

OSTEOPROTECTIVE EFFECTS OF 'ANTI-DIABETIC' POLYHERBAL MIXTURE IN TYPE 1 DIABETIC RATS

Aleksandra PETROVIĆ*, Višnja MADIĆ, Marina JUŠKOVIĆ,
Ljubiša ĐORĐEVIĆ, Perica VASILJEVIĆ

Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš,
Višegradska 33, 18000 Niš, Serbia

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Bone loss leading to osteopenia and osteoporosis is a frequent secondary complication of diabetes. This study aimed to evaluate the value of a traditionally used 'anti-diabetic' polyherbal mixture as a possible remedy for the prevention of this complication. Diabetes was induced in Wistar female rats with a single intraperitoneal injection of alloxan monohydrate. The animals with blood glucose higher than 20 mmol/L for 14 consecutive days were considered diabetic. For the next 14 days, animals were treated with two concentrations of the polyherbal mixture (10 and 20 g of dry plant material/kg). Bone histopathology was evaluated using the H&E and Masson's trichrome staining. Alloxan-induced diabetes triggered bone histological changes characteristic for the development of osteopenia and osteoporosis and treatment with the polyherbal decoction restored these histopathological changes of the bones to the healthy animal level. At the same time, treatment with these tested doses has shown no adverse effects. These findings suggest that this mixture might be used as a remedy for the prevention of diabetic bone loss.

Key words: diabetes, osteoprotective, polyherbal mixture, traditional medicine

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease where lack of insulin and/or insulin resistance results in constant hyperglycaemia, accumulation of advanced glycation end-products (AGE), excessive production of reactive oxygen species (ROS), and chronic inflammation [1-3]. Chronic hyperglycaemia, together with elevated levels of AGE and ROS cause a decrease in bone mineralization and osteocyte differentiation as well as an increase in apoptosis of osteocytes, decrease of bone collagen and, finally, accelerated bone resorption [1,4-12]. Over time, these pathological conditions may result in the development of osteopenia and osteoporosis [13-16]. As a consequence, there is an increased risk of bone fractures in the diabetic compared to the non-

*Corresponding author: e-mail: aleksandra.petrovic2@pmf.edu.rs

diabetic population, which is an additional burden to their already reduced quality of life [17-19].

Bone density is reduced by 22 to 37% in type 1 diabetic patients [20]. Although there were some cases where a reduction in bone density and diabetes is diagnosed at the same time, usually, the severity of osteoporosis is directly proportional to the elapsed time since the onset of diabetes [21-23]. Subsequently, the prevalence of osteoporosis rapidly increases with age in both type 1 and type 2 diabetic patients and this incidence is higher in menopausal women than in men [24].

For centuries, medicinal plants have been used as ethnopharmacological remedies in the treatment of osteopenia and osteoporosis [25]. However, many studies have shown that, when addressing complex diseases, treatments based on polyherbal mixtures lead to a better therapeutic outcome than treatments with a single medicinal plant [26,27]. Similarly, one of the most commonly used polyherbal mixtures in the Balkan countries, which is made of blackberry (*Rubus fruticosus* L., *Rosaceae*) and blueberry (*Vaccinium myrtillus* L., *Ericaceae*) leaves, tormentil (*Potentilla erecta* (L.) Rauschel, *Rosaceae*) roots, St. Benedict's herb (*Geum urbanum* L., *Rosaceae*) aerial parts and kidney bean (*Phaseolus vulgaris* L., *Fabaceae*) pods [28], has much lower toxicity and higher anti-toxicity than its ingredients when used alone [29]. Moreover, our previous study showed that this polyherbal mixture has high hypoglycemic and anti-inflammatory activities due to its high antioxidant properties due to the synergistic effect of its active compounds, i.e., isoquercetin, rutin, quercitrin, arbutin, hyperoside, astragaloside, trifolin, as well as gallic, quinic and caftaric acid. [30]. However, when used in high concentrations, this traditionally used 'anti-diabetic' polyherbal preparation [28,29] shows low levels of nephrotoxicity and hepatotoxicity [30].

Having in mind that maintenance of normal glycaemic values and regulation of oxidative stress may prevent the development of osteopenia and osteoporosis [31,32] this study aimed to further assess both the other potential adverse effects if used in high concentrations, as well as the potential osteoprotective activity of the traditionally used polyherbal mixture through evaluation of its effects on the histopathological changes of healthy and diabetic female rats bones.

MATERIALS AND METHODS

Collection and extraction of plant material

V. myrtillus, *P. erecta*, *R. fruticosus*, *G. urbanum* and *P. vulgaris* were collected in Serbia during 2017 (Stara Planina Mt., Vlasina, Sićevo Gorge, Gornja Koritnica and Niška Banja, respectively) and taxonomically identified. Voucher specimens were deposited at the herbarium collection of the Faculty of Science and Mathematics, University of Niš, under the accession numbers HMN 13717–13721.

The plant material was dried in dark conditions at room temperature for 2-3 weeks and finely ground afterwards. The 'anti-diabetic' polyherbal mixture was prepared

according to the traditionally used recipe, i.e., *V. myrtillus* leaves, *P. erecta* roots, *R. fruticosus* leaves, *G. urbanum* aerial parts and *P. vulgaris* pods were thoroughly mixed [28]. The extract was prepared according to traditional use, i.e., in the form of a decoction. 100 g of the herbal mixture was boiled in 1000 mL of distilled water until half of the liquid evaporated. The obtained extract was filtered, and the rest of the solvent was removed using a vacuum evaporator (IKA RV10 Rotary Evaporator with HB10 Bath, Gaithersburg).

Animals

Sixty female Wistar rats 10 weeks old (200-250 g) were obtained from the Institute of Biomedical Research, Medical Faculty, Niš, Serbia. The animals were treated according to the principles of the Care and Use of Laboratory Animals and approved by the Ethical Committee of the Faculty of Medicine, the University of Niš (No. 323-07-01762/2019-05/5). Animals were maintained under standard husbandry conditions with a temperature of $23 \pm 2^\circ\text{C}$, relative humidity $55 \pm 10\%$ and 12/12 h light/dark cycle. Animals were fed with standard rat food pellets and water provided *ad libitum*.

Evaluation of polyherbal mixture effect on healthy animals' bone structure

The potential effect of polyherbal mixture histopathological changes of the healthy animals' bones was evaluated by following the principles of OECD 407 (Repeated Dose 28-day Oral Toxicity (OECD 2018)), as previously described [30]. Briefly, twenty healthy animals were randomized into four treatment groups of five rats each as follows:

- Group HC: normoglycemic rats that received 1 ml of water only,
- Group H-10: normoglycemic rats that received 1 ml of polyherbal mixture decoction (10 g of dry plant material/kg),
- Group H-20: normoglycemic rats that received 1 ml of polyherbal mixture decoction (20 g of dry plant material/kg),
- Group H-40: normoglycemic rats that received 1 ml of polyherbal mixture decoction (40 g of dry plant material/kg).

Animals were treated every morning for 28 consecutive days, *per os*. The bodyweight was recorded daily.

Evaluation of polyherbal mixture effect on diabetic animals' bone structure

As previously described, diabetes was induced by a single intraperitoneal injection of the freshly prepared alloxan monohydrate dissolved in ice-cold saline (150 mg/kg), while healthy control received PBS only [30]. To prevent hypoglycaemia, 1 h after the alloxan injection, the rats have been treated with a 5% glucose solution over the

next 48 h, *ad libitum* [33]. Fourteen days after the alloxan monohydrate injection, the animals with blood glucose >20 mmol/L (n=25) were considered diabetic. Animals were randomized into six treatment groups of five rats each as follows:

- Group DC: diabetic rats that received 1 ml of water only,
- Group D-10: diabetic rats that received 1 ml of polyherbal mixture decoction (10 g of dry plant material/kg),
- Group D-20: diabetic rats that received 1 ml of polyherbal mixture decoction (20 g of dry plant material/kg),
- Group I: diabetic rats that received insulin glargine (13 IU/kg),
- Group M: diabetic rats that received 1 ml of metformin dissolved in water (150 mg/kg),
- Group HC: normoglycemic rats that received 1 ml of water only.

Water, metformin and the herbal mixture were administered by oral gavage, and insulin by intraperitoneal injection, every morning for 14 consecutive days. The bodyweight was recorded daily.

Histopathological studies

Twenty-four hours after the last treatment, animals were sacrificed using intraperitoneal injection of a high dose (300 mg/kg) of ketamine (10% Ketamidol, Richter pharma, Austria) followed by cardiopuncture and removal of blood. The whole left femurs were collected, washed with ice-cold saline, fixed in 10% formalin, and decalcified in EDTA (10%, pH 7.4). The distal metaphyses were embedded in paraplast, cut into 5 µm thick tissue sections (Leica RM2125 RT, Germany), and subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining [34]. Ten sections per sample of cortical bone were imaged using a Leica microscope (Leica DM2500, Germany) at 400× magnification, while the whole bone sections were imaged using Leica Stereo Microscope (Leica M205 C, Germany) at 20x magnification, and analyzed by ImageJ software (NIH, USA).

Having in mind that one of the key factors in the development of diabetic osteoporosis is the bone loss evolved as a consequence of impaired osteocyte function [35-37], possible osteoprotective effects of the herbal mixture were evaluated using comparative analysis of four histological parameters: the size of the bone area (B.Ar.), osteocyte lacunar density (Os.Lac.D.), size of the osteocyte lacunar area and bone collagen deposition [35,38].

The size of the bone area was calculated using a formula:

$$B.Ar. = Tt.B.Ar. - B.Ma.Ar.$$

where Tt.B.Ar. was the size of the total bone area and B.Ma.Ar. was the size of the bone marrow area and expressed in mm².

Osteocyte lacunar density was expressed as total lacunar number (Tt.L.N.) per mm² of B. Ar. Size of the osteocyte lacunar area was expressed in μm² and bone collagen depositions in %.

Statistical analysis

Statistical analysis was done using GraphPad Prism 5 (GraphPad Software, La Jolla California USA). All experiments were done in pentaplicate and 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. Statistical differences were accepted if p was less than 0.05.

RESULTS

The effect of the polyherbal mixture on healthy animals bone histology

Treatment with the tested polyherbal mixture decoction produced no changes in the size of the healthy animals bone area (Figure 1C) or osteocyte lacunar area (Figure 1E).

Additionally, treatment with tested decoction did not have any effect on experimental animals bone collagen deposition, as shown in Figure 2C.

However, animals treated with the highest tested concentration (40 g/kg) had increased osteocyte lacunar density (748.79 ± 41.57 /mm²) compared to the untreated healthy controls (673.01 ± 16.94 /mm²), as shown in Figure 1D.

The effect of the polyherbal mixture on diabetic animals bone histology

As shown in Figure 3C, size of the bone area was significantly decreased ($p < 0.01$) in untreated diabetic animals (3.25 ± 0.51 mm²) compared to the healthy control ones (4.71 ± 0.61 mm²). Administration of polyherbal mixture only slightly increased the bone area (3.92 ± 0.21 and 3.92 ± 0.51 mm², respectively) compared to the diabetic controls, while treatments with insulin and metformin restored this parameter to the healthy animal level (4.73 ± 0.48 and 4.23 ± 0.56 mm², respectively).

Moreover, osteocyte lacunar density was extremely decreased ($p < 0.001$) in the DC group of animals (402.36 ± 28.34 /mm²) compared to the HC group (673.01 ± 16.94 /mm²). Both treatments with insulin and with a higher concentration of polyherbal decoction (20 g/kg) significantly increased osteocyte lacunar density (572.92 ± 21.21 and 554.11 ± 27.34 /mm², respectively) compared to the DC group and brought it to the healthy animal level (Figure 3D).

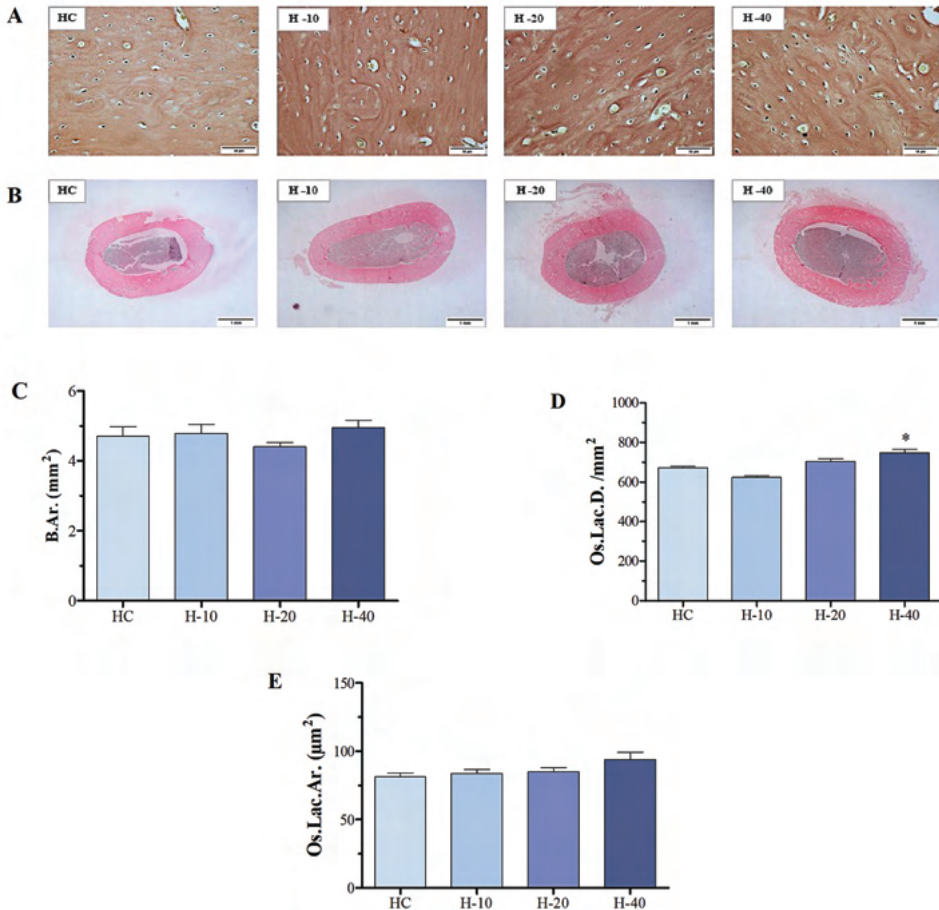


Figure 1. ‘Anti-diabetic’ polyherbal mixture effects on healthy experimental animals cortical bone: **(A)** H&E stained sections of femoral cortical bone. Scale bar 50 µm. Magnification 400x; **(B)** H&E stained femoral cortical bone cross-section. Scale bar 1 mm. Magnification 20x; **(C)** Effect of polyherbal mixture treatment on the femoral cortical bone area; **(D)** Effect of polyherbal mixture treatment on osteocyte lacunar density; **(E)** Effects of polyherbal mixture treatment on osteocyte lacunar area; HC: Healthy control; H-10: Polyherbal mixture 10 g/kg; H-20: Polyherbal mixture 20 g/kg; H-40: Polyherbal mixture 40 g/kg; B.Ar.: Bone Area; Os.Lac.D.: Osteocyte Lacunar Density; Os.Lac.Ar.: Osteocyte Lacunar Area. Data were expressed as the mean ± standard deviation, n=5. *p<0.05 compared to the HC group.

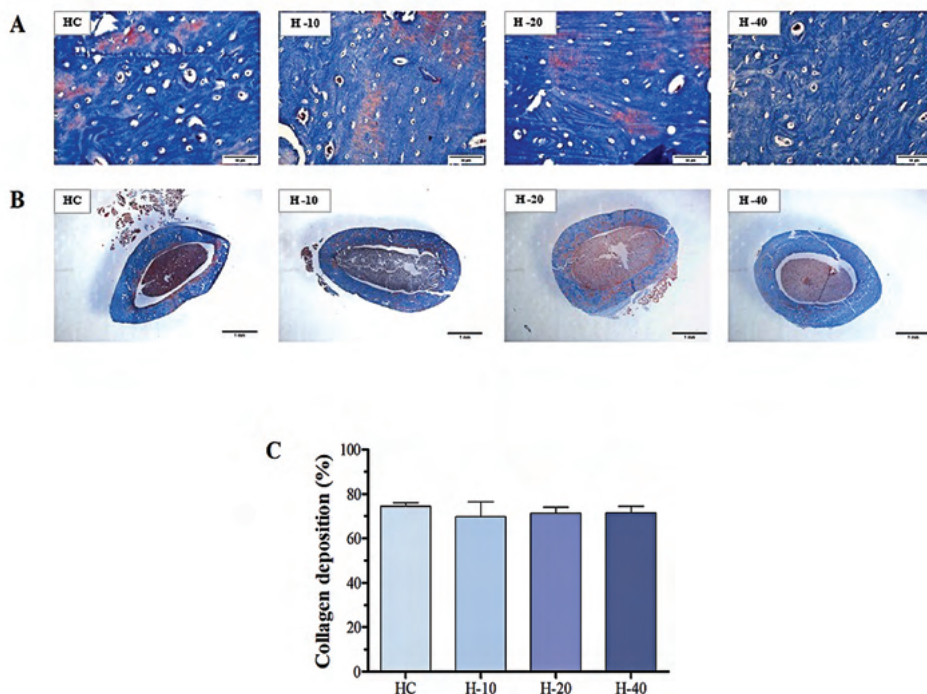


Figure 2. ‘Anti-diabetic’ polyherbal mixture effects on healthy experimental animals bone collagen: **(A)** Masson’s trichrome stained sections. Scale bar 50 μ m magnification 400x; **(B)** Masson’s trichrome stained femoral cortical bone cross-section. Scale bar 1 mm. Magnification 20x; **(C)** HC: Healthy control; H-10: Polyherbal mixture 10 g/kg; H-20: Polyherbal mixture 20 g/kg; H-40: Polyherbal mixture 40 g/kg. Data were expressed as the mean \pm standard deviation, n=5.

Additionally, the size of the osteocyte lacunar area was significantly decreased ($p < 0.001$) in the untreated diabetic group ($66.05 \pm 2.62 \mu\text{m}^2$) compared to the healthy one ($81.52 \pm 5.89 \mu\text{m}^2$). Administration of the herbal mixture in both tested concentrations completely normalized the osteocyte lacunar area (81.02 ± 6.41 and 83.75 ± 7.17 , in D-10 and D-20 experimental groups, respectively), while treatments with insulin and metformin did not have any effect on this parameter (Figure 3E).

At the same time, the deposition of collagen in the bone tissue was significantly decreased in untreated diabetic animals ($52.24 \pm 1.58\%$) compared to the healthy ones ($74.57 \pm 3.52\%$). Fourteen days of administration of the lower tested dose (10 g/kg) of polyherbal mixture significantly increased deposition of bone collagen ($70.84 \pm 4.88\%$) compared to the diabetic control, while treatment with a higher dose (20 g/kg) completely restored this parameter ($75.59 \pm 6.91\%$) to the healthy animal level. In the same manner, treatments with both metformin and insulin increased the deposition of collagen in diabetic animals bones (73.77 ± 9.78 and $85.61 \pm 4.27\%$, respectively), compared to the DC group (Figure 4C).

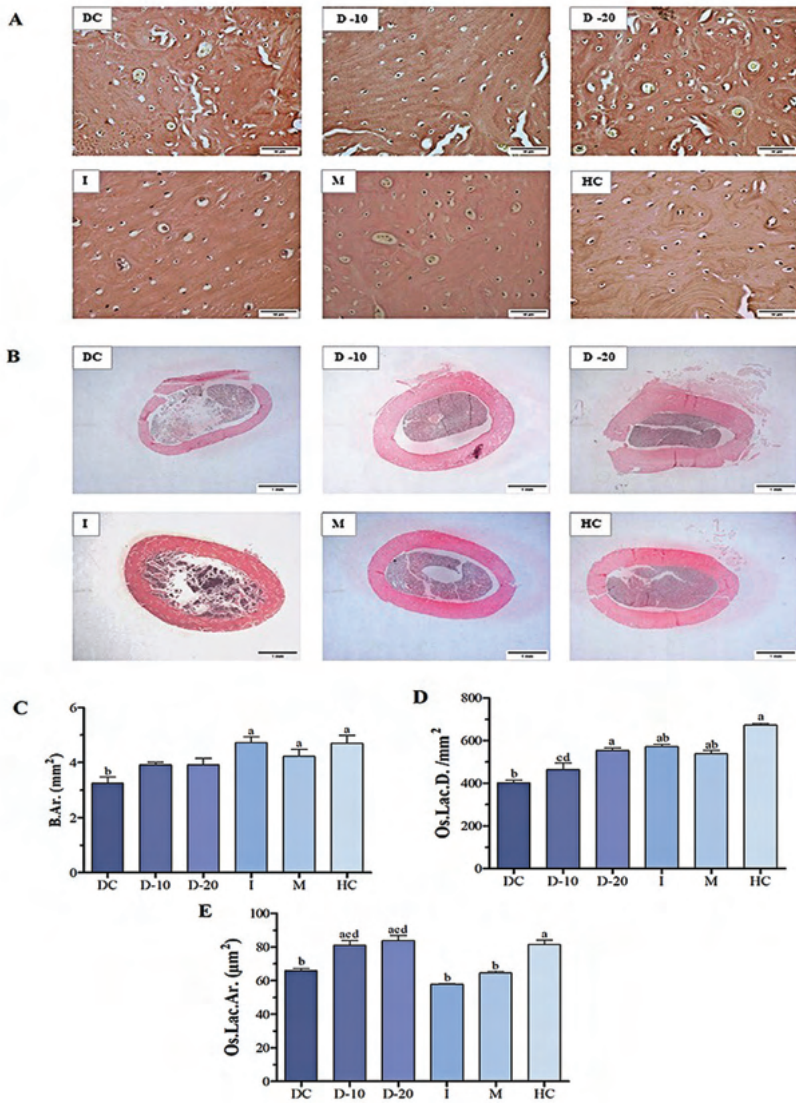


Figure 3. ‘Anti-diabetic’ polyherbal mixture effects on diabetic experimental animals cortical bone: **(A)** H&E stained sections of femoral cortical bone. Scale bar 50 µm. Magnification 400x; **(B)** H&E stained femoral cortical bone cross-section. Scale bar 1 mm. Magnification 20x; **(C)** Effect of polyherbal mixture treatment on the femoral cortical bone area; **(D)** Effect of polyherbal mixture treatment on osteocyte lacunar density; **(E)** Effects of polyherbal mixture treatment on osteocyte lacunar area; DC: Diabetic control; I: Insulin; M: Metformin; D-10: Polyherbal mixture 10 g/kg; D-20: Polyherbal mixture 20 g/kg; HC: Healthy control; B.Ar.: Bone Area; Os.Lac.D.: Osteocyte Lacunar Density; Os.Lac.Ar.: Osteocyte Lacunar Area. Data were expressed as the mean ± standard deviation, n=5. ^ap<0.05 compared to the DC group; ^bp<0.05 compared to the HC group; ^cp<0.05 compared to the I group; ^dp<0.05 compared to the M group.

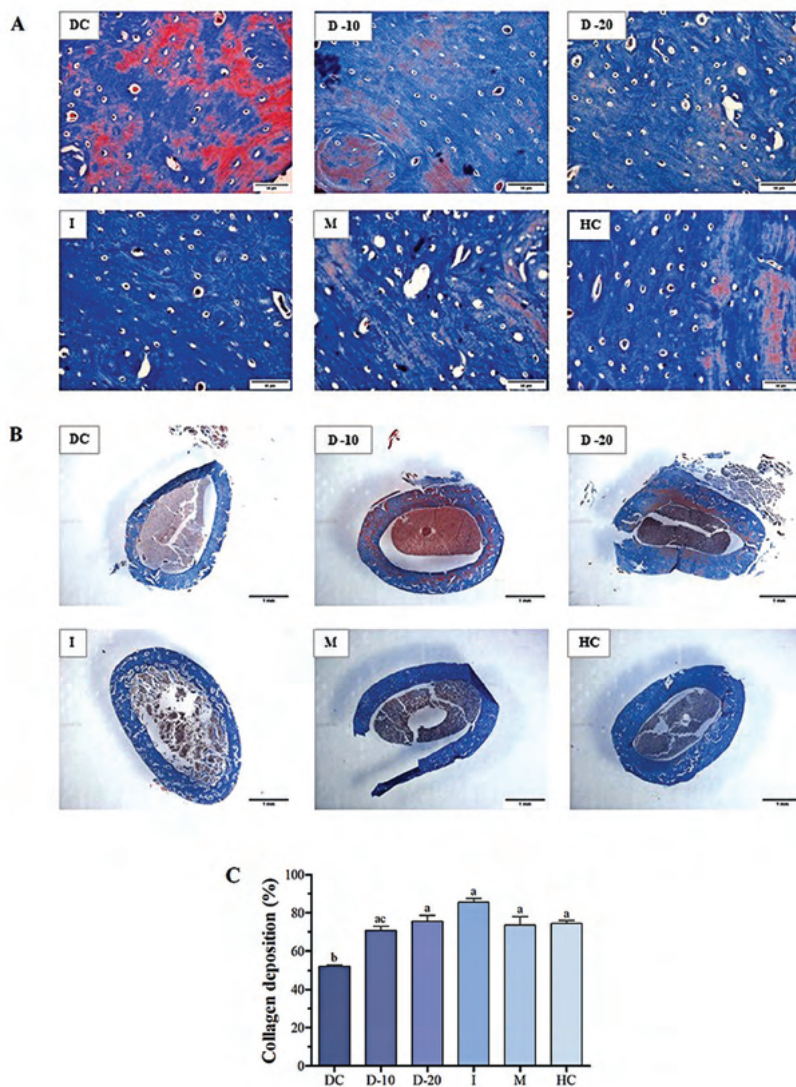


Figure 4. ‘Anti-diabetic’ polyherbal mixture effects on diabetic experimental animals bone collagen: **(A)** Masson’s trichrome stained sections. Scale bar 50 μ m magnification 400x; **(B)** Masson’s trichrome stained femoral cortical bone cross-section. Scale bar 1 mm. Magnification 20x; **(C)** DC: Diabetic control; I: Insulin; M: Metformin; D-10: Polyherbal mixture 10 g/kg; D-20: Polyherbal mixture 20 g/kg; HC: Healthy control. Data were expressed as the mean \pm standard deviation, n=5. ^ap<0.05 compared to the DC group; ^bp< 0.05 compared to the HC group; ^cp<0.05 compared to the I group; ^dp<0.05 compared to the M group.

DISCUSSION

This study aimed to evaluate the value of a traditionally used 'anti-diabetic' polyherbal mixture as a possible remedy for diabetic bone loss prevention.

Histopathological analysis of healthy animals bones treated with low and middle concentration (10 and 20 g/kg) of the polyherbal mixture showed no changes in any of the analyzed histological parameters (Figure 1C, 1E, Figure 2C). However, four weeks of treatment with the highest tested concentration (40 g/kg) increased osteocyte lacunar density which may lead to the increase in bone density and the development of osteopetrosis (Figure 1D) [39-41]. These findings are an additional proof that this polyherbal mixture, as the majority of medicinal plants, when applied in high doses may cause adverse effects [42], not only in terms of hepatotoxicity and nephrotoxicity [30] but also as a potential trigger of osteopetrosis. Because of that, for the evaluation of potential osteoprotective affect the doses of 10 and 20 g/kg of the polyherbal mixture have been chosen.

The osteoprotective effect of the traditionally used herbal mixture was evaluated using the alloxan-induced diabetic rat model. This model is appropriate because alloxan-induced oxidative stress inhibits differentiation of osteoblasts into osteocytes which results in the decreased number of osteocytes per bone area [4,36,37,43,44], that closely resembles the situation seen in human diabetic osteopenia [35,37].

Histopathological analysis of diabetic animals bones showed that treatment with alloxan significantly decreased osteocyte lacunar density compared to the healthy animals ($p < 0.001$), indicating that the model was successful. Interestingly, two weeks of treatment with the 'anti-diabetic' polyherbal mixture was even more effective than treatment with metformin in normalizing the density of osteocyte lacunae (Figure 3D). These results are in concordance with the study of Domazetovic and associates [41] where treatment with *V. myrtillus* extract protected both osteocytes and their precursors from oxidative stress. Additionally, gallic acid, one of the most abundant bioactive compounds in this polyherbal mixture [30] is well known for its anti-osteoporotic activities [40].

Moreover, the size of the osteocyte lacunar area was extremely decreased in the untreated diabetic group of animals compared to the healthy ones ($p < 0.001$), while 14 days of treatment with both tested concentrations of the herbal mixture completely restored this pathological change and brought it to the healthy animal level. Having in mind that chronic hyperglycaemic environment may cause changes in both shape and size of osteocyte lacunar area [40], observed osteoprotective mechanism of the tested polyherbal mixture can be explained by its outstanding hypoglycemic activity observed in our previous study [30].

Interestingly, treatment with the highest tested concentration of herbal mixture restored both the size of the bone area as well as the density of osteocyte lacunae to the healthy animal level, similarly to the diabetic control groups treated with

insulin in metformin (Figure 3C, 3D). Having in mind the fact that both insulin and metformin have proven osteoprotective effects, where lack of available insulin leads to the inactivation of osteoblasts and subsequent osteopenia [45-48], while metformin stimulates proliferation of osteoblasts [49], inhibits activation of osteoblasts, and increases bone mineral density through stimulation of osteoprotegerin and inhibition of RANKL expression [50, 51], we might notice the potential of the tested herbal mixture as a herbal remedy for diabetic osteoporosis.

Additionally, we have observed a decreased level of bone collagen in untreated diabetic animals (Figure 4C), which is one of the well-known characteristics of alloxan-induced diabetic rats [19,52,53]. By the end of the experiment, treatment with polyherbal mixture completely normalized the deposition of bone collagen in all experimental animals. We noticed that the herbal mixture (20 g/kg) was more successful in stimulating the synthesis of collagen compared to metformin, which is known as a drug capable of stimulating the synthesis of collagen type I and bone matrix [54].

Having in mind that decoction of this herbal mixture is characterized not only with a high content of polyphenols and flavonoids but also with the presence of even 10 biologically active compounds, i.e. isoquercetin, rutin, quercitrin, arbutin, hyperoside, astragaloside, trifolin as well as gallic, quinic and caftaric acid [30], the results of this study are in agreement with previous studies where many naturally derived polyphenols, due to the high antioxidative activity [55-58] are well known for osteoprotective activities [59-61]. Namely, isoquercetin, the most abundant bioactive compound in the tested decoction [30], decreases the elevated level of oxidative stress in diabetic rats [62] and has anti-osteoporotic effect in ovariectomised rats [63]. 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- κ B activation [2,64,65]. Supplementation with quercetin and arbutin ameliorates bone loss by reducing bone resorption [25,31,32,66-68] through the suppression of osteoclasts activity and the stimulation of osteoblast activity [69-73]. Hyperoside protects osteoblasts from oxidative stress [74,75], while astragaloside stimulates differentiation of osteoblasts [76]. Additionally, quinic acid promotes osteoblasts differentiation through activation of the Wnt/ β -catenin pathway, while caftaric acid modulates RunX2 and RANKL/OPG signalling pathways [77].

To the best of our knowledge, this is the first report on the *in vivo* evaluation of this 'anti-diabetic' polyherbal preparation osteoprotective effect.

CONCLUSION

Treatment with the 'anti-diabetic' polyherbal mixture decoction was effective in the amelioration of histopathological changes of the bones in an alloxan-induced diabetic rat model. This finding suggests that this mixture might be a candidate for possible future usage as a remedy for the prevention of diabetic osteopenia/osteoporosis. However, more studies are needed.

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Authors' contributions

AP carried out literature research, designed a study, did the microscopy and histopathology, analyzed the results and drafted the manuscript. VM conceived the study, carried out the *in vivo* experiments and drafted the manuscript. MJ participated in plant collecting and taxonomy. LjĐ confirmed the histopathological analysis. PV supervised of all the work. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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OSTEOPROTEKTIVNI EFEKTI „ANTIDIJABETSKE“ BILJNE MEŠAVINE NA TIP 1 DIJABETIČNOM MODELU PACOVA

Aleksandra PETROVIĆ, Višnja MADIĆ, Marina JUŠKOVIĆ,
Ljubiša ĐORĐEVIĆ, Perica VASILJEVIĆ

Gubitak koštane mase, koji dovodi do osteopenije i osteoporoze, česta je sekundarna komplikacija dijabetesa. Cilj ovog istraživanja je procena vrednosti tradicionalno korišćene "antidijabetske" biljne mešavine kao mogući lek za prevenciju ove komplikacije. Dijabetes je indukovao kod ženki pacova Wistar ubrizgavanjem aloksan-monohidrata intraperitonealno. Životinje sa nivoom šećera u krvi većim od 20 mmol/L uzastopno 14 dana, smatrane su dijabetičnim. Tokom narednih 14 dana životinje su tretirane dve koncentracijama biljne mešavine (10 i 20 g suvog biljnog materijala/kg). Dijabetične kontrolne grupe tretirane su insulinom glarginom (13 IU/kg), metforminom (150 mg/kg) i vodom. Zdrava kontrolna grupa je tretirana vodom. Histopatološka analiza kostiju procenjena je na osnovu hematoksilin-eozin i Mason-trihromnog bojenja. Dijabetes indukovao aloksanom je pokrenuo histopatološke promene na kostima karakteristične za nastanak osteopenije i osteoporoze, a tretman dekoktom biljne mešavine je te histopatološke promene vratio na fiziološki nivo. Takođe, tretman ovim testiranim dozama ne pokazuje toksične efekte. Na osnovu zapažanja biljna mešavina bi se mogla koristiti u prevenciji gubitka koštane mase kod dijabetičara.