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Research article

EFFECTS OF A POLYHERBAL MIXTURE ON FEMORAL AND TIBIAL MICROARCHITECTURE IN RATS WITH DIABETIC OSTEOPOROSIS

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The femur and tibia are prone to the development of osteopenia and osteoporosis, particularly in individuals with diabetes, who are more susceptible than healthy individuals. This study aimed to evaluate the effects of a polyherbal mixture composed of *Centaurium erythraea* aerial parts, *Cichorium intybus* roots, and *Potentilla erecta* rhizomes on the microarchitecture of these two bones in non-diabetic and diabetic rats. Diabetes was induced in female Wistar rats by a single intraperitoneal injection of alloxan monohydrate. Animals were treated with four concentrations of the polyherbal mixture – 2.5, 5, 10, and 15 g of dry herbal weight /kg of animal, by oral gavage. Non-diabetic controls received water. Diabetic control groups were treated with water, insulin-glargine (13 IU/kg), or glimepiride (1 mg/kg), respectively. The answer to the therapy was assessed via HbA1c test and pathohistological evaluation of bone tissue. H&E staining was used to evaluate the cortical bone area and osteocyte lacunar area in the epiphysis and the cortical bone, while Masson's trichrome staining was used for the determination of collagen level and trabecular area in the epiphysis. Neither of the tested concentrations caused any pathological changes in the bones of healthy animals. The highest tested concentration of the polyherbal mixture showed greater efficacy than insulin and glimepiride in improving HbA1c levels and ameliorating microarchitectural alterations induced by alloxan, with more pronounced effects observed in the femur than in the tibia. These findings suggest the osteoprotective potential of this polyherbal mixture in an experimental model of diabetes-induced osteoporosis.

Keywords: diabetes, femur, osteoporosis, polyherbal mixture, tibia

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INTRODUCTION

Diabetic osteoporosis is a secondary form of osteoporosis characterized by increased bone fragility, structural degradation, and high fracture risk. It is one of the most common secondary complication of diabetes, a chronic metabolic disorder affecting approximately 589 million people worldwide, with nearly 30% of patients developing this particular complication [1-5].

Although the standard method for osteoporosis diagnosis and prognosis is primarily based on the evaluation of bone mineral density (BMD), when addressing such a multifactorial condition such as diabetic osteoporosis and avoiding the so-called "diabetic paradox," in which individuals with diabetes and completely normal BMD values exhibit a higher tendency toward fractures, a more detailed analysis of bone microarchitecture, such as structural organization of trabecular and cortical compartments, including their relative proportions and ultrastructural characteristics [6,7], as well as assessing the possible connection of these parameters with primary diabetic problems such as hyperglycemic environment, is necessary [3,6-8].

Thus, in diabetic osteoporosis development, the changes in the bone microarchitecture can be attributed to the elevated level of Advanced Glycation End Products (AGEs) on bone matrix, collagen glycosylation, elevated level of ROS and inflammation in diabetes [9,10], where lower extremities are more affected with these microarchitecture changes.

Namely, as primary weight-bearing structures, the lower extremities endure substantial mechanical forces and experience significant mechanical stress [11,12]. Moreover, structural differences between the bones of the lower extremities, particularly the greater proportion of trabecular area in the femur compared with the tibia, are associated with higher metabolic activity and increased cellular turnover, especially within the femoral epiphysis. [13,14]. This site-specific skeletal response is important both for understanding the pathophysiology of diabetic osteoporosis and to evaluate the differences in therapeutic response.

Although standard antidiabetic pharmacotherapy effectively normalizes glycemia, it often fails to fully address chronic inflammation, increased oxidative stress, and the overproduction of AGEs, the key contributors to the development of diabetic osteoporosis. Consequently, increasing attention has been directed toward investigating the therapeutic potential of medicinal plants in the management of diabetes and its secondary complications. This interest is well justified, as phytochemicals exhibit considerable potential to modulate dysregulated cellular metabolism, enhance cell survival, and strengthen antioxidant defense mechanisms [15,16]. In the context of diabetic osteoporosis, even greater therapeutic benefits have been reported when herbal preparations are used in combination rather than as single agents [17-19].

Our previous study demonstrated that a polyherbal mixture composed of *Centaurium erythraea* (Rafn) aerial parts, *Cichorium intybus* (L.) roots, and *Potentilla erecta* (L.)

Räuschel rhizomes possesses a significant hypoglycemic and antioxidant potential. Moreover, several bioactive constituents identified in this polyherbal formulation [19], including rutin, caftaric acid, hyperoside, isoquercetin, have already been reported to exert protective effects against diabetic osteoporosis through a variety of mechanisms [20-28].

However, the effect of this polyherbal mixture on bone tissue is still unknown.

Thus, this study aimed to evaluate both the safety of the polyherbal mixture – specifically, its potential adverse effects in healthy animals – as well as its osteoprotective activity, by assessing its impact on diabetes-induced microarchitectural changes in both the femur and tibia.

MATERIALS AND METHODS

Plant material collection and extraction

Plant species *C. intybus*, and *C. erythrea* were collected at Stara Planina Mt., Serbia (43 21.943 N; 22 46.000 E), and *P. erecta* was collected on the Vlasina plateau, Serbia (42 43.940 N; 22 19.533 E) during 2020 season. After they were taxonomically identified, voucher specimens were deposited at the herbarium collection of the Faculty of Science and Mathematics, University of Niš; numbers HMN: 14454 – 14460. After 2-3 weeks of drying at room temperature, in dark conditions, the plant material was used for preparing the polyherbal mixture according to the traditional recipe [29]. The polyherbal mixture was prepared by mixing *C. intybus* roots, *C. erythrea* aerial parts, and *P. erecta* rhizomes (15, 15, and 70 g, respectively). The decoction was prepared according to traditional use, i.e. 100 g of the herbal mixture was boiled in 1000 mL and concentrations of 2.5, 5, 10, and 15 g of dry plant material per kg of animal weight (g/kg) were adequately prepared, as described in our previous study [19].

Animals

Female Wistar rats (220-270g) were obtained from the Institute of Biomedical Research, Medical Faculty, Niš, Serbia. According to the principles of the Care and Use of Laboratory Animals, they were kept under standard husbandry conditions: standard rat feed and water were provided *ad libitum,* temperature of 23 ± 2 °C, 12/12 h, light/dark cycle, relative humidity of 55 ± 10%. The experiment was approved by the Ethical Committee of the Faculty of Medicine, the University of Niš (No. 323-07-08987/2022-05).

Healthy animal study

To determine potential histopathological changes, we conducted the toxicity study following the principles of OECD 407 (Repeated Dose 28-day Oral Toxicity (OECD

2018)) [30], as described by Petrović et al. (2024) [19]. Four groups (H-2.5, H-5, H-10, and H-15) were treated with tested polyherbal mixture decoction (2.5, 5, 10, and 15 g/kg, respectively), and the fifth group, i.e., healthy controls (H–C), was treated with water, by oral gavage, for the 28 days. The doses of evaluated polyherbal mixture decoctions varied from the minimal to the maximal ones used in ethnopharmacology [29], adapted to the animal testing model [17-19, 31].

Diabetic animal study

As previously described, diabetes was induced by a single intraperitoneal injection of freshly prepared alloxan monohydrate dissolved in ice-cold saline (150 mg/kg), while non-diabetic controls (ND-C) received PBS only. To prevent hypoglycemia, rats were treated with a 5% glucose solution 1 h after the alloxan injection and allowed to drink it *ad libitum* for the following 48 h [19]. Fourteen days after alloxan monohydrate injection, animals with blood glucose levels >20 mmol/L were considered to have developed stable chemically induced diabetes [19]. Diabetic animals were randomly assigned to six treatment groups of five rats each. For the following 14 days, animals in groups D-2.5, D-5, D-10, and D-15 received the polyherbal mixture at doses equivalent to 2.5, 5, 10, and 15 g of dry plant material/kg, respectively. Control groups included diabetic animals treated with glimepiride (1 mg/kg; group D-G), insulin glargine administered intraperitoneally (13 IU/kg; group D-I), and water alone for the diabetic control (group D-C) and non-diabetic control groups (group ND-C). All treatments, except insulin, were administered by oral gavage.

Analysis of tissue samples

At the end of the experiment, following overnight fasting, animals were anesthetized with 10% Ketamidor, and euthanized by cardiopuncture. Blood samples obtained via cardiac puncture were collected in EDTA tubes and levels glycated hemoglobin (HbA1c) were analyzed immediately using NycoCard method.

Left femurs and tibias were isolated, washed in ice-cold saline, fixed in 10% formalin and decalcified in 10% EDTA (pH 7.4). Tissue sections were prepared by standard protocol, by embedding tissues in paraplast, cutting them into 5 μm thick sections (Leica RM2125 RT, Germany), and subjecting them to Hematoxylin and Eosin (H&E) and Masson's trichrome staining. For each sample, 10 visual fields at x400 magnification of compact bone and epiphysis were imaged, while the whole bone sections were imaged at x20 magnification using Leica Stereo Microscope (Leica M205 C, Germany), and analyzed by ImageJ software (NIH, USA).

The pathophysiological analysis focused on the following parameters: cortical bone area, osteocyte lacunar area in the cortical bone and the epiphysis, trabecular area in the epiphysis, and bone collagen deposition. All the parameters were analyzed according to the previously described method [18].

Statistical analysis

Statistical analysis was done by GraphPad Prism 5 (GraphPad Software, La Jolla California USA). All experiments were done in pentaplicate, i.e., five animals per tested group were used (n=5). Data were expressed as the mean ± standard deviation. The differences between the controls and the individual dosage groups of the tested extract were analyzed by the one-way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison Test. If p was less than 0.05, the results were considered statistically significant.

RESULTS

Healthy animal study

As shown in Table 1, treatment with the polyherbal mixture at any of the tested concentrations did not produce any statistically significant changes in the levels of glycated hemoglobin compared with untreated healthy controls.

Table 1. HbA1c level in healthy animals

Group	HbA1c (mmol/mol)
H-C	49.62 ± 2.25
H-2.5	43.87 ± 0.87
H-5	41.59 ± 0.74
H-10	36.74 ± 1.37
H-15	41.03 ± 1.88

H-C: healthy control; **H-2.5:** polyherbal mixture 2.5 g/kg; **H-5:** polyherbal mixture 5 g/kg; **H-10**: polyherbal mixture 10 g/kg; **H-15:** polyherbal mixture 15 g/kg. All data were expressed as the mean ± standard deviation, n=5.

In addition, the results of the histopathological analysis showed that none of the tested concentrations of the polyherbal mixture caused any significant changes in any of the tested parameters compared to untreated healthy controls, in either the femoral or tibial cortical bone area (Figure 1 (V)), trabecular area in the epiphysis (Figure 1 (VI)), or osteocyte lacunar area in both the cortical bone and epiphysis (Figure 2 (VI, VII)). Similarly, as shown in Figure 1 (VII), no significant differences in collagen deposition were observed in either femoral or tibial bone tissue across experimental groups.

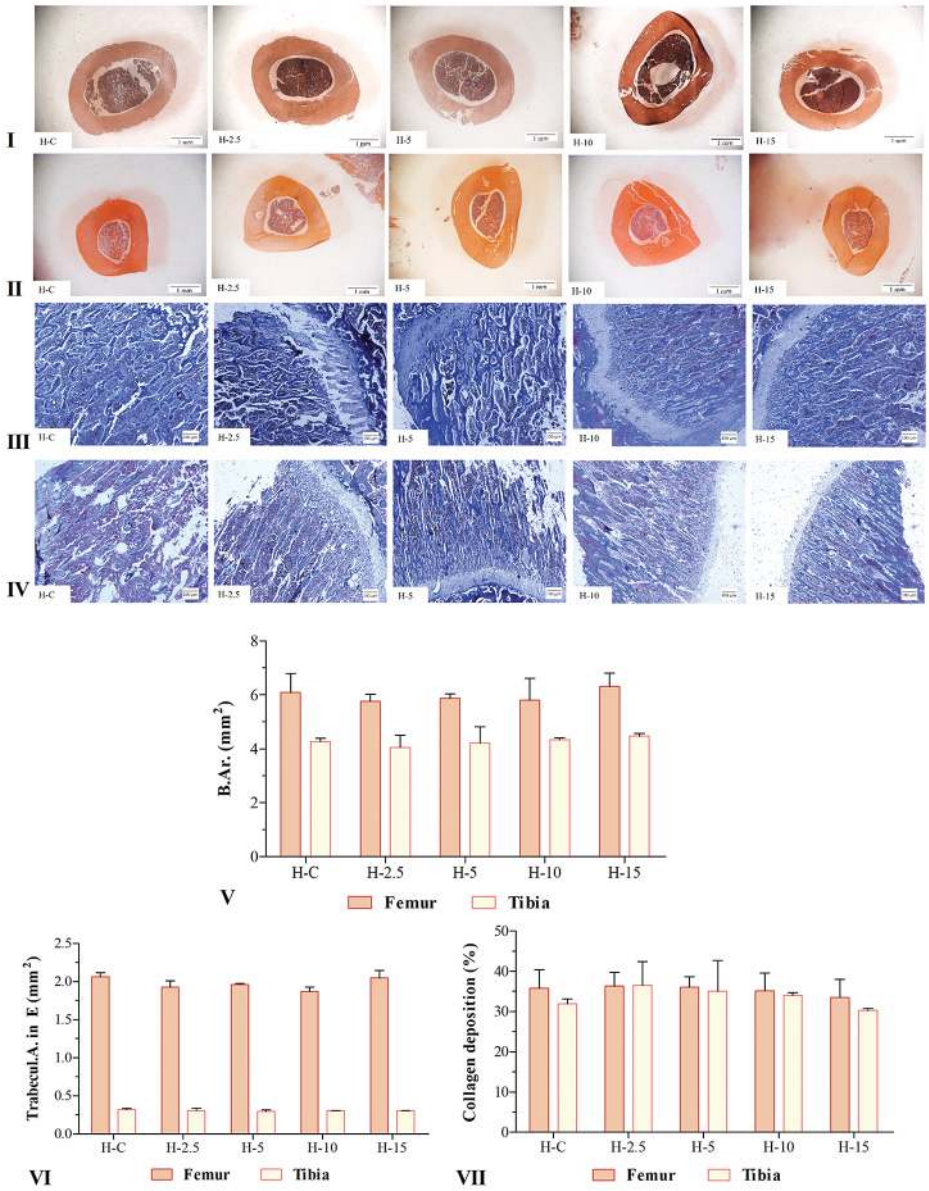


Figure 1. The polyherbal mixture effects on the cortical bone area, trabecular area and collagen deposition in the epiphysis of healthy animals: **(I)** Femoral compact bone cross-section. Stained by H&E. Scale bar 1 mm. Magnification 20x; **(II)** Tibial compact bone cross-section. Stained by by H&E. Scale bar 1 mm. Magnification 20x; **(III)** Femoral epiphysis cross-section. Stained by Masson's trichrome. Scale bar 1 mm. Magnification 50x; **(IV)** Tibial epiphysis cross-section. Stained by Masson's trichrome. Scale bar 1 mm. Magnification 50x; **(V)** Histopathological analysis of the cortical bone area of femur and tibia; **(VI)** Histopathological analysis of the trabecular area in epiphysis of femur and tibia. **(VII)** Histopathological analysis of collagen deposition in epiphysis of femur and tibia. **H-C:** healthy control; **H-2.5:** the polyherbal mixture 2.5 g/kg; **H-5:** the polyherbal mixture 5 g/kg; **H-10:** the polyherbal mixture 10 g/kg; H-15: the polyherbal mixture 15 g/kg. B.Ar.: Bone Area. **Trabecul. A in E:** Trabecular area in the epiphysis. All data were expressed as the mean ± standard deviation, n=5.

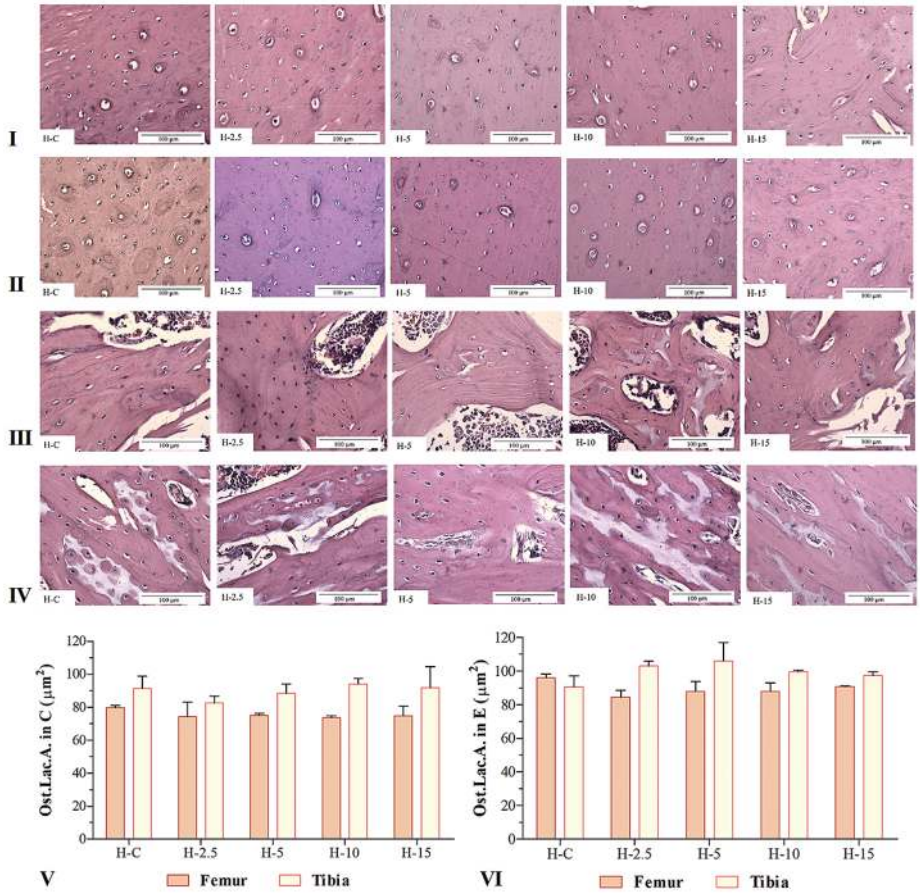


Figure 2. The polyherbal mixture effects on osteocyte lacunar area in cortical bone and epiphysis of the femur and tibia of healthy animals: **(I)** Femoral cortical bone cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(II)** Tibial cortical bone cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(III)** Femoral epiphysis cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(IV)** Tibial epiphysis cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(V)** Histopathological analysis of the cortical bone lacunar area of the femur and tibia; **(VI)** Histopathological analysis of the lacunar area in epiphysis of femur and tibia. **H-C:** healthy control; **H-2.5:** the polyherbal mixture 2.5 g/kg; **H-5:** the polyherbal mixture 5 g/kg; **H-10:** the polyherbal mixture 10 g/kg; **H-15:** the polyherbal mixture 15 g/kg. **Ost. Lac. A. in C**: Osteocyte Lacunar are of Cortical bone. **Ost. Lac. A. in E:** Osteocyte Lacunar Area in Epiphysis. All data were expressed as the mean ± standard deviation, n=5.

Diabetic animal study

As presented in Table 2, glycated hemoglobin levels were significantly increased ($p < 0.001$) in untreated diabetic controls (231.54 ± 3.83%) compared to non-diabetic controls (45.52 ± 0.76%). Treatment with the polyherbal mixture decreased HbA1c values in a dose-dependent manner. The most pronounced ameliorative effect compared to untreated diabetic controls ($p < 0.001$) was observed in animals treated with the higher tested doses of the polyherbal extract (groups D-10 and D-15), with

HbA1c values of 91.26 ± 0.89% and 54.60 ± 0.53%, respectively. Treatment with lower tested doses of the polyherbal mixture (2.5 and 5 g/kg), as well as treatment with insulin (163.30 ± 6.23%) and treatment with glimepiride (181.50 ± 5.55%) slightly decreased glycated hemoglobin levels compared to untreated diabetic controls.

Table 2. HbA1c level in diabetic animals.

Group	HbA1c (mmol/mol)
D-C	231.54 ± 3.83^{b}
D-2.5	172.76 ± 6.02^{b}
D-5	162.70 ± 5.36^{b}
D-10	91.26 ± 0.89^{a}
D-15	54.60 ± 0.53^{ad}
D-I	163.30 ± 6.23^{b}
D-G	181.50 ± 5.55^{b}
ND-C	45.52 ± 0.76^{acd}

D-C: diabetic control. **ND-C:** non-diabetic control; **D-2.5:** polyherbal mixture 2.5 g/kg; **D-5:** polyherbal mixture 5 g/kg; **D-10:** polyherbal mixture 10 g/kg; **D-15:** polyherbal mixture 15 g/kg; **D-I:** insulin; **D-G:** glimepiride. Data were expressed as the mean ± standard deviation (n = 5). $^{a}\mathbf{p} < 0.001$ compared to the D-C group; $^{b}\mathbf{p} < 0.001$ compared to the ND-C group; $^{c}\mathbf{p} < 0.001$ compared to the D-I group; $^{d}\mathbf{p} < 0.001$ compared to the D-G group.

Histopathological evaluation showed that cortical bone areas of both examined bones were significantly decreased ($p < 0.001$) in diabetic controls (3.38 ± 0.19, and 3.26 ± 0.21 mm², in the femur and tibia, respectively) compared to non-diabetic controls (5.70 ± 0.54, and 4.31 ± 0.06 mm², in the femur and tibia, respectively) (Figure 3 (III)). Treatment with higher doses of the polyherbal mixture significantly increased cortical bone areas compared to diabetic controls ($p < 0.001$) in both femur (5.09 ± 0.43, and 5.96 ± 0.66 mm², in D-10 and D-15 groups, respectively) and tibia (4.14 ± 0.28, and 4.31 ± 0.19 mm², in D-10 and D-15 groups, respectively), the values similar to those observed in non-diabetic controls (Figure 3 (III)).

Compared to untreated diabetic controls, treatment with glimepiride slightly increased cortical bone areas in both femur and tibia (4.96 ± 0.57, and 3.71 ± 0.45 mm², respectively), while insulin treatment increased the cortical bone area only in the femur (4.07 ± 0.57 mm²), while simultaneously decreasing the cortical bone area of the tibia (2.84 ± 0.76 mm²) (Figure 3 (III)).

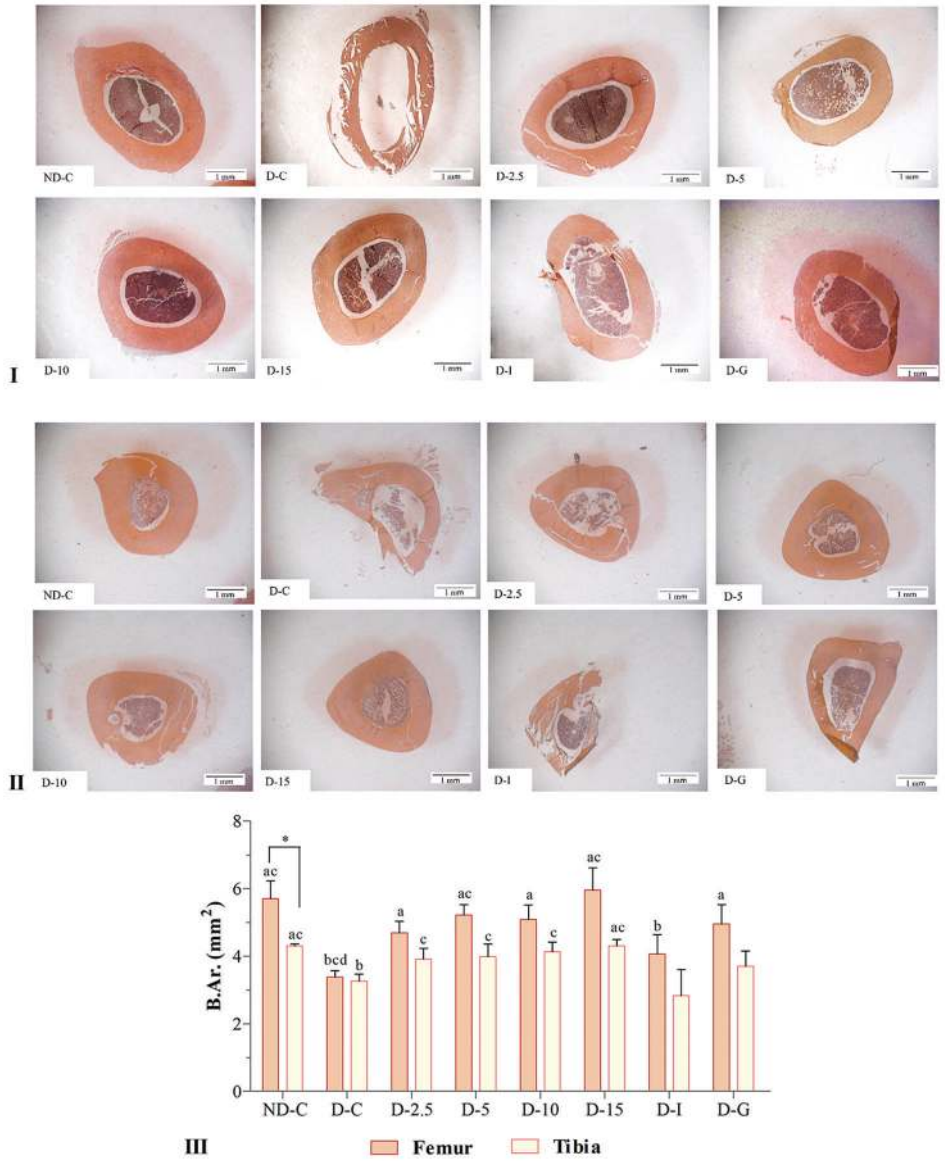


Figure 3. The polyherbal mixture effects on the cortical bone area of the femur and tibia of diabetic animals: **(I)** Femoral compact bone cross-section. Stained by H&E. Scale bar 1 mm. Magnification 20x; **(II)** Tibial compact bone cross-section. Stained by H&E. Scale bar 1mm. Magnification 20x; **(III)** Histopathological analysis of the cortical bone area of the femur and tibia. **D-C:** diabetic control; **ND-C:** non-diabetic control; **D-2.5:** the polyherbal mixture 2.5 g/kg; **D-5:** the polyherbal mixture 5 g/kg; **D-10:** the polyherbal mixture 10 g/kg; **D-15:** the polyherbal mixture 15 g/kg; **D-I:** insulin; **D-G:** glimepiride. B.Ar.: Bone Area. All data were expressed as the mean ± standard deviation, n=5. ^{a}**p** < 0.001 compared to the D-C group; ^{b}**p** < 0.001 compared to the ND-C group; ^{c}**p** < 0.001 compared to the D-I group; ^{d}**p** < 0.001 compared to the D-G group ***p** < 0.001 among femur and tibia.

Osteocyte lacunar area in both the cortical bone (Figure 4 (III)) and epiphysis (Figure 5 (III)) of femur in diabetic controls (40.98 ± 3.12, and 36.36 ± 3.11 μm^2, in the cortical bone and epiphysis, respectively) were significantly decreased ($p < 0.001$) compared to non-diabetic controls (79.53 ± 1.28, and 97.57 ± 2.92 μm^2, in the cortical bone and epiphysis, respectively).

The same pattern appears in the tibia, as well. Namely, the osteocyte lacunar area in the cortical bone (Figure 4 (III)) and epiphysis of tibia epiphysis (Figure 5 (III)) in diabetic controls (32.00 ± 3.86, and 35.23 ± 2.29 μm^2), were significantly decreased ($p < 0.001$) compared to healthy animals (82.75 ± 11.85, and 87.70 ± 6.14 μm^2, in the cortical bone and epiphysis, respectively).

Treatment with higher tested doses of the polyherbal mixture increased the osteocyte lacunar area in both femur in tibia cortical area compared to diabetic controls ($p < 0.001$), where osteocyte lacunar area in the cortical bone area in D-10 group was 74.07 ± 2.88 and 74.32 ± 5.28 μm^2, and in D-15 group – 83.6 ± 0.98 and 82.75 ± 8.40 μm^2, in the femur and tibia respectively (Figure 4 (III), and Figure 5 (III)).

As shown in Figure 5 (III), only the highest tested concentration of the polyherbal mixture (15g/kg) slightly increased the osteocyte lacunar area in femur and tibia epiphysis – 75.69 ± 1.57 and 78.23 ± 5.01 μm^2, respectively.

The standard medical treatments with insulin and glimepiride showed some protective effects, but still suboptimal compared to the D-15 group, on osteocyte lacunar area in cortical bone (70.34 ± 0.64, and 53.78 ± 1.49 μm^2) with $p < 0.001$ in comparison with D-C, D-I and D-G (Figure 4 (III)), and epiphysis (64.07 ± 1.49, and 56.84 ± 1.52 μm^2 of femur bone), with $p < 0.001$ in comparison with D-C, ND-C, D-I and D-G (Figure 5 (III)). Likewise, the osteocyte lacunar area in cortical bone (48.67 ± 1.23, and 55.60 ± 1.29 μm^2) and epiphysis (55.60 ± 1.29, and 54.35 ± 1.49 μm^2) of tibia in a group tested with insulin and glimepiride were slightly increased ($p < 0.001$ in comparison with D-C, and ND-C) (Figure 4 (III), and Figure 5 (III)).

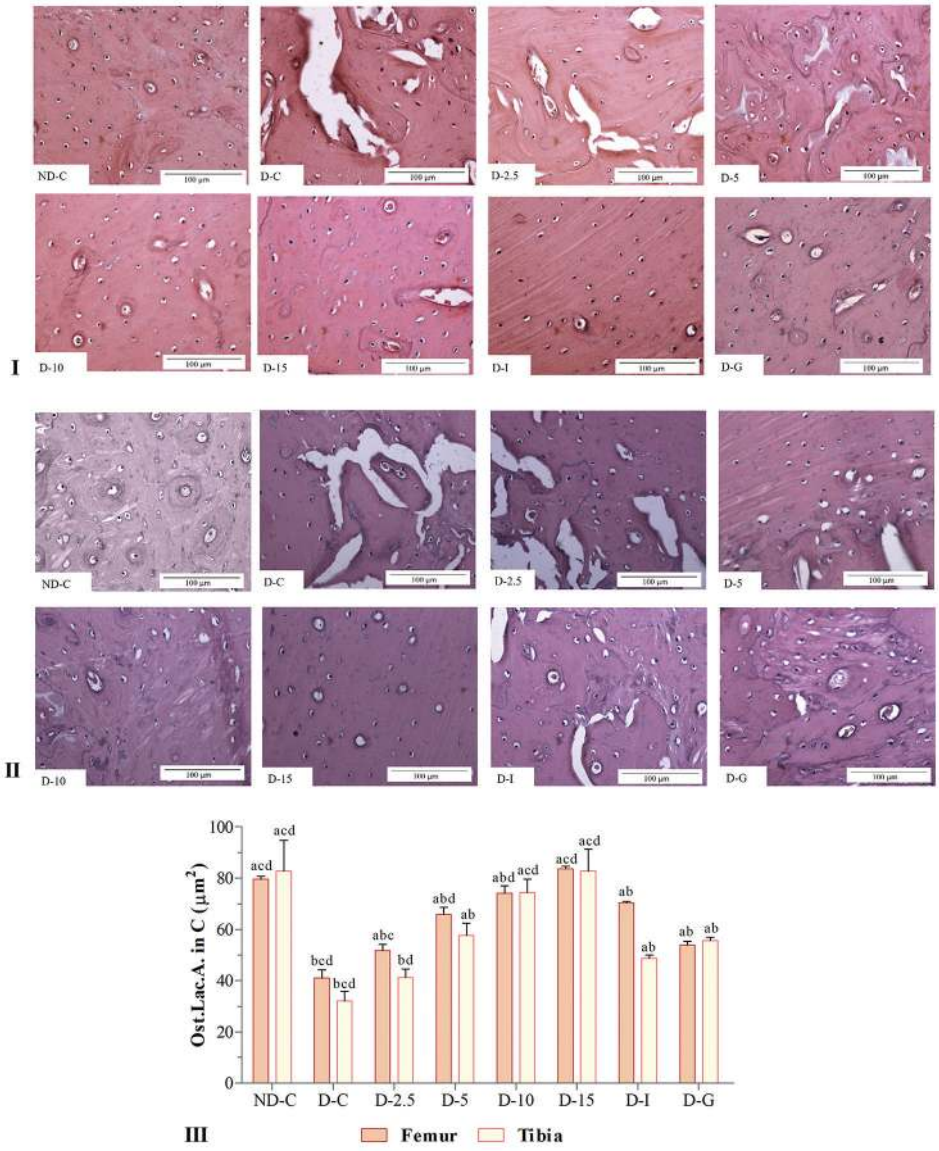


Figure 4. The polyherbal mixture effects on the cortical bone osteocyte lacunar area of the femur and tibia of diabetic animals: **(I)** Femoral cortical bone cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(II)** Tibial cortical bone cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(III)** Histopathological analysis of the lacunar area of cortical bone of femur and tibia. **D-C:** diabetic control. **ND-C:** non-diabetic control; **D-2.5:** polyherbal mixture 2.5 g/kg; **D-5:** polyherbal mixture 5 g/kg; **D-10:** polyherbal mixture 10 g/kg; **D-15:** polyherbal mixture 15 g/kg; **D-I:** insulin; **D-G:** glimepiride. **Os.Lac.A. in C:** Osteocyte Lacunar Area in Cortical bone. All data were expressed as the mean ± standard deviation, n=5. ^{a}**p** < 0.001 compared to the D-C group; ^{b}**p** < 0.001 compared to the ND-C group; ^{c}**p** < 0.001 compared to the D-I group; ^{d}**p** < 0.001 compared to the D-G group.

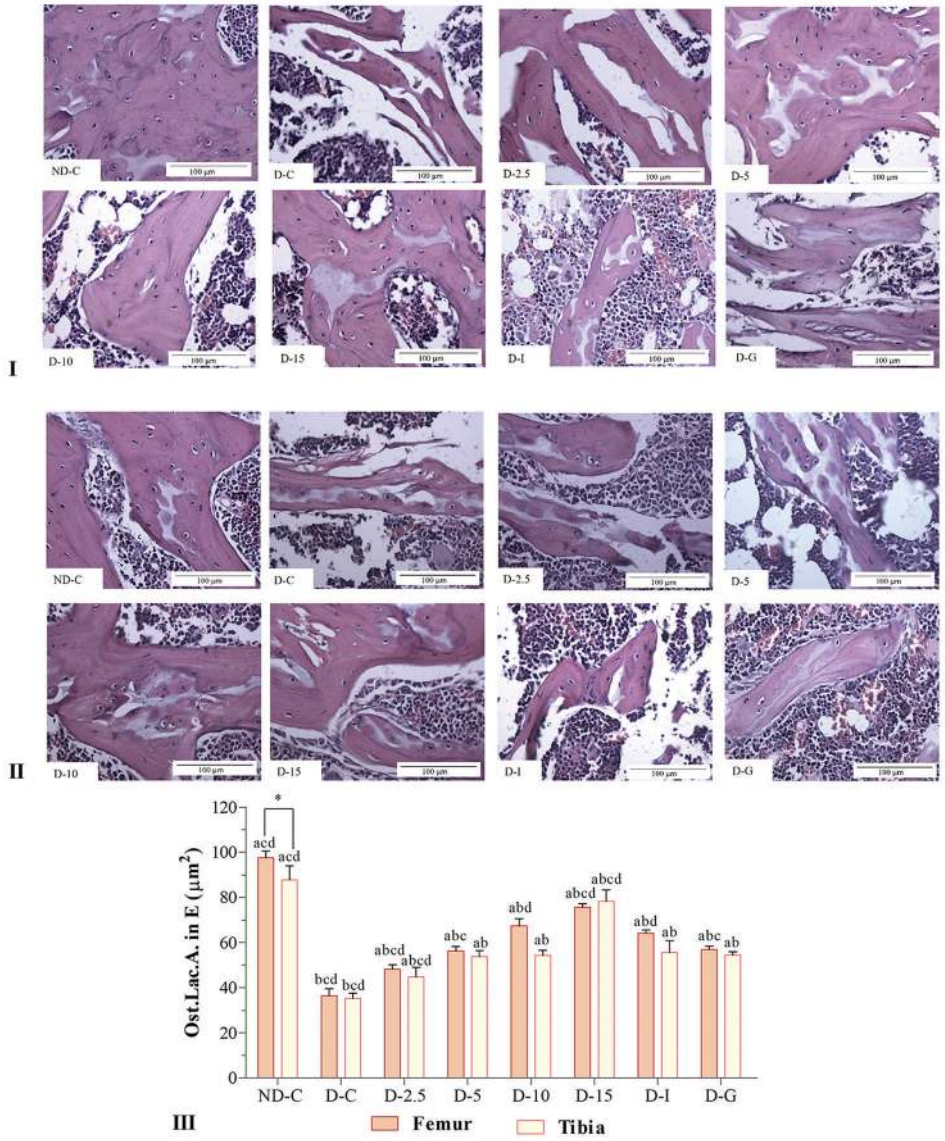


Figure 5. The polyherbal mixture effects on osteocyte lacunar area in epiphysis of femur and tibia of diabetic animals: **(I)** Femoral epiphysis cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(II)** Tibial epiphysis cross-section. Stained by H&E. Scale bar 100 μm. Magnification 400x; **(III)** Histopathological analysis of the lacunar area of epiphysis of femur and tibia. **D-C:** diabetic control. **ND-C:** non-diabetic control; **D-2.5:** polyherbal mixture 2.5 g/kg; **D-5**: polyherbal mixture 5 g/kg; **D-10:** polyherbal mixture 10 g/kg; **D-15:** polyherbal mixture 15 g/kg; **D-I:** insulin; **D-G:** glimepiride. **Os.Lac.A. in E:** Osteocyte Lacunar Area in Epiphysis. All data were expressed as the mean ± standard deviation, n=5. $^{a}\mathbf{p} < 0.001$ compared to the D-C group; $^{b}\mathbf{p} < 0.001$ compared to the ND-C group; $^{c}\mathbf{p} < 0.001$ compared to the D-I group; $^{d}\mathbf{p} < 0.001$ compared to the D-G group; $^{*}\mathbf{p} < 0.001$ among femur and tibia.

The trabecular area in the epiphysis of both femur and tibia, was significantly decreased in diabetic controls (0.73 ± 0.18 and 0.13 ± 0.02 mm^2) in comparison with non-diabetic controls (2.06 ± 0.05 and 0.33 ± 0.03 mm^2) ($p < 0.001$) (Figure 6 (III)).

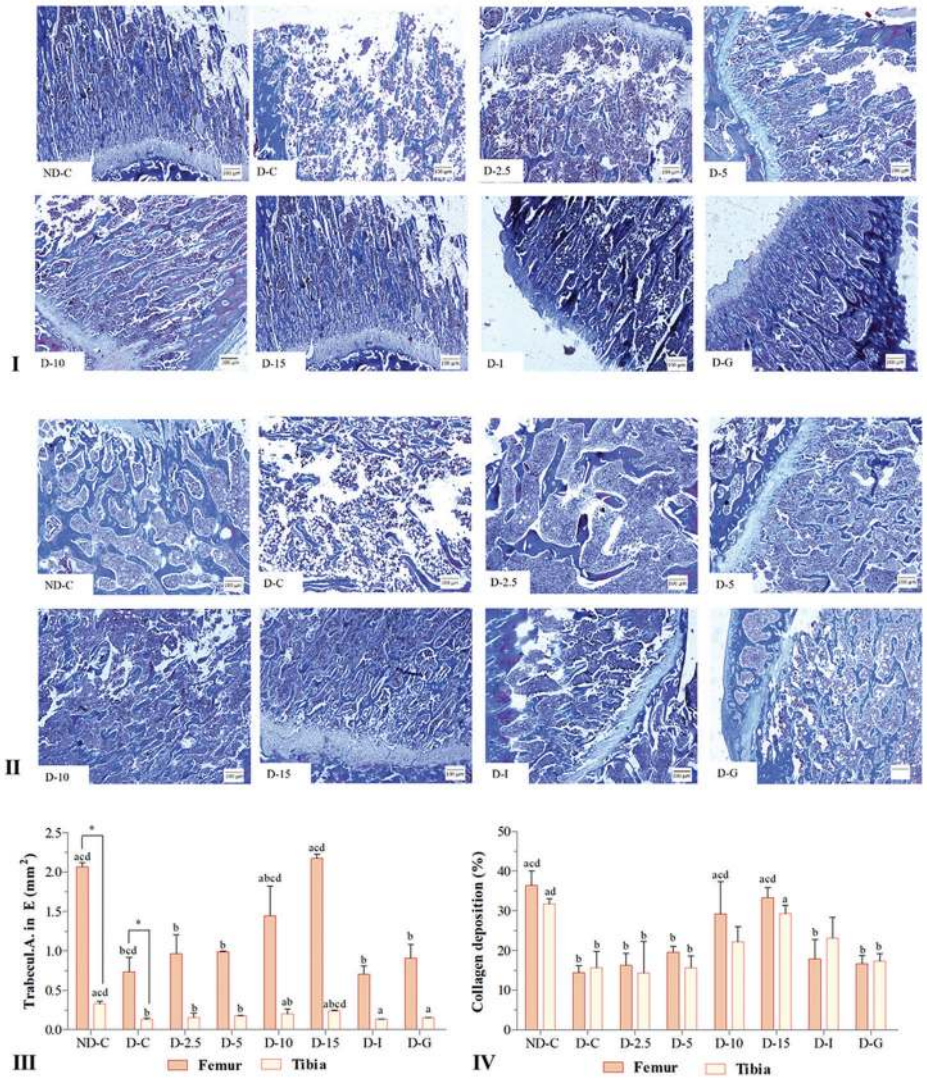


Figure 6. The polyherbal mixture effects on trabecular area in epiphysis and collagen deposition of diabetic animals: **(I)** Femoral epiphysis cross-section. Stained by Masson's trichrome. Scale bar 100 μm. Magnification 50x; **(II)** Tibial epiphysis cross-section. Stained by Masson's trichrome. Scale bar 100 μm. Magnification 50x; **(III)** Histopathological analysis of the trabecular area in the epiphysis of femur and tibia. **(IV)** Histopathological analysis of collagen deposition in the epiphysis of the femur and tibia. **D-C:** diabetic control. **ND-C:** non-diabetic control; **D-2.5:** polyherbal mixture 2.5 g/kg; **D-5:** polyherbal mixture 5 g/kg; **D-10:** polyherbal mixture 10 g/kg; **D-15:** polyherbal mixture 15 g/kg; **D-I:** insulin; **D-G:** glimepiride. **Trabecul. A in E:** Trabecular area in the epiphysis. All data were expressed as the mean ± standard deviation, n=5. $^{a}p < 0.001$ compared to the D-C group; $^{b}p < 0.001$ compared to the ND-C group; $^{c}p < 0.001$ compared to the D-I group; $^{d}p < 0.001$ compared to the D-G group; $^{*}p < 0.001$ among femur and tibia.

The polyherbal mixture treatments induced a concentration-dependent increase in this parameter, with the highest tested concentration (15 g/kg) displaying the values of trabecular area in the femur epiphysis (2.17 ± 0.05 mm^2) comparable with the ND-C group ($p < 0.001$ in comparison with D-C), while the tibial epiphysis mildly increased this parameter (0.24 ± 0.01 mm^2), but still statistically significant in comparison with D-C, and ND-C ($p < 0.001$). Insulin and glimepiride treatments showed a significant effect on the femur (0.71 ± 0.10 and 0.91 ± 0.18 mm^2, respectively) in comparison with D-C on this parameter ($p < 0.001$). However, in the tibia, neither insulin nor glimepiride treatments produced significant effects compared to diabetic controls, but rather, this particular parameter was significantly reduced compared to non-diabetic controls ($p < 0.001$) (Figure 6 (III)).

Collagen depositions in the extracellular matrix in the cortical bone of femur and tibia was significantly lower in D-C (14.37 ± 1.81, and 15.66 ± 4.03%) in comparison with ND-C (36.38 ± 3.64, and 31.69 ± 1.35%) with $p < 0.001$. As shown in Figure 6 (IV), treatments with the polyherbal mixture induced a concentration-dependent upregulation of collagen synthesis, with the results recorded in a group treated with the 15 g/kg decoction concentration showing that collagen depositions were significantly increased in both femoral and tibial bones (33.35 ± 2.52, and 29.26 ± 2.06%, respectively) ($p < 0.001$ in comparison with D-C). Insulin and glimepiride treatments were significantly reduced in comparison with ND-C ($p < 0.001$). Both treatments on the femoral bone (17.89 ± 4.87, and 16.67 ± 2.06%, in D-I and D-G, respectively) showed a slight, but still inferior effect on collagen deposition in comparison with D-10, and D-15 groups ($p < 0.001$), while, insulin and glimepiride treatments (23.09 ± 5.16, and 17.32 ± 1.85%, in D-I and D-G, respectively) on a tibial bone, increased collagen deposition, but with no statistical significance in comparison with D-C (Figure 6 (IV)).

DISCUSSION

This study aimed to assess the therapeutic potential of a traditionally used polyherbal mixture composed of *C. erythraea* aerial parts, *C. intybus* roots, and *P. erecta* rhizomes in mitigating diabetic osteoporosis. The effects were assessed through histopathological analysis of collagen content and trabecular bone microarchitecture. Furthermore, treatment sensitivity was evaluated by comparing the responses of the femur and tibia. The efficacy of this antidiabetic herbal formulation was also compared with that of conventionally used antidiabetic pharmaceuticals in preventing the development of this common secondary complication of diabetes.

The osteoprotective efficacy of this polyherbal formulation was investigated using an alloxan-induced diabetic rat model. Alloxan monohydrate, a well-established diabetogenic agent, was selected based on our previous studies demonstrating its suitability for inducing not only hyperglycemia but also a spectrum of secondary diabetic complications [17-19]. Mechanistically, alloxan exerts pronounced cytotoxic

effects through selective apoptosis of pancreatic *β*-cells, leading to insulin deficiency and persistent hyperglycemia. In addition, its high systemic toxicity promotes excessive generation of reactive oxygen species and enhances the formation of advanced glycation end products, thereby contributing to widespread structural and functional alterations across multiple organs. Moreover, it is well known that the alloxan-induced oxidative stress impairs osteogenic differentiation by inhibiting the maturation of osteoblasts into osteocytes, resulting in a reduced osteocyte area per bone area. This pathological profile closely parallels the microarchitectural and cellular alterations observed in human diabetic osteopenia, thereby supporting the translational relevance of this experimental model [32-36].

However, the first step in the evaluation of any potential therapeutic agent is the assessment of its safety. Our previous research demonstrated that polyherbal formulations, when administered at high concentrations, may exert adverse effects, such as increased osteocyte lacunar area, elevated bone mineral density, and the potential development of osteopetrosis [18]. Since none of the tested concentrations of this polyherbal mixture caused significant changes in any of the evaluated parameters in either the femur or the tibia (Figures 1 (V, VI, VII) and 2 (V, VI), or in glycemic values (Table 1), all four doses of the polyherbal mixture were selected for the assessment of its potential osteoprotective effect.

Histopathological analysis of the femurs and tibias of diabetic animals showed that alloxan treatment significantly reduced the cortical bone area, osteocyte lacunar area, trabecular area in the epiphysis, and bone collagen content compared to healthy controls ($p < 0.001$), confirming the successful establishment of the experimental model (Figures 3 (III), 4 (III), 5 (III) and 6 (III, IV)). Interestingly, treatment with the highest tested concentration of the polyherbal mixture (15 g/kg) was more effective than treatment with glimepiride and treatment with insulin in ameliorating these alterations in both the femur and tibia model (Figures 3 (III), 4 (III), 5 (III) and 6 (III, IV)) [33,37].

Having in mind that chronic hyperglycemia often leads to alterations in both the shape and size of the osteocyte lacunar area [38], as well as to changes in other analyzed parameters of alloxan-induced experimental diabetic osteoporosis [39-41], the osteoprotective effect of the tested polyherbal mixture can be attributed to its pronounced hypoglycemic activity observed in our previous study [19] and further confirmed by the results of glycated hemoglobin analysis (Table 2), as well as to the presence of even 21 bioactive compounds, including *p*-hydroxybenzoic acid and its derivatives, catechin and its derivatives, epicatechin, isoquercetin, hyperoside, rutin, quercetin derivatives, as well as caffeic acid and its derivatives, detected in our previous research [19,42].

Since it is well known that bioactive compounds such as polyphenols modulate diabetic osteoporosis through inhibition of excessive ROS generation, attenuation of inflammation, reduction of caspase activity in osteoblasts with consequent

enhancement of their survival, and modulation of osteoclast differentiation via suppression of bone resorption markers such as RANKL, as well as stimulation of osteogenic markers associated with osteoblast differentiation and bone matrix mineralization, including Runx2, type I collagen, and ALP [43], these findings are in accordance with our previous study, which demonstrated strong antioxidant and anti-inflammatory properties of this polyherbal mixture – where administration of its extract at a concentration of 15 mg/kg completely normalized serum ALP levels [19].

Furthermore, the mechanisms of action of the major bioactive constituents present in the tested polyherbal mixture have been well documented in previous studies. For example, hydroxybenzoic acid, the most abundant polyphenol in the mixture, exerts osteoprotective effects through activation of the ERK, AKT, PI3K, and ERα signaling pathways [27]. Quercetin, hyperoside, and caffeic acid inhibit genesis of osteoclasts and promote their apoptosis through suppression of the RANKL/RANK/NF-κB and MAPK signaling pathways, while simultaneously protecting osteoblasts and stimulating their proliferation, differentiation, and calcium mineralization [20-24]. In addition, isoquercetin enhances the expression of VEGF and β-catenin, which cooperate in bone matrix renewal by promoting osteoblast survival and suppressing osteoclast activity through induction of apoptosis [25,26]. Moreover, rutin increases bone density by inhibition of osteoclast activity via inhibition of receptor activator of nuclear factor kappa-B ligand (RANKL), by decreasing the elevated level of ROS and through inhibition of NF-κB activation [27,44,45].

In addition, alterations in collagen structure contribute to increased bone fragility [10]. In our study, the cortical bone of the femur exhibited a higher level of collagen reduction than the tibia. The polyherbal mixture treatment positively regulated collagen production in the dose-depended manner, demonstrating the most profound effects within the group treated with the 15 g/kg of polyherbal mixture decoction (Figure 6 (IV)), in both femur and tibia, which confirm these phytochemicals potential to influence collagen production as well [46].

Interestingly, standard pharmacological treatments only modestly ameliorated the progression of diabetic osteoporosis, with effects comparable to those observed in animals receiving lower doses of the polyherbal mixture (2.5 and 5 g/kg), most likely due to their lower hypoglycemic activity in this experimental model (Table 2) and their predominantly direct effects on the bone remodeling process. Namely, it has been shown that glimepiride promotes osteogenic differentiation in rat osteoblasts via the PI3K/Akt/eNOS pathway [47–51], while, similarly to our findings, the study by Nyman and associates reported that insulin stimulates the proliferation of osteoblasts and their differentiation into osteocytes, restores mineralization, improves bone architecture, and increases ALP levels. However, it does not have effect on the increase in the surface area of both bones, especially in the tibia due to faster metabolism, mineralization and vascularization of the femur [52].

Thus, the observed differences in therapeutic response between standard pharmacological treatments and the highest tested dose of the polyherbal mixture may be attributed to the synergistic action of the mixture's bioactive compounds, which simultaneously ameliorates hyperglycemic conditions in this experimental model (Table 1) and exerts direct regenerative effects on bone tissue, as demonstrated in previous studies [20–28,46].

The observed differences in treatment response between cortical bone (Figure 3 (III)) and the epiphysis (Figure 4 (III)), where the osteocyte lacunar area was more effectively restored in the cortical bone than in the epiphysis, as well as the differential responses of the femur and tibia to polyherbal mixture treatment during the development of diabetic osteoporosis, particularly the more pronounced normalization of the trabecular area in the femoral epiphysis compared to the tibial epiphysis (Figure 6 (III)), may be explained by differences in tissue sensitivity, biomechanical loading, cortical-to-trabecular bone ratio, regional RANK expression, bone perfusion, and metabolic activity [53,54].

Namely, the trabecular bone in the epiphysis is highly metabolically active and remodels rapidly, making the epiphyseal region particularly susceptible to bone loss in osteoporosis compared to cortical bone in the diaphysis, and it deeply rely on vascularization [55], that may be compromised in diabetic conditions [56].

Alloxan-induced diabetes led to a greater degradation of bone tissue in the femur than in the tibia, as evidenced by the significantly higher ratio of cortical bone surface area between the healthy and diabetic control groups in the femur compared with the tibia (Figure 3 (III)). The more pronounced regenerative effect of the highest tested concentration of the polyherbal mixture observed in the femur compared with the tibia can be attributed to the larger cortical bone area, faster renewal of osteocyte lacunae, and growth of trabeculae with newly formed bone tissue, as well as to better vascularization and mineralization of the bone matrix, which is facilitated by the specific orientation of collagen fibers providing greater strength to the femur than to the tibia (Figures 3 (III), 4 (III), 5 (III) and 6 (III, IV)) [53,54,57,58]. However, considering the smaller trabecular bone surface area together with the higher number of differentiated osteocytes in the tibial epiphyses (Figures 5 (III) and 6 (III)), a longer treatment period may be required to fully reach the regenerative potential of the polyherbal mixture in both bones [13,14,54].

CONCLUSION

This study showed that treatment with the polyherbal mixture significantly improved several bone microarchitectural parameters in diabetic rats compared with diabetic controls, with effects varying between bone types and regions. Although the femur was more severely affected by hyperglycemia, it exhibited more pronounced responses in cortical bone parameters following treatment. While the observed improvements

did not consistently reach the levels observed in non-diabetic controls, these findings suggest that the polyherbal mixture may represent a promising adjunctive approach for further investigation in experimental models of diabetes-associated bone deterioration.

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Authors' contributions

BM wrote the paper and conducted the literature research. AP conducted the literature research, designed the study, performed the in vivo experiment, carried out microscopy and histopathology, analyzed the results, and reviewed the paper. VM performed the in vivo experiment and reviewed the paper. NM performed the in vivo experiment. BZ collected plant material, conducted the taxonomy work, and reviewed the paper. LjĐ confirmed the histopathological analysis and reviewed the paper. PV supervised all of the work.

Declaration of conflicting interests

The authors declare no actual, potential, or perceived conflicts of interest for this article.

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EFEKTI BILJNE MEŠAVINE NA MIKROARHITEKTURU FEMURA I TIBIJE KOD PACOVA SA DIJABETESNOM OSTEOPOROZOM

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Femur i tibija kod osoba sa dijabetesom pokazuju veću podložnost razvoju osteopenije i osteoporoze, jer su ove osobe osetljivije na pomenute promene od osoba koje dijabetes nemaju. Cilj ove studije bio je da se ispita efekat biljne mešavine napravljene od nadzemnog dela biljke *Centaurium erythraea*, korena vrste *Cichorium intybus* i rizoma vrste *Potentilla erecta* na mikroarhitekturu ove dve kosti na pacovima bez dijabetesa i sa dijabetesom. Dijabetes je indukovan injektiranjem jedne doze aloksan monohidrata intraperitonealno ženkama pacova soja *Wistar*. Životinje su tretirane biljnom mešavinom u četiri koncentracije - 2.5, 5, 10, and 15 g suve biljne mase/kg životinje oralnom gavažom. Životinje bez dijabetesa dobijale su vodu. Dijabetične kontrolne grupe tretirane su vodom, odnosno insulin-glarginom (13 IU/kg) i glimepiridom (1 mg/kg). Odgovor na terapiju procenjen je određivanjem nivoa HbA1c i patohistološkom analizom koštanog tkiva. Na osnovu H&E-om bojenja analizirana je površina kortikalnog dela kostiju i površine lakuna osteocita u epifizama i kortikalnom delu kostiju, dok je Mason-trihromnim bojenjem dokazano prisustvo kolagena i izmerena površina trabekula u epifizama. Nijedna testirana koncentracija nije dovela do patoloških promena na kostima zdravih životinja. Biljna mešavina se pri najvećoj testiranoj koncentraciji pokazala efikasnijom od insulina i glimepirida u poboljšanju nivoa HbA1c i ublažavanju mikroarhitektonskih promena izazvanih aloksanom, sa snažnije izraženim efektima na femuru nego li na tibija. Dobijeni rezultati studije ukazuju na to da na eksperimentalnom modelu osteoporoze indukovane dijabetesom biljna mešavina pokazuje osteoprotektivni potencijal.