Exploring Indigenous Legumes of Indonesia: Pigeon Pea (*Cajanus cajan*) and Evaluating Its Impacts on Bone Tissue Structure in Female Rats

ABSTRACT

Aims: The aim of the research was to analyze isoflavone levels and the estrogenic potential of pigeon pea (*Cajanus cajan*) on bone tissue structure preclinically.
Study Design: Analysis of genistein and daidzein pigeon pea powder using the HPLC method. Preclinical tests were carried out using rat. 24 female white Sprague Dawley rats aged 8-9 months body weight at the start of treatment 150-180 grams. Rats were divided into 3 treatment groups. Control (P_0), treatment given a solution of pigeon pea seed powder 24 grams : 24 ml of distilled water (P_1), and treatment given a solution of pigeon pea seed powder 8 grams : 24 ml of distilled water. The solution administration treatment was for 36 days. On the 37th day, surgery was performed and blood and bones were taken. Blood 17 β were analyzed using the HPLC method. HE staining bone micro technique.
Result: Genistein and daidzein pigeon pea were 247.89828 µg/g and 188.61309 µg/g respectively. 17β-estradiol P_0 level 237.231 pg/ml; P_1 603.926 pg/ml P_2 486.153 pg/ml. Increased proliferation of osteoblasts and fibroblasts in bone tissue structures.
Conclusion: Genistein and daidzein are isoflavone compounds found in pigeon pea seeds. Genistein and daidzein can increase 17β-estradiol levels. Pigeon pea seeds have the potential to act as natural estrogen.

Keywords: pigeon pea, osteoblast, daidzein, genistein

1. INTRODUCTION

Osteoporosis in women is a factor caused by decreased estrogen levels, this results in a decrease in bone mineral density (BMD) **(Levin et al., 2018; Gosset, et al., 2021).** Estrogen deficiency causes rapid bone loss within 2-3 years post menopause **(Gosset et al., 2021).** The use of hormone replacement therapy (HRT) is a choice for many menopausal women to overcome this **(Gambacciani & Levancini, 2014; Eastell et al., 2019; Rozenberg et al., 2020; Gosset et al., 2021).**

Continuous use of hormone replacement therapy (HRT) for a long time can pose health risks, making the condition of osteoporosis increasingly complex. Therefore, there is a need for preventive measures and good practices in dealing with osteoporosis. Efforts to prevent osteoporosis can be made by administering natural ingredients containing estrogen **(QIN, et al., 2005; Martiniakova et al., 2020; Karimi et al., 2024).** The use of natural ingredients is a safer and more effective preventative measure than treatment using synthetic ingredients **(Pohl & Kong Thoo Lin, 2018; Scott et al., 2020; Karimi et al., 2024).**

The diversity of local Leguminoceae plants in Indonesia is a wealth of biological natural resources that have not been utilized optimally for health. Leguminoceae are plants that are grouped as phytoestrogens, because they contain high levels of isoflavone components **(Yoo et al., 2005; Nikolić et al., 2017; Das et al., 2020; Whitten et al., 2020).** Isoflavones as a compound with a chemical structure similar to 17 β estradiol can bind to estrogen receptors **(Vitale et al., 2013; Křížová et al., 2019; Fuentes & Silveyra, 2019).** The largest components of isoflavones are daidzein, genistein, glycitein, and coumestrol which are thought to be found in Leguminoceae which are distributed in plants and their products **(Nikolić et al., 2917; Whitten et al., 2020; Sun et al., 2022).**

The chemical structure of isoflavones is similar to 17 β estradiol, so its physiological properties resemble the hormone estrogen. Based on its physiological properties, isoflavones have been widely developed in the food and health sectors. Isoflavone can be used for prevention and treatment, as Hormone Replacement Therapy (HRT) **(Larkin et al., 2008; Pabich, & Materska, 2019).** Isoflavones can be used for the treatment of bone fragility, prevention of osteoporosis in postmenopausal women, therapy and prevention of reduction in bone density in postmenopausal women **(Perna et al., 2016; Lambert et al., 2017; Akhlaghi et al., 2019; Sansai et al., 2020).**

Pigeon pea (*Cajanus cajan*), one of Indonesia's local Leguminoceae, grows abundantly in the lowlands, at an altitude of around 1,800 – 2,000 m. Pigeon pea nutrition 22.6 g/100 g, with glutamic acid, aspartic acid and lysine **(Putra et al., 2021).** The abundance of pigeon peas has not been explored much, the health benefits of pigeon peas have not been explored.

Therefore, pigeon pea is suspected to contain isoflavone compounds, it is necessary to carry out an in-depth analysis, so that pigeon peas, a local bean in Indonesia, can be developed as a natural estrogenic ingredient, especially for preventing osteoporosis. Based on these problems, an analysis is needed to promote natural alternatives side by side reduced dependence on synthetic hormones at affordable solutions.The aim of this study was to analyze isoflavone levels and the estrogenic potential of pigeon pea (*Cajanus cajan*) in bone tissue structure preclinically. It is hoped that the research results will show that pigeon pea can be developed to prevent osteoporosis.

2. MATERIALS AND METHODS

Dried black pigeon pea seeds were obtained from pigeon pea plantations in Ponorogo Regency, East Java, Indonesia. Identification based on Number 0269/Taxo-Plant/Biology/XI/2023

2.1 Making pigeon pea seed powder

500 grams of pigeon pea seeds were dried in an incubator at 60^{o}C for 3 days, then ground using a blender, then ground using a 40 mesh sieve. The fine dry powder of pigeon pea was weighed by total weight.

2.2 Making pigeon pea seed extract

100 grams of pigeon pea powder was dissolved in 96% ethanol, the solution was homogenized with a vortex for 10 minutes. The solution was left for 30 minutes. Then filtered using Whatman filter paper no. 42, the filtrate was taken. The filtrate that was obtained was then centrifuged at a speed of 8000 rpm for 20 minutes, then 1 ml of the supernatant was taken and then dissolved in 10 ml of distilled water.

2.3 Analysis of genistein and daidzein from pigeon pea seed extract using HPLC method

Pigeon pea seed powder extract was purified through solid phase extraction using HPLC Shimadzu C18 Sep-Pak, system controller: SCL 10 AVP; solvent delivering unit LC 20 AT; column oven CTO 10 ASVP; SPD 20A UV-Vis detector; column temperature: 25^{o}C; mobile phase: acetonitrile 20% in acetic acid 3%; mobile phase method: isocratic; flow rate: 0.8 ml/min; injection volume: 20 µl detector wavelength: 261 nm; run time: 60 minutes.

2.4 Serum 17β-estradiol analysis HPLC method

On the 37th day, blood was drawn. The 17β-estradiol analysis procedure was according to **Gatti et al., (1998)** and **Yamada et al., (2002).**

Grouping of experimental animals
Rats were divided into three treatment groups. The first group was a control (P_0), the second group (P_1) was given a solution of pigeon pea seed powder in the ratio of seed powder: distilled water (24 grams of powder:24 ml of distilled water) and the third group (P_2) was given a solution of pigeon pea seed powder in the ratio of seed powder:distilled water (8 grams of powder:24 ml of distilled water).

Treatment of experimental animals
24 female white Sprague Dawley rats aged 10-12 months (premenopause phase) were obtained from the Animal Breeding Unit Blitar, East Java, body weight at the start of treatment 150-180 grams, kept in group cages in the Biosciences experimental animal laboratory at Brawijaya University. Rats were kept in group cages at room temperature (± 27° C), humidity 50-60%, 12 hour light cycle, daily feeding before treatment at 07.00 am, milk pellet composition A with a composition consisting of 12% water, 16% crude protein , crude fat 3-7%, crude fiber 8%, ash 10%, calcium 0.9%-1.2%, phosphorus 0.6%-1% with raw materials yellow corn, wheat bran, SBM, molasses, palm olein, essential amino acids, essential minerals, premixes, and vitamins. Providing water ad libitum. Husk replacement and cage maintenance are carried out every day. Rats were treated by direct induction into the stomach using a gavage tube with pigeon pea solution once a day for 36 days. On the 37th day of the rat, blood was drawn, surgery and bones were taken.

Making histological preparations for HE staining
Includes the stages of fixation, washing, dehydration, clearing, infiltration, blocking, trimming, sectioning, mounting and staining. Procedure for making histological preparations **(Feldman & Wolfe, 2014; Sampedro-Carrillo, 2022).** Changes in bone tissue structure were observed using an optilab microscope.

Data analysis
The data obtained, namely data on genistein and daidzein levels from the HPLC test, were analyzed based on the chromatogram results, sample curve area and retention time. Data on bone tissue structure preparations were analyzed for changes in bone tissue structure, osteoblasts and osteoclasts.

3. RESULTS AND DISCUSSION

The results of High Performance Liquid Chromatography (HPLC) analysis of isoflavones (genistein and daidzein) showed genistein and daidzein levels of 247.89828 µg/g and 188.61309 µg/g, respectively. The results of the HPLC analysis are in Figure 1.

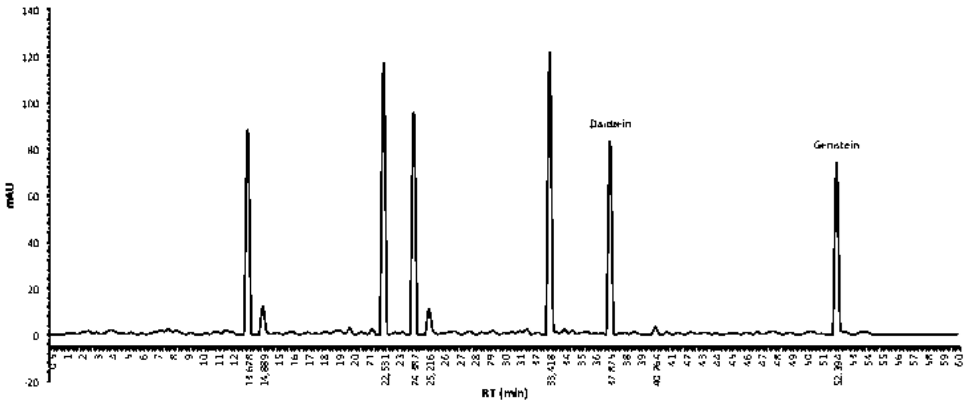


Figure 1. HPLC with specifications: Shimadzu, injection volume 20 µl, running time 60 minutes, wavelength detector 261 nm, column temperature 25°C, column C 185 µm Shimadzu 120x4.6 mm, mobile phase acetonitrile 20% in acetic acid 3% , Flow rate: 0.8 ml/min. HPLC pigeon pea contained genistein 247,898 µg/g and daidzein 188,613 µg/g.

HPLC analysis Figure 1 and Table 1, based on sample weight, retention time, and sample curve area.

Table 1. HPLC analysis of pigeon pea seed extract

Sample name	Sample weight (g)	RT (min)	Sample curve area	Result (µg/g)	Compound
Pigeon pea	5,0003	37,875	83,28266	188,61309	Daidzein
	5,0003	52,394	74,22387	247,89828	Genistein

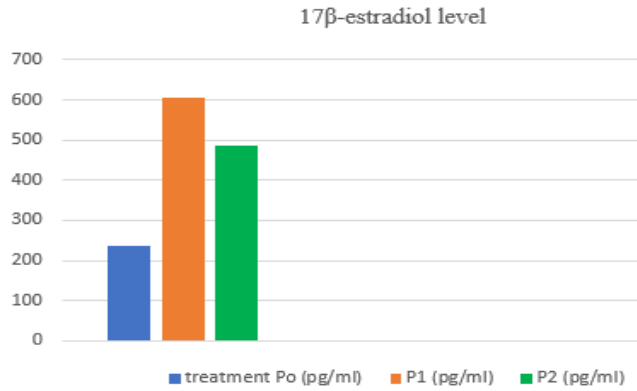
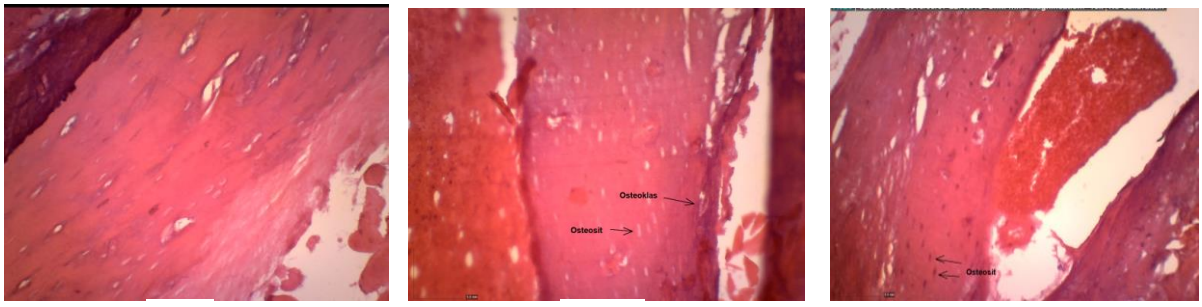


Figure 2: HPLC analysis of 17β-estradiol for each treatment was P_0 237.231 pg/ml; P_1 603.926 pg/ml P_2 486.153 pg/ml

Isoflavones, lignans, stilbens, koumestans have an aromatic ring structure similar to 17 β-estradiol **(Dixon, 2004; Ahmad Hairi et al., 2018; Lee, & Tseng, 2020; Chavda et al., 2024),** this is often referred to as estrogen like molecules **(Vaya & Tamir, 2004; Kiyama, 2023).** Therefore, these compounds are often called phytoestrogens. Genistein and daidzein are the main isoflavones, which are aglycones that are widely distributed in plants of the Leguminoceae family **(Chavda et al., 2024).**

Genistein and daidzein are thought to be able to bind to estrogen receptors on ER α and ER β in the body system **(Dhananjaya et al., 2012; Chan et al., 2018).** The phytoestrogen compounds genistein and daidzein have a mechanism similar to estradiol so they can provide potency in organ systems. Figure 3 shows that giving pigeon pea powder solution to mice for 36 days was able to increase the proliferation of osteoblasts and fibroblasts in bone tissue structures.



P_0 P_1 P_2

Figure 3. Bone tissue structure of white rat, HE staining, 100x

P_0 The structure of bone tissue in normal treatment; P_1 Osteoblasts experience proliferation and increase in fibroblast tissue; P_2 Osteoblasts experience proliferation although not all of them proliferate, there are some osteoclasts.

One way ANOVA statistical analysis shows that the significance value (Sig.) 0.004 is smaller than the α value 0.01, this means that Ho is rejected (Table 2). The results of this statistical analysis stated that given a solution of pigeon pea seed powder had a very significant effect on the number of osteoblasts.

Table 2. One way anova analysis of given solution of pigeon pea seed powder to osteoblasts

	Sum of Squares	*Df*	*Mean Square*	*F*	*Sig.*
Between Groups	452.177	2	220.094	7.273	.004
Within Groups	641.458	21	30.261		
Total	1093.635	23			

Osteoblasts are part of the mesenchymal cells that are responsible for bone formation and development. Osteoblasts produce osteoprotegerin (OPG) as a receptor to prevent bone loss, while 17β-estradiol can stimulate OPG production **(Rickard et al., 2003; An et al., 2016).** Genistein and daidzein pigeon pea solution can increase the binding of estrogen receptors so that they can increase bone mineral density (BMD) **(Abdi et al., 2016; Nayeem et al., 2019).** The hormone estrogen can inhibit bone resorption, suppress the production of IL-1, IL-6 and TNFα, and inhibit RANK-RANKL interactions by stimulating stromal cells to produce OPG **(Drugarin, et al., 2003; Hooshiar, et al., 2022).**

Genistein and daidzein in pigeon pea are phytoestrogens, and can act as substitutes for the hormone estrogen. Therefore, these two phytoestrogen compounds can act as estrogen hormones, especially as estrogen replacement therapy in menopausal conditions to prevent osteoporosis.

4. CONCLUSION

Genistein and daidzein pigeon pea were 247.89828 µg/g and 188.61309 µg/g respectively. 17β-estradiol P_0 level 237.231 pg/ml; P_1 603.926 pg/ml P_2 486.153 pg/ml. Giving pigeon pea can increase 17β-estradiol levels in mice, this can cause proliferation of osteoblasts and fibroblasts of bone tissue structures. Pigeon pea has the potential to act as a natural estrogen.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

This article based on our research and did not used any AI technology

REFERENCES

Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. *Histopathology: methods and protocols*, 31-43.

Gatti, R., Gotti, R., Gioia, M. G., & Cavrini, V. (1998). HPLC analysis of pharmaceutical estrogens in raw materials and dosage forms. *Journal of pharmaceutical and biomedical analysis*, *17*(2), 337-347.

Yamada, H., Yoshizawa, K., & Hayase, T. (2002). Sensitive determination method of estradiol in plasma using high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography B*, *775*(2), 209-213.

Sampedro-Carrillo, E. A. (2022). Sample preparation and fixation for histology and pathology. *Immunohistochemistry and Immunocytochemistry: Methods and Protocols*, 33-45

Hooshiar, S. H., Tobeiha, M., & Jafarnejad, S. (2022). Soy isoflavones and bone health: Focus on the RANKL/RANK/OPG pathway. *BioMed research international*, *2022*(1), 8862278.

Drugarin, D., Drugarin, M., Negru, S., & Cioace, R. (2003). RANKL/RANK/OPG molecular complex-control factors in bone remodeling. *TMJ*, *53*(3-4), 297-302.

Dixon, R. A. (2004). Phytoestrogens. *Annu. Rev. Plant Biol.*, *55*(1), 225-261.

Chavda, V. P., Chaudhari, A. Z., Balar, P. C., Gholap, A., & Vora, L. K. (2024). Phytoestrogens: Chemistry, potential health benefits, and their medicinal importance. *Phytotherapy Research*.

Nayeem, F., Chen, N. W., Nagamani, M., Anderson, K. E., & Lu, L. J. W. (2019). Daidzein and genistein have differential effects in decreasing whole body bone mineral density but had no effect on hip and spine density in premenopausal women: A 2-year randomized, double-blind, placebo-controlled study. *Nutrition Research*, *68*, 70-81.

Abdi, F., Alimoradi, Z., Haqi, P., & Mahdizad, F. (2016). Effects of phytoestrogens on bone mineral density during the menopause transition: a systematic review of randomized, controlled trials. *Climacteric*, *19*(6), 535-545.

An, J., Yang, H., Zhang, Q., Liu, C., Zhao, J., Zhang, L., & Chen, B. (2016). Natural products for treatment of osteoporosis: The effects and mechanisms on promoting osteoblast-mediated bone formation. *Life sciences*, *147*, 46-58.

Rickard, D. J., Monroe, D. G., Ruesink, T. J., Khosla, S., Riggs, B. L., & Spelsberg, T. C. (2003). Phytoestrogen genistein acts as an estrogen agonist on human osteoblastic cells through estrogen receptors α and β. *Journal of cellular biochemistry*, *89*(3), 633-646.

Chan, K. K., Siu, M. K., Jiang, Y. X., Wang, J. J., Leung, T. H., & Ngan, H. Y. (2018). Estrogen receptor modulators genistein, daidzein and ERB-041 inhibit cell migration, invasion, proliferation and sphere formation via modulation of FAK and PI3K/AKT signaling in ovarian cancer. *Cancer cell international*, *18*, 1-14.

Dhananjaya, K., Sibi, G., Mallesha, H., Ravikumar, K. R., & Awasthi, S. (2012). Insilico studies of daidzein and genistein with human estrogen receptor α. *Asian Pacific Journal of Tropical Biomedicine*, *2*(3), S1747-S1753.

Lee, T. Y., & Tseng, Y. H. (2020). The potential of phytochemicals in oral cancer prevention and therapy: a review of the evidence. *Biomolecules*, *10*(8), 1150.

Kiyama, R. (2023). Estrogenic flavonoids and their molecular mechanisms of action. *The Journal of Nutritional Biochemistry*, *114*, 109250.

Vaya, J., & Tamir, S. (2004). The relation between the chemical structure of flavonoids and their estrogen-like activities. *Current Medicinal Chemistry*, *11*(10), 1333-1343.

Ahmad Hairi, H., Jamal, J. A., Aladdin, N. A., Husain, K., Mohd Sofi, N. S., Mohamed, N., ... & Shuid, A. N. (2018). Demethylbelamcandaquinone B (Dmcq B) is the active compound of Marantodes pumilum var. alata (Blume) Kuntze with osteoanabolic activities. *Molecules*, *23*(7), 1686.

Das, S., Sharangi, A. B., Egbuna, C., Jeevanandam, J., Ezzat, S. M., Adetunji, C. O., ... & Onyeike, P. C. (2020). Health benefits of isoflavones found exclusively of plants of the fabaceae family. *Functional Foods and Nutraceuticals: Bioactive Components, Formulations and Innovations*, 473-508.

Nikolić, I. L., Savić, G. I. M., Tačić, A. D., & Savić, I. M. (2017). Classification and biological activity of phytoestrogens: A review. *Advanced technologies*, *6*(2), 96-106.

Whitten, P. L., Kudo, S., & Okubo, K. K. (2020). Isoflavonoids. In *Handbook of plant and fungal toxicants* (pp. 117-137). CRC Press.

Křížová, L., Dadáková, K., Kašparovská, J., & Kašparovský, T. (2019). Isoflavones. *Molecules*, *24*(6), 1076.

Fuentes, N., & Silveyra, P. (2019). Estrogen receptor signaling mechanisms. *Advances in protein chemistry and structural biology*, *116*, 135-170.

Vitale, D. C., Piazza, C., Melilli, B., Drago, F., & Salomone, S. (2013). Isoflavones: estrogenic activity, biological effect and bioavailability. *European journal of drug metabolism and pharmacokinetics*, *38*, 15-25.

Sun, W., Shahrajabian, M. H., & Cheng, Q. (2022). Bioactive ingredients of legumes. In *Functional foods and nutraceuticals in metabolic and non-communicable diseases* (pp. 371-382). Academic Press.

Levin, V. A., Jiang, X., & Kagan, R. (2018). Estrogen therapy for osteoporosis in the modern era. *Osteoporosis International*, *29*, 1049-1055.

Gosset, A., Pouillès, J. M., & Trémollieres, F. (2021). Menopausal hormone therapy for the management of osteoporosis. *Best Practice & Research Clinical Endocrinology & Metabolism*, *35*(6), 101551.

Gosset, A., Pouillès, J. M., & Trémollieres, F. (2021). Menopausal hormone therapy for the management of osteoporosis. *Best Practice & Research Clinical Endocrinology & Metabolism*, *35*(6), 101551.

Rozenberg, S., Al-Daghri, N., Aubertin-Leheudre, M., Brandi, M. L., Cano, A., Collins, P., ... & Harvey, N. C. (2020). Is there a role for menopausal hormone therapy in the management of postmenopausal osteoporosis?. *Osteoporosis international*, *31*, 2271-2286.

Gambacciani, M., & Levancini, M. (2014). Hormone replacement therapy and the prevention of postmenopausal osteoporosis. *Menopause Review/Przegląd Menopauzalny*, *13*(4), 213-220.

Eastell, R., Rosen, C. J., Black, D. M., Cheung, A. M., Murad, M. H., & Shoback, D. (2019). Pharmacological management of osteoporosis in postmenopausal women: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, *104*(5), 1595-1622.

Martiniakova, M., Babikova, M., & Omelka, R. (2020). Pharmacological agents and natural compounds: available treatments for osteoporosis. *Journal of Physiology & Pharmacology*, *71*(3).

QIN, L., ZHANG, G., SHI, Y., LEE, K., & LEUNG, P. (2005). Prevention and treatment of osteoporosis with traditional herbal medicine. In *Current topics in osteoporosis* (pp. 513-531).

Karimi, S. M., Bayat, M., & Rahimi, R. (2024). Plant-derived natural medicines for the management of osteoporosis: A comprehensive review of clinical trials. *Journal of Traditional and Complementary Medicine*, *14*(1), 1-18.

Scott, S. E., Rozin, P., & Small, D. A. (2020). Consumers prefer “natural” more for preventatives than for curatives. *Journal of Consumer Research*, *47*(3), 454-471.

Pohl, F., & Kong Thoo Lin, P. (2018). The potential use of plant natural products and plant extracts with antioxidant properties for the prevention/treatment of neurodegenerative diseases: in vitro, in vivo and clinical trials. *Molecules*, *23*(12), 3283.

Larkin, T., Price, W. E., & Astheimer, L. (2008). The key importance of soy isoflavone bioavailability to understanding health benefits. *Critical reviews in food science and nutrition*, *48*(6), 538-552.

Lambert, M.N.T.; Hu, L.M.; Jeppesen, P.B. A systematic review and meta-analysis of the effects of isoflavone formulations against estrogen-deficient bone resorption in peri- and postmenopausal women. Am. J.Clin. Nutr. 2017, 106, 801–811.

Pabich, M., & Materska, M. (2019). Biological effect of soy isoflavones in the prevention of civilization diseases. *Nutrients*, *11*(7), 1660.

Putra, I. D., Marsono, Y., & Indrati, R. (2021). Effect of simulated gastrointestinal digestion of bioactive peptide from pigeon pea (Cajanus cajan) tempe on angiotensin-I converting enzyme inhibitory activity. *Nutrition & Food Science*, *51*(2), 244-254.

Akhlaghi, M.; Ghasemi Nasab, M.; Riasatian, M.; Sadeghi, F. Soy isoflavones prevent bone resorption and loss, a systematic review and meta-analysis of randomized controlled trials. Crit. Rev. Food Sci. Nutr. 2020, 60, 2327–2341.

Perna, S.; Peroni, G.; Miccono, A.; Riva, A.; Morazzoni, P.; Allegrini, P.; Preda, S.; Baldiraghi, V.; Guido, D.; Rondanelli, M. Multidimensional Effects of Soy Isoflavone by Food or Supplements in Menopause Women: A Systematic Review and Bibliometric Analysis. Nat. Prod. Commun. 2016, 11, 1733–174.

Sansai, K.; Na Takuathung, M.; Khatsri, R.; Teekachunhatean, S.; Hanprasertpong, N.; Koonrungsesomboon, N. Effects of isoflavone interventions on bone mineral density in postmenopausal women: A systematic review and meta-analysis of randomized controlled trials. Osteoporos. Int. 2020, 31, 1853–1864.