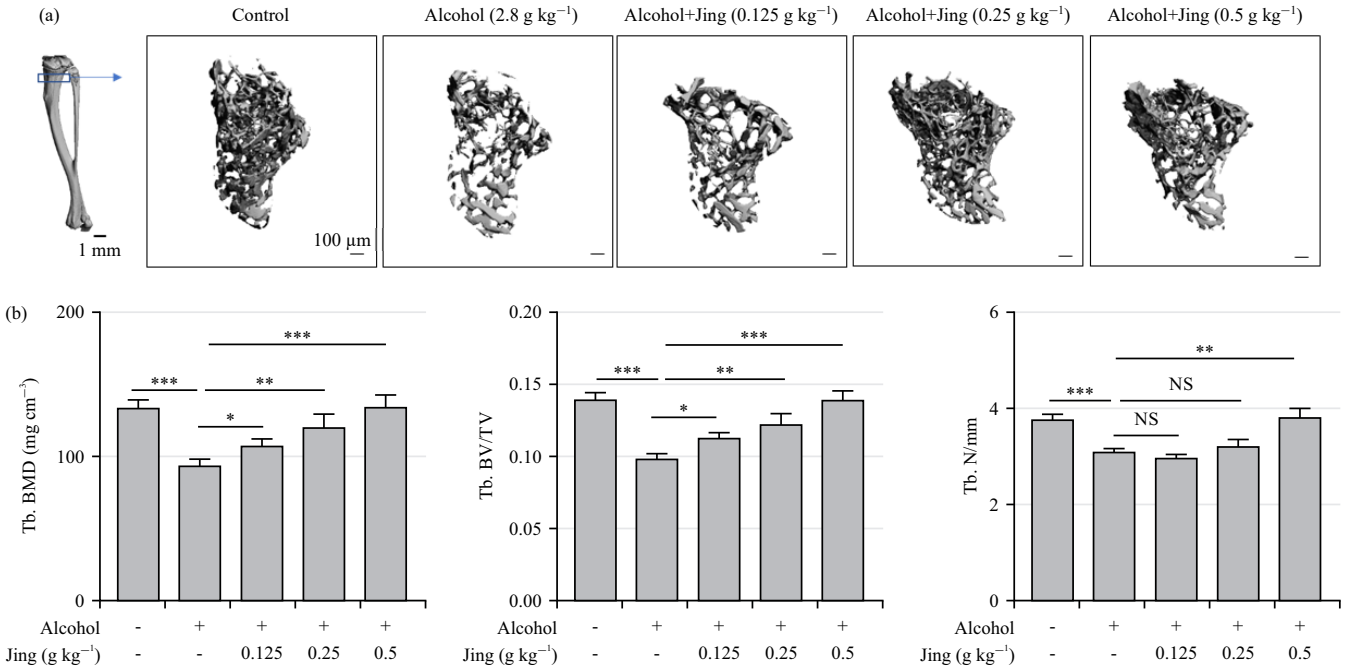

Fig. 3(a-b): An herbal extract (Jing extract) prevents chronic alcohol consumption-induced osteopenia in young adult female Balb/c mice, (a) Representative µ-CT 3-D microstructures of trabecular bone were obtained from female mice with alcohol gavage (alcohol)±Jing extract or without alcohol (DDW control) and (b) Quantitative analysis of Trabecular Bone Mineral Density (Tb. BMD)

(a) Bars represent 100 µm. The 3-D microstructural properties of the tibia were calculated using software supplied by the manufacturer and (b) 2.8 g kg^{-1} of alcohol, n = 14, vs control, n = 18, ***p<0.001, t-test; alcohol+0.125 g kg^{-1} of Jing extract, n = 6, vs alcohol, *p<0.05; alcohol+0.25 g kg^{-1} of Jing extract, n = 16 vs alcohol, **p<0.01; alcohol+0.5 g kg^{-1} of Jing extracts, n = 8, vs alcohol, ***p<0.001, t-test); the quantitative analysis of tibia Trabecular Relative Bone Volume (Tb. BV/TV) (*p<0.05, **p<0.01, ***p<0.001; control, n = 18; alcohol, n = 9; alcohol+0.125 g kg^{-1}, n = 6, +0.25 g kg^{-1}, n = 12, +0.5 g kg^{-1}, n = 8, of Jing extract; t-test); the quantitative analysis of tibia trabecular number per mm (Tb. N) (***p<0.001, **p<0.01 and NS: Not significant; n is same as BV/TV; t-test)

Herbal extract prevents chronic alcohol-induced bone loss in Balb/c mice: In present investigation, the study aimed to determine the efficacy of an herbal extract in mitigating the bone loss induced by chronic alcohol consumption in Balb/c female mice. To establish the model for alcohol-induced osteopenia, mice were administered a dose of 2.8 g kg^{-1} of alcohol via oral gavage for a duration of 40 days. As depicted in Fig. 2a, this regimen led to significant bone loss in Balb/c female mice. Subsequently, mice were assessed the impact of the herbal extract, specifically Jing extract from traditional Chinese medicine, on alcohol-induced osteopenia. In this experiment, 2 months old Balb/c female mice were orally administered 2.8 g kg^{-1} of alcohol via gavage, with or without varying doses of the herbal extract (Jing extract) at 0.125, 0.25 or 0.5 g kg^{-1}, as outlined in Fig. 3a. After 40 days of oral administration of alcohol, with or without the herbal extract, the mice were sacrificed for bone morphological analysis through µ-CT. Figure 3 revealed that, the three-dimensional microstructures of trabecular bone (Fig. 3a) and the quantitative analysis of tibial microstructural properties (Fig. 3b), encompassing Trabecular Bone Mineral Density (Tb. BMD), Trabecular Relative Bone Volume (Tb. BV/TV) and trabecular number per mm (Tb. N), indicated a dose-dependent preventive effect of the herbal extract on alcohol-induced bone loss in Balb/c female mice. Current data demonstrated that the administration of 2.8 g kg^{-1} of alcohol via oral gavage induced substantial bone loss in Balb/c mice following 40 days of treatment (Fig. 3). However, all three doses of the herbal extract-low, middle and high-ameliorated the trabecular bone damage caused by chronic alcohol consumption. Additionally, all three doses mitigated the alcohol-induced reductions in Bone Mineral Density (Tb. BMD) and relative Bone Volume or Bone Volume fraction (BV/TV) (Fig. 3b). Furthermore, the high dose of the herbal extract also alleviated the decrease in trabecular number (Tb. N) induced by alcohol (Fig. 3b).

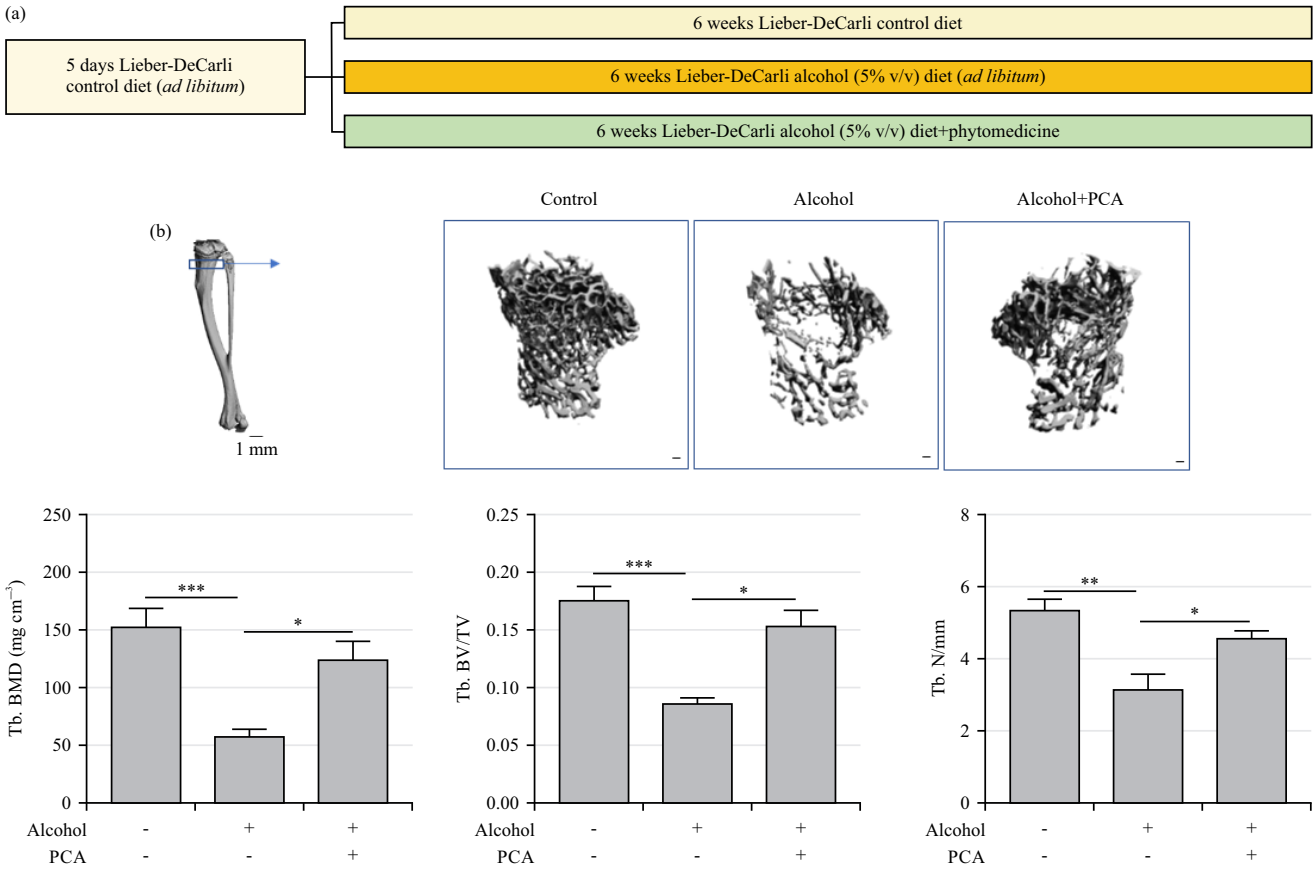

Fig. 4(a-b): Protocatechuic acid (PCA) prevents chronic alcohol consumption-induced osteopenia in C57BL6 mice, (a) Overview of the chronic alcohol Lieber-DeCarli liquid diet feeding procedure and (b) µ-CT analysis of proximal tibia

Scanned regions proximal to the growth plate were indicated with the box. Representative 3-D microstructures of trabecular bone were obtained from proximal tibia of C57BL6 male mice with Lieber-Decarli 5% alcohol diet (alcohol) ± PCA (50 mg kg^{-1}) or Lieber-Decarli control diet (Control) for 6 weeks; bar in bottom right-hand represents 100 µm. The quantitative µ-CT data of proximal tibia Tb. BMD, Tb. BV/TV and Tb. N (alcohol, n = 7; Control, n = 9; alcohol+PCA, n = 4) demonstrated that supplementation of the Lieber-DeCarli alcohol liquid diet with 50 mg kg^{-1} PCA was able to attenuate trabecular bone loss in C57BL/6J mice (*p<0.05, **p<0.01, ***p<0.001 and t-test). There are no differences in cortical BMD among the experimental groups (data not shown)

Protocatechuic acid alleviates chronic alcohol-induced bone loss in C57BL6 mice: Present study aimed to investigate whether protocatechuic acid (PCA), a component found in the Jing herbal extract, mitigates alcohol-induced osteopenia in C57BL/6J male mice (8 weeks old) fed with the Lieber-DeCarli alcohol diet. Following six weeks of dietary intervention, during which C57BL/6J mice were fed either the Lieber-DeCarli liquid control diet or the Lieber-DeCarli alcohol diet containing 5% v/v alcohol, with or without the inclusion of the phytomedicine candidate PCA at a dosage of 50 mg kg^{-1} (Fig. 4a), the study analyzed bone microstructures in the tibia using µ-CT (Fig. 4b). Current data demonstrated that the alcohol liquid diet-induced trabecular bone loss in the tibias of male C57BL/6J mice (Fig. 4b). Intriguingly, the supplementation of the Lieber-DeCarli alcohol liquid diet with 50 mg kg^{-1} PCA significantly attenuated this trabecular bone loss in male C57BL/6J mice (Fig. 4b). These findings highlight PCA as one of the potential phytomedicines within the herbal extract.

Protocatechuic acid mitigates alcohol-induced osteopenia in Balb/c mice: This study aimed to evaluate the preventive effects of protocatechuic acid (PCA) on alcohol-induced bone loss in Balb/c female mice, following a similar experimental approach as detailed in Fig. 3 for the herbal extract. Current study involved a comparative analysis of PCA, administered at a dosage of 50 mg kg^{-1} and vitamin D_3, delivered at a dosage of 2000 IU kg^{-1}. The objective was to assess the individual impacts of PCA and vitamin D_3 on alcohol-induced osteopenia in 2 months old female Balb/c mice. Over a period of 40 days,

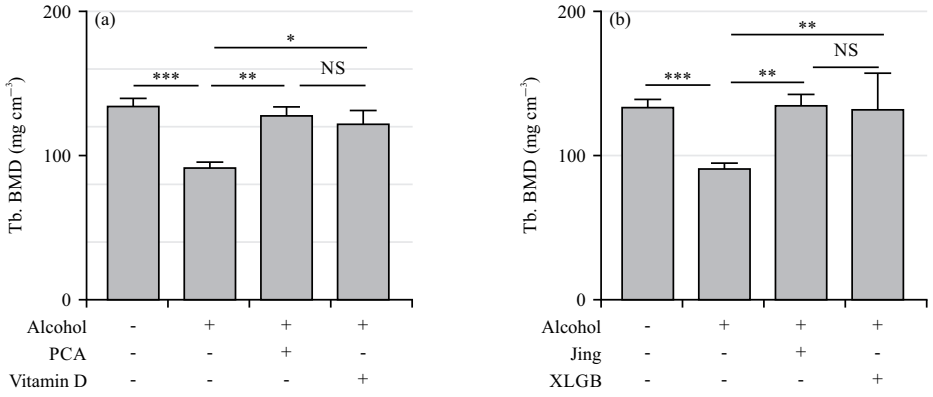

Fig. 5(a-b): Effects of PCA and herbal extract on alcohol-induced osteopenia in Balb/c mice, (a) Balb/c female mice (2 months old) were orally administrated 2.8 g kg^{-1} body weight of alcohol with or without 50 mg kg^{-1} PCA or 2000 IU kg^{-1} of vitamin D_3 (Vit. D); after 40 days treatment, both PCA and vitamin D_3 significantly prevented alcohol-induced osteopenia and (b) Balb/c female mice (2 months old) were orally administrated 2.8 g kg^{-1} body weight of alcohol with or without 0.5 g kg^{-1} Jing extract or 0.4 g kg^{-1} of Xian-Ling-Gu-Bao (XLGB)

(a) **p<0.01, alcohol+PCA, n = 5, vs alcohol, n = 9; *p<0.05, vitamin D+alcohol, n = 5, vs alcohol; PCA vs vitamin D3, NS: Not significant and t-test and (b) After 40 days, both Jing extract and XLGB significantly prevented alcohol-induced osteopenia in Balb/c female mice (***p<0.001, alcohol, n = 9, vs control, n = 18; **p<0.01, alcohol+Jing, n = 8, vs alcohol, t-test; alcohol+XLGB, n = 4 vs alcohol, **p<0.01; Jing vs XLGB, NS: Not significant and Mann-Whitney test)

mice were administered either 2.8 g kg^{-1} of alcohol per body weight alone or in combination with either 50 mg kg^{-1} of PCA or 2000 IU kg^{-1} of vitamin D_3 (Vit. D) via gavage in Balb/c female mice. Results demonstrated significant protective effects of both PCA and vitamin D_3 against alcohol-induced osteopenia in these mice (Fig. 5a). Remarkably, the bone health of mice treated with alcohol in conjunction with either PCA or vitamin D_3 exhibited substantial improvements compared to those treated solely with alcohol. It is worth mentioning that no significant difference was observed between the effects of 50 mg kg^{-1} of PCA and 2000 IU kg^{-1} of vitamin D_3 (Fig. 5a).

Herbal extracts (Jing) and Xian-Ling-Gu-Bao (XLGB) in preventing alcohol-induced osteopenia: In our experiments (Fig. 5b), we employed XLGB as the control for Traditional Chinese Medicine (TCM) anti-osteoporotic treatment. After a 40 days period of oral administration involving 2.8 g kg^{-1} of alcohol, either with or without 0.5 g kg^{-1} of Jing extract or 0.4 g kg^{-1} of XLGB, we observed that both Jing extract and XLGB significantly prevented alcohol-induced osteopenia in 2 months old female Balb/c mice. Importantly, their preventive effects did not exhibit significant differences (Fig. 5b). These findings underscore the potential of both PCA and Jing extract as promising interventions for mitigating the detrimental consequences of chronic alcohol consumption on bone health.

DISCUSSION

Traditional Chinese Medicine (TCM) has been practiced over thousands of years for the treatment and symptom management of a wide range of medical conditions, including osteoporosis and alcoholism. For thousands of years, guided by TCM principles, alcohol has been combined with TCM herbal ingredients to create Chinese herbal Liqueur, which serve both preventive and therapeutic purposes[38,39]. The herbal extract (Jing extract) utilized in this study is derived from a Traditional Chinese Medicine (TCM) herbal formula found in the renowned Chinese Herbal Liqueur, Chinese Jing Liqueur[19,22-24]. Europe also has a longstanding tradition of consuming medicinal wines, including Absinthe, Chartreuse, Bénédictine, Dubonnet, Strega, Galliano, Gin, Kräuterlikör, Becherovka and Unicum, etc. Given that alcoholic osteoporosis primarily stems from diminished bone formation rather than heightened bone resorption[17,19], anabolic anti-osteoporotic agents that stimulate bone formation, such as vitamin D and estrogen, are likely to prevent alcohol-induced bone loss as reported by researchers[30,37]. The TCM formulas have a longstanding history of use in preventing and treating osteoporosis and the phytochemicals derived from TCM formulas hold significant potential for the development of novel anti-osteoporotic drugs[39-41]. A key molecular mechanism of TCM anti-osteoporotic drugs lies in their ability to promote osteoblast-mediated bone formation[42], rendering TCM a promising candidate for therapy targeting alcoholic osteoporosis.

To investigate the potential of the herbal extract (Jing extract) and protocatechuic acid (PCA), a component of the herbal extract, in ameliorating alcohol-induced osteopenia in murine models (Fig. 1), the study initially focused on determining the optimal dosage and duration for oral alcohol administration in Balb/c female mice. Among the various oral alcohol dosages tested, namely 0.7, 1.4 and 2.8 g kg^{-1} of body weight administered over a 40 days period, it was observed that the 0.7 g kg^{-1} dosage significantly increased Bone Mineral Density (BMD), the 1.4 g kg^{-1} alcohol dosage did not induce significant bone loss, while the 2.8 g kg^{-1} alcohol dosage resulted in substantial bone loss in Balb/c female mice (Fig. 2). Epidemiological studies suggested that light to moderate alcohol consumption have a beneficial or neutral impact on bone health and chronic excessive drinking leads to a loss of bone mass and increases the risk of osteoporosis[13-18]. Current findings from a female murine alcohol model in this study align with these epidemiological observations and our prior analysis involving male mice[19]. Doses exceeding 2.8 g kg^{-1} or longer durations (50 days) led to unacceptable mortality rates in the alcohol group and a shorter duration (30 days) at the 2.8 g kg^{-1} dose did not result in significant alcohol-induced bone loss (data not shown). As a result, this study selected the 2.8 g kg^{-1} alcohol dosage with a 40 days duration for further investigation into the effects of the herbal extract and PCA on alcohol-induced osteopenia.

This study shows that the herbal extract (Jing extract) effectively mitigates alcohol-induced osteopenia in Balb/c female mice (Fig. 3), which confirmed previous findings in male mice[19]. The three doses of Jing extract, 0.125, 0.25 or 0.5 g kg^{-1}, ameliorated the trabecular bone damage caused by chronic alcohol consumption (Fig. 3), implying that the potential of this TCM extract in preventing alcohol-induced osteoporosis faced by long-term drinkers. Xian-Ling-Gu-Bao (XLGB) capsule is an effective traditional Chinese medicine prescription used to prevent and treat osteoporosis in China[31-33]. The XLGB was used as the TCM antiosteoporotic medicine control in current experiments. The current study showed that both Jing extract and XLGB significantly prevented alcohol-induced osteopenia in Balb/c female mice and their preventing effects have no significant difference (Fig. 5). These findings highlight the potential of Jing extract as a promising intervention to mitigate the detrimental effects of chronic alcohol consumption on bone health.

There are several different murine models of alcohol-induced bone loss regarding alcohol administration, including oral alcohol administration by gavage[43,44], alcohol in drinking water[45,46], intraperitoneal (IP) administration of alcohol by injection[44] and Liber-DeCarli liquid diet feeding[29,30,47]. While there are both benefits and drawbacks associated with various methods of alcohol administration, utilizing oral alcohol administration via gavage in animals proves to be a superior model for mimicking intermittent drinking patterns observed in humans. In this study, we employed oral alcohol administration via gavage in Balb/c female mice as the murine models for alcohol-induced bone loss, aiming to investigate the impacts of the herbal extract (Fig. 3) and PCA (Fig. 5) on alcoholic osteoporosis. The C57BL/6J mice voluntarily drink alcohol-laden liquids preferentially and are widely used in alcohol-liquid diet experiments[29,48]. In the second model of alcohol-induced bone loss of this study, we used C57BL/6J mice with the Lieber-DeCarli alcohol liquid diet to investigate whether protocatechuic acid (PCA), a component found in the herbal extract, mitigates alcohol-induced osteopenia (Fig. 4). In both oral alcohol administration by gavage model (Fig. 5) and Lieber-DeCarli alcohol liquid diet model (Fig. 4), PCA significantly attenuated trabecular bone loss in mice. These findings highlighted PCA as one of the potential phytomedicines within the herbal extract.

Among the TCM herbs in the herbal extract used in this study, *Astragalus*, *Cistanche deserticola*, *Dioscorea polystachya*, *Lycium barbarum*, *Epimedium*, *Cinnamomum cassia*, *Syzygium aromaticum*, *Angelica sinensis* and *Curculigo orchioides* (Fig. 1). Protocatechuic acid (PCA) was detected in *Angelica sinensis*[49], *Cinnamomum cassia*[50], *Syzygium aromaticum*[51], *Dioscorea polystachya*[52] and *Lycium barbarum*[53]. Protocatechuic acid (PCA), a natural phenolic compound in various plant-based foods and traditional herbal medicines, has a wide array of health benefits[25-28]. In an animal model of postmenopausal osteoporosis, oral supplementation with PCA (20 mg/kg/day) significantly ameliorated the ovariectomized-mediated bone loss and changes in bone biomechanical properties in female ICR mice[54]. In a lipopolysaccharide (LPS)-induced mouse model of inflammatory bone loss, oral administered 25 mg kg^{-1} of PCA attenuates bone loss in ICR male mice[55]. The PCA significantly increased intracellular mineralization in MSCs *in vitro*[56]. Previous study demonstrated that PCA significantly alleviates the inhibitory effects of alcohol on osteoblastogenesis[19]. Further *in vivo* investigations are needed to explore the mechanism by which PCA ameliorates alcohol-induced bone loss resulting from decreased bone formation or increased bone resorption.

Based on Lipinski's drugability in which poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight is greater than 500 and the calculated Log P (Partition coefficient) is greater than 5[57], the drug-like property parameters of PCA include 3 H-bond donors, 4 H-bond acceptors, 154.1 of molecular weight and 0.903 of calculated Log P, suggested that PCA is a promising drug candidate. Botanical-based natural products are an important resource for medicinal drug discovery and continue to provide diverse pharmacophores with therapeutic potential against human diseases[58]. In this study, current findings highlight PCA as one of the potential phytomedicines within the herbal extract. However, further investigations are warranted to ascertain whether the remaining natural products identified in the herbal extract possess similar potential as phytomedicines in our mouse model of osteopenia.

CONCLUSION

This study demonstrates that a TCM extract and protocatechuic acid, an emerging phytomedicine within the herbal extract, effectively mitigate alcoholic osteoporosis in murine models. Natural products derived from TCM herbs have made substantial contributions to pharmacotherapies and remain an invaluable resource for drug discovery. This study demonstrates the potential of therapeutic natural products in preventing alcohol-induced osteoporosis faced by long-term drinkers.

SIGNIFICANCE STATEMENT

Traditional Chinese Medicine (TCM) has treated various diseases, including bone diseases and alcoholism. Chronic excessive drinking results in alcohol-induced bone diseases, including osteoporosis, which increases fracture risk. Current study showed that a TCM herbal extract and protocatechuic acid (PCA), a phytomedicine within the TCM herbal extract, effectively mitigate chronic excessive alcohol consumption-induced osteoporosis in mice, implying that traditional medicinal plants have the therapeutic potential of preventing chronic excessive drinking-induced bone diseases.

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