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The effect of pomegranate juice consumption on bone histomorphometric parameters with the use of an animal model*

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The aim of the study was to evaluate the effect of pomegranate juice consumption on bone histomorphometric parameters using an animal model. The animals (rats) were divided into 7 experimental groups. The groups differed in the concentration of pomegranate juice administered. The control group received only water, the other groups were supplied with commercial or freshly squeezed pomegranate juice at concentrations of 10%, 25% and 50%. To study the structure of the proximal end of the tibia, a Skyscan 1174 X-ray microtomography device, equipped with a 1.3Mp FW VDS camera, was used. The proximal end (metaphysis) of the left tibia was examined. The following bone tissue parameters were analysed: bone mineral density (BMD) (mm3), the number of trabeculae (Tb.N) (mm-1), trabecular separation (Tb.Sp) (mm) and trabecular thickness (Tb. Th) (mm), BS/BV(mm-1). It was found that 25% pomegranate juice, both freshly squeezed and commercial, significantly increased bone density (BMD), increased trabecular thickness (Tb.TH), and reduced the distance between trabeculae (Tb.SP) compared to the control group and groups receiving juice at other concentrations. The highest BMD, the thickest trabeculae and the smallest distance between trabeculae were observed after the application of fresh 25% pomegranate juice. Thus, it can be concluded that pomegranate juice, especially freshly squeezed, improves bone density.

KEY WORDS: anatomy / bone development / histomorphometry / BMD / pomegranate juice / animal model

The pomegranate fruit was used for centuries in ancient cultures for medicinal purposes. In addition to being a source of vitamins C, E, B and A, it also contains magnesium, calcium, potassium, phosphorus, silicon, and iodine as well as significant amounts of protein, folic acid, fiber and beta-carotene [Wang *et al.* 2018]. Furthermore, it is a valuable source of flavonoids, which are found in a wide range of plant foods and have the greatest potential among dietary components in promoting bone health beyond calcium and vitamin D. Recent epidemiological studies have shown that flavonoid intake has a stronger relationship with bone structure and metabolism than overall fruit and vegetable intake [Yeung *et al.* 2020a, 2021ab, 2022, 2023]. Bioactive flavonoids are assessed for properties beyond their chemical antioxidant capacity, including anti-inflammatory effects [Cooper and Horbańczuk 2004, Horbańczuk *et al.* 2008, Cooper *et al.* 2008, Huminiecki *et al.* 2017, 2018, Tewari *et al.* 2017b, Yeung *et al.* 2018, 2019, 2020ab, Mozos *et al.* 2018, 2021, Wang *et al.* 2020, Pieczyńska *et al.* 2020, Chao *et al.* 2021, Chopra *et al.* 2022,]. Some have been reported to enhance bone formation and inhibit bone resorption through their action on cell signaling pathways that influence osteoblast and osteoclast differentiation [Viuda-Martos *et al.* 2010].

Osteoporosis and osteoporotic fractures are major public health problems. Osteoporotic fractures impact quality of life and lead to an increased financial burden on our healthcare systems. Fracture-related burden is expected to increase over the coming decades [Borgström *et al.* 2020]. They are conditioned by many factors, including a reduction in bone mineral density (BMD) [Acevedo *et al.* 2018, Nuti *et al.*

2019]. In view of that, the search for factors enhancing BMD increase or stabilization seems justified [Adamczyk *et al.* 2020, Charuta *et al.* 2012]. Such factors include, among other things, genetics, nutritional factors including n-3 [Horbańczuk *et al.* 1998, 2007, Pomianowski *et al*. 2009, Jóźwik *et al*. 2010, Strzałkowska *et al*. 2010, Tewari *et al.* 2017].

Recently, the nutritional and pharmaceutical properties of pomegranate PG have been of a growing interest to researchers [Wang *et al.* 2018]. A pomegranate has been found to have anti-inflammatory and antioxidant [Cervantes-Anaya *et al.* 2022] as well as antineoplastic properties [Sharma *et al.* 2017, Nasser *et al.* 2020, Henning *et al.* 2019, Paller *et al.* 2017]. In addition, it positively affects the cardiovascular system [Danesi *et al.* 2017, Barati *et al.* 2004, Aviram *et al.* 2004, Sahebkara *et al.* 2017]. PG has been successfully used in a number of medical conditions to obtain enhanced therapeutic results [Saeed *et al.* 2018].

There is evidence that pomegranate juice may have a beneficial impact on patients at risk of SARS-CoV-2 [Saleem *et al.* 2022]. Furthermore, researchers have reported that pomegranate juice is a rich source of polyphenols, and its consumption prevents bone mass loss [Mori-Okamoto *et al.* 2004]. Ammar *et al.* [Ammar *et al.* 2017] emphasize the positive effects of pomegranate juice on osteoarticular regeneration after strengths training. Pomegranate juice supplementation can potentially alleviate oxidative stress by increasing antioxidant response assessed acutely and up to 48 hours after an intense weightlifting training. Also Shuid [Shuid *et al.* 2013], in his review, highlighted the importance of a pomegranate in preventing bone mass loss in musculoskeletal diseases. The results reported by Malihezaman Monsefi [Monsefi *et al.* 2012] suggested that pomegranate might improve bone formation. Thus, the aim of the study was to assess the influence of pomegranate juice consumption on bone histomorphometric parameters using an animal model.

Material and methods

The experiment was condacted on Wistar (male) rats aged 10 weeks were kept in cages that met the statutory requirements (in concordance with the 2006 Journal of Laws, No 50 item 368).

The method of euthanasia of the animals: decapitation, after anesthesia. The method of anesthesia: agent: ketamine, the method of administration: intraperitoneal, dose 100 mg/kg body weight. Xylazine, the method of administration: intraperitoneal, dose 10 mg/kg body weight. Pentobarbital sodium, route of administration: intraperitoneal: dose 200 mg/kg body weight. Measures to alleviate suffering: anesthesia.

The animals were kept under standard husbandry conditions with constant access to feed and water. The animals were kept under controlled environmental conditions. These included a constant temperature of 23±2°C, a regular light cycle 12/12 hours, air humidity of 40-60%, and the number of air changes in the farm room was 20. The animals were fed a standard feed (Altromin Spezialfutter GmbH & Co) dedicated for the maintenance of animals with an energy value of 3625.85 kcal/kg.

After a 2-week quarantine, the animals were divided into 7 experimental groups (6 individuals each). The groups differed in the type of pomegranate juice served:

Group 1 (Control) - received only water;
Group 2 - received commercial pomegranate juice with a concentration of 10%;
Group 3 - received commercial pomegranate juice with a concentration of 25%;
Group 4 - received commercial pomegranate juice with a concentration of 50%;
Group 5 - received freshly squeezed pomegranate juice with a concentration of 10%;
Group 6 - received freshly squeezed pomegranate juice with a concentration of 25%;
Group 7 - received freshly squeezed pomegranate juice with a concentration of 50%.

The animals had their drinkers changed twice a day at 8.00 a.m. and 4.00 p.m. After two months of the experiment, the animals were euthanized and the material was collected for the studies. All the procedures were conducted in accordance with institutional guidelines and in compliance with national and international laws and policies. The study was approved by the relevant Local Ethical Committee for Experiments on Animals in Warsaw in Resolution No 27/200.

The same environmental conditions and the same feed as during the quarantine period were maintained throughout the experiment. The studied material was the proximal end of the left tibia. The measurement site was determined separately for each bone. The study area was the VOI area. The 3D volume measurement was based on the marching cubes volume model of the VOI mm3. A Skyscan 1174 X-ray microtomograph (Bruker, Belgium) with a 1.3 Mp FW VDS camera was used to study the bone structure (Fig. 1). The resolution of the scan was 1024 x 1024 (image pixel size: 21.74 µm). The intensity and voltage of the lamp were 50 KV and 850 µA, respectively. The exposure duration was 2400 ms. The rotation angle was 0.7° with 3 frames per rotation. A 0.5 mm thick aluminum filter was used. The scanning time was 50 minutes. The proximal segments of the left tibial bones were analyzed. For that purpose, appropriate ROIs were distinguished. The following software was used

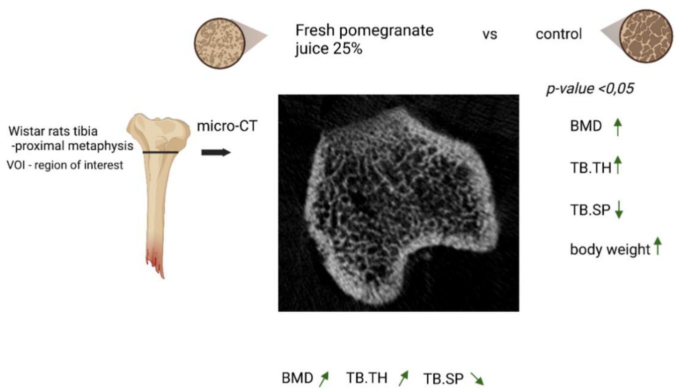


Fig 1. Image from a computer tomography (a Skyscan 1174 X-ray microtomography). The photo shows a cross-section of a rat's bone (proximal end of the tibia). Visible spongy creature inside the bone. The figure was created with BioRender.com, license no MJ25HPP52N.

for the study: Software=Version 1.5 (build 7), NRecon Version 1.7.4.2 reconstruction software, CT Analyser, version: 1.18.4.0 software.

The following morphological parameters of the osseous tissue were analyzed: 3D morphometric parameters integrated for the whole volume of interest (VOI).

Designations of the analyzed parameters: BMD (Bone Mineral Density, mm^3), BS/BV (bone surface/volume ratio, mm^{-1}), TB.TH (trabecular thickness, mm), TB.N (trabecular number), TB.SP (trabecular separation, mm).

Statistical analysis

The results were statistically processed using one-way analysis of variance ($p \leq 0.05$). The significance of differences was checked with the use of Tukey's test at $p \leq 0.05$.

All calculations were performed using STATISTICA 13.0 software.

Results and discussion

The research presented in Table 1 showed the effect of administered pomegranate juice on increasing bone mineral density (BMD), which was statistically confirmed at $p \leq 0.05$ in the investigated groups. In the individuals that drank only water, the mean bone mineral density was the lowest compared to the other experimental groups. The highest bone mineral density (BMD) was found in the group of rats that drank fresh pomegranate juice at a concentration of 25%. In this case, the mineral density ranged from 0.89 to 1.105 mm^3.

The bone density of the animals fed the 25% commercial juice was not significantly different from the bone mineral density of those that drank 25% fresh pomegranate juice.

Rats that drank 50% pomegranate juice, both commercial and freshly squeezed, showed a significant increase in the bone density, in relation to the control group. There was also an increase in the bone density relative to the control group of the rats consuming fresh or commercial 10 % pomegranate juice. BMD values of rats watered with 10% and 50% juice were lower compared to the group of rats that consumed 25% fresh pomegranate juice (Tab. 1). Thus, consumption of 25% fresh pomegranate juice had the greatest effect on increasing bone density (BMD).

The next bone histomorphometric parameter analyzed was bone trabecular thickness (Tb.TH). It was found that the type of pomegranate juice consumed had a significant effect on bone trabecular thickness (Tab. 1).

The type of pomegranate juice consumed did not significantly affect the number of trabeculae compared to the control group and the mean number of trabeculae ranged from 0.0237 to 0.0244 (Tab. 1).

The next bone histomorphometric parameter analyzed was bone trabecular thickness (Tb.TH). The analysis showed that the type of juice administered had an effect on increasing the thickness of bone trabeculae. The thickest bone trabeculae were observed in individuals from the group that was given fresh pomegranate juice with a

Table 1. Average values of bone histomorphometric parameters in the investigated rats groups

Juice	BMD mean±SD (n=6)	TB.N mean±SD (n=6)	TB.TH mean±SD (n=6)	TB.Sp mean±SD (n=6)	BS/BV mean±SD (n=6)	Body weight mean±SD (n=6)
Control (Group 1)	0.770±0.059[d]	0.0237±0.0011[a]	4.238±0.238[c]	38.3±2.51[a]	0.333±0.038[c]	119.83±1.32[b]
Commercial pomegranate juice 10% (Group 2)	0.927±0.075[bc]	0.0237±0.001[a]	5.061±0.452[b]	34.93±1.01[bc]	0.346±0.05[bc]	120.67±1.21[b]
Commercial pomegranate juice 25%(Group 3)	0.978±0.069[ab]	0.0226±0.0014[a]	5.274±0.585[ab]	35.88±1.79[bc]	0.389±0.038[ab]	120.5±1.64[b]
Commercial pomegranate juice 50%(Group 4)	0.884±0.015[c]	0.0228±0.001[a]	5.374±0.217[ab]	37.85±2.46 [ab]	0.383±0.042[ab]	121.5±0.83[ab]
Fresh pomegranate juice 10%(Group 5)	0.954±0.037[b]	0.0220±0.0008[a]	5.115±0.291[b]	34.97±2.31[c]	0.365±0.04[ab]	119.83±0.98[b]
Fresh pomegranate juice 25%(Group 6)	0.988±0.052[a]	0.0241±0.0013[a]	5.696±0.665[a]	34.61±1.24[c]	0.392±0.023[ab]	121.0±2.36[ab]
Fresh pomegranate juice 50%(Group 7)	0.918±0.077[bc]	0.0244±0.0012[a]	5.544±0.439[ab]	33.81±1.03[c]	0.401±0.047[a]	122.3±1.36[a]

Bone mineral density BMD values (mm^3) (total for compact and spongy substance), the number of trabeculae (TB.N), the trabecular thickness (TB.TH) values (mm), the trabecular separation (TB.SP) values (mm), the bone surface/volume ratio (BS/BV) values (mm^{-1}), body weight values (g) values for groups that were given water (Group1; n=6), commercial juice with different concentrations (Group 2-4; n=6) and fresh pomegranate juice with different concentrations (Group 5-7; n=6).
[abcd]Means bearing the same letters are not significantly different at $p<0.05$.

concentration of 25% to drink. Bone trabecular thickness ranged from 5.044 to 6.352 with a mean equal to and standard deviation of SD = 0.665. A similar bone trabecular thickness was also observed in the groups that were given fresh pomegranate juice with a concentration of 50%, and commercial juice with concentrations of 25 and 50%. The lowest trabeculae thickness was observed in individuals of the control group. The thickness of the trabeculae in this group ranged from 4.122 to 4.722 and the variation did not exceed 6% (V = 5.61%). The administration of fresh pomegranate juice with a concentration of 10% and commercial juice with a concentration of 10% increased the thickness of the bone trabeculae compared to the control group, but decreased their thickness compared to the group that consumed fresh 25% pomegranate juice (Tab. 1). The smallest mean distance between the trabeculae (TB.SP) was observed in the bones of individuals when fed fresh pomegranate juice at a concentration of 50% (33.81), fresh pomegranate juice at a concentration of 25% (34.61) and fresh pomegranate juice at a concentration of 10% (34.97) and was significantly smaller than that observed in the control group (38.30) and in the group consuming commercial juice at a concentration of 50%. The concentration of commercial juice did not differentiate the distance between trabeculae. The administration of commercial juice with a concentration of 50% caused the distance between trabeculae not to be significantly different from that observed in the control group (Tab. 1).

The highest value of the BS/BV parameter was obtained after the use of fresh pomegranate juice with a concentration of 50% (0.401). This value was significantly higher than that obtained in the control group (0.333) and in the group consuming commercial juice with a concentration of 10% (0.346). The use of commercial juice with a concentration of 10% did not differentiate this characteristic compared to the control group (Tab. 1).

It was found that the type of juice added differentiated body weight. The addition of freshly squeezed pomegranate juice at concentrations of 25% and 50% and commercial juice at 50% increased the body weight of individuals compared to that obtained in individuals that were supplied with water , commercial juice at concentrations of 10 and 25% and freshly squeezed juice at 10% (Tab. 1).

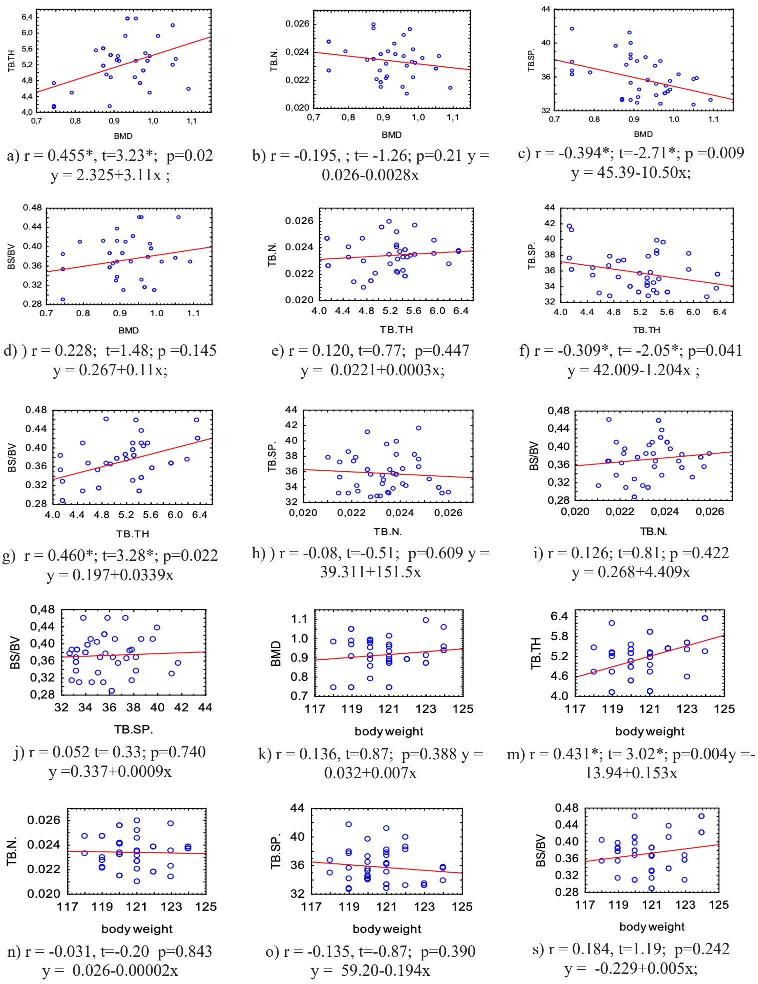
Regression analysis showed a relationship between BMD and trabecular thickness (TB.H) trabecular separation (TB.SP) and VOI. As mineral density increased by mm^3, trabecular thickness increased by 3.11 mm, separation decreased by 10.5 mm (Fig. 2).

The thickness of the trabeculae was significantly negatively correlated with the separation (TB. SP.) As their thickness increased, the distance between them decreased. If the thickness of the trabeculae increased by 1 mm then their distance decreased by 1.204 mm. The thickness of the trabeculae was positively correlated with BS/BV parameter. As the thickness of the trabeculae increased by 1 mm, the BS/BV values increased by 0.0339 mm^{-1} (Fig. 2).

No correlation was found between the number of trabeculae and their separation and the BS/BV parameter (Fig. 2).

Body weight was only significantly correlated with trabecular thickness (TB.TH). As body weight increased by 1 g, trabecular thickness decreased by an average of 0.194 mm (Fig. 2).

At present, pomegranate fruit and its products are consumed worldwide and various health benefits are attributed to them [Khomich *et al.* 2019]. Pomegranate juice is one of the main products of pomegranate processing, high in a complex of polyphenolic compounds with high antioxidant activity and beneficial neuroprotective effects [Matthews *et al.* 2019, Siddarth *et al.* 2020]. A portion of industrially produced pomegranate juice is reported to provide an average of 15% of the recommended daily intake of potassium, 5% of magnesium and about 10% of copper.



a) r = 0.455*, t=3.23*; p=0.02 y = 2.325+3.11x ;

b) r = -0.195, ; t= -1.26; p=0.21 y = 0.026-0.0028x

c) r = -0.394*; t=-2.71*; p =0.009 y = 45.39-10.50x;

d)) r = 0.228; t=1.48; p =0.145 y = 0.267+0.11x;

e) r = 0.120, t=0.77; p=0.447 y = 0.0221+0.0003x;

f) r = -0.309*, t= -2.05*; p=0.041 y = 42.009-1.204x ;

g) r = 0.460*; t=3.28*; p=0.022 y = 0.197+0.0339x

h)) r = -0.08, t=-0.51; p=0.609 y = 39.311+151.5x

i) r = 0.126; t=0.81; p =0.422 y = 0.268+4.409x

j) r = 0.052 t= 0.33; p=0.740 y =0.337+0.0009x

k) r = 0.136, t=0.87; p=0.388 y = 0.032+0.007x

m) r = 0.431*; t= 3.02*; p=0.004y =-13.94+0.153x

n) r = -0.031, t=-0.20 p=0.843 y = 0.026-0.00002x

o) r = -0.135, t=-0.87; p=0.390 y = 59.20-0.194x

s) r = 0.184, t=1.19; p=0.242 y = -0.229+0.005x;

Fig. 2. The analysis of the relationship between BMD and TB.TH (a), BMD and TB.N (b), BMD and TB.SP (c), BMD and BS/BV (d); TB.TH and TB.N (e), TB.TH and TB.SP (f), TB.TH and BS/BV (g); TB.N and TB.SP (h), TB.N and BS/BV (i), TB.SP and BS/ BV (j), body weight and BMD (k), body weight and TN.TH (m), body weight and TB.N (n), body weight and TB.SP (o), body weight and BS/BV (s). The significance of the regression coefficient was evaluated using Student's t-test (t; df=40). Statistically significant values are marked (*) for p≤0.05.

Polyphenolic compounds (ellagitannins, anthocyanins, ellagic acid) and minerals (potassium, magnesium, copper) are of the greatest importance in pomegranate juice in terms of providing the body with micronutrients and small bioactive substances that have a significant effect on bone formation and maintaining the osseous tissue in a good condition.

Taking this into account, in order to investigate the effect of pomegranate juice on the osseous tissue structure, we conducted an experiment on a rat model (the proximal end of the tibia), with the administration of fresh pomegranate juice and a commercially available pomegranate juice. Our studies using CT scans showed a positive effect of pomegranate juice on bone parameters. Both freshly squeezed and commercial juice significantly increased the bone mineral function compared to the control group. The best results were obtained after the consumption of 25% juice.

The mean BMD value in the group receiving fresh juice with a concentration of 25% was the highest, but was not statistically different from that obtained in the group receiving commercial juice with the same concentration.Consumption of 50% and 10% juice significantly increased BMD values compared to the control group, but significantly decreased compared to the group receiving 25% juice. These findings partly agree with the research by Mori-Okamoto *et al*. [2004], Monsefieg *et al*. [2012], Shuid *et al*. [2013] and Spilmont *et al*. [2014] who proved that pomegranate extract increased BMD and prevented bone loss.

Our study also found that the administration of pomegranate juice affected the distribution of the distance between the trabeculae and the distribution of the thickness of the trabeculae in comparison to the control group, but did not affect the trabeculae. Mori-Okamoto *et al*. [2004] obtained results similar to ours. Bone volume and number of bone trabeculae were shown to increase significantly and the separation of the trabeculae decreased in the pomegranate-treated group compared with the control group.The health-promoting properties of the juice were published by Shuid and Mohamed [2013], who recommended the use of pomegranate to reduce bone loss in severe musculoskeletal diseases, and Mori-Okamoto *et al*. [2004] proved that pomegranate juice inhibited bone turnover stimulated by ovariectomy, and therefore is effective in menopausal bone loss conditions in women.Animal studies suggested that commonly consumed fruit rich in antioxidants had a marked effect on bone, as indicated by higher bone mass, trabecular bone volume, number, and thickness and lower trabecular separation by enhancing bone formation and suppressing bone resorption, resulting in greater bone strength. Such osteoprotective effects seemed to be mediated via antioxidant or anti-inflammatory pathways and their downstream signalling mechanisms, leading to osteoblast mineralization and osteoclast inactivation [Spilmont *et al*. 2014]. This was confirmed by a study by Deyhim et al.2005 in which citrus juice (orange and grapefruit) was proven to prevent osteoporosis in rats after orchidectomy. He found that drinking citrus juice had a positive effect on serum antioxidant status and bone strength. Such osteoprotective effects seemed to be mediated via antioxidant or anti-inflammatory pathways and their downstream signaling mechanisms, leading to osteoblast mineralization and osteoclast inactivation [Deyhim *et al*. 2008]. In future studies, randomized controlled trials are warranted to extend the bone-protective activity of fruit and its bioactive compounds. Mechanistic studies are needed to distinguish the role of phytochemicals and other components in the bone protection offered by fruit. Advanced imaging technology will determine effective doses of phytochemicals and their metabolites in improving bone mass, microarchitectural integrity and bone strength, which is a critical step in translating the benefits of fruit consumption.

The results reported here suggest that pomegranate juice, especially when administered at 25%, improves bone condition.

Conclusion

The research showed that pomegranate juice increased bone mineral density (BMD) and positively affected bone histomorphometric parameters. Pomegranate juice administration increased bone mineral density compared to the control group. The highest bone mineral density (BMD) was found in the group of rats that drank fresh or commercial pomegranate juice with a concentration of 25%. Rats that drank either 50% or 10% pomegranate juice, both commercial and freshly squeezed, had an increase in bone density relative to the control group, but a decrease compared to the 25% juice group. Similar to BMD, bone trabecular thickness was dependent on the type and concentration of pomegranate juice consumed. The addition of pomegranate juice increased the thickness of the trabeculae compared to the control group, and the differences between the control group and the others were statistically proven ($p \leq 0.05$). The thickest bone trabeculae were observed in individuals from the group that was given fresh pomegranate juice with a concentration of 25% to drink. In the group receiving juice (fresh and commercial) with a concentration of 10%, the thickness of the trabeculae was significantly lower than that obtained in the group consuming 25% fresh juice, but greater than in the control group.

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Conflicts of Interest: The authors declare no conflict of interest.

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