Antiosteoporosis Effects of 70% Ethanolic Extract Combination of Dayak Onion Bulbs (*Eleutherine bulbosa* (Mill.) Urb) and Cowpea (*Vigna unguiculata* (L.) Walp.) on the Hypoestrogen Rats

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ABSTRACT

Introduction: Cowpea and Dayak onion bulbs extracts were proven to have an effect on bone, cause by the content of daidzein and eleutherinol which has been known to have an affinity to bind with estrogen receptor selectively. The objective of this study to determine the effects of Dayak onion bulbs and cowpea extract combination on the level of bone calcium, bone weight, and the scoring of bone marrow fat in the hypoestrogen rats compared with the single dose of cowpea extract. **Material and Methods**: This study used 32 female, white Sprague-Dawley rats were divided into eight groups. The SHAM group was given CMC 0.5%; the negative group was given CMC 0.5%, the positive group was given raloxifene 1.08 mg/200 g BW, cowpea group was given cowpea extract 100 mg/200 BW. The dose variation was given 70% ethanolic extract combination of Dayak onion bulbs and cowpea with four doses variation 36 mg/200 g; 100 mg/200 g BW, 18 mg/200 g; 100 mg/200 g BW, 9 mg/200 g; 100 mg/ 200 g BW, and 4.5 mg/200 g; 100 mg/200 g BW. Ovariectomy was performed on all groups except the SHAM to obtain the condition of hypoestrogen. Four weeks after the surgery, the rats were treated with an oral administration of extract daily for 28 days. Determination of the levels of tibia bone calcium, tibia bone weight, and the scoring of tibia bone marrow fat counted on day 29. **Results:** The result showed that Dayak onion bulbs and cowpea extract combination were able to elevate the level of bone calcium and bone weight significantly, and reduced the scoring of bone marrow fat higher than a single dose of cowpea extract. **Conclusions:** The results showed that Dayak onion bulbs and cowpea extract combination could be developed to be a drug for osteoporosis in the future.

Key words: Antiosteoporosis, Bone calcium, Bone marrow fat, Bone weight, Cowpea, Dayak onion bulbs, Raloxifene.

INTRODUCTION

Osteoporosis is a bone metabolic disease characterized by reduced bone mass, disruption of bone microarchitecture, and increased bone fragility resulting in fracture risk. Bone metabolism may be impaired due to various factors, such as lack of estrogen, low intake of calcium and vitamin D, and the side effects of some drugs. The lack of estrogen after menopause increases the risk of osteoporosis. Menopause is a normal and natural event which is defined as the permanent cessation of ovulation and hence menstruation due to ovarian failure.[1] Hypoestrogen conditions lead to increased osteoclast activity resulting in bone loss that indicated a low level of bone calcium.[2] Deficiency of estrogen could increase the bone marrow fat resulting in impaired bone formation.[3]

Estrogen is the women sex hormone that produced mainly by ovarian.[4] Estrogen affects stimulation of bone formation and limits bone resorption, affecting lipid profile and vascular endothelial, liver and cardiovascular system. The protective effects of estrogen in bone are due to many mechanisms. For example, repression of pro-osteoclastic cytokines, such as IL-1, IL- 6, IL-7, and TNFα, in osteoblasts have been well documented to promote increased bone mass. The physiological functions of estrogenic compounds are modulated largely by the estrogen receptors subtypes alpha (ERα) and beta (ERβ). ERα is present mainly in the mammary gland, uterus, ovary, bone, liver, and adipose tissue. By contrast, ERβ is found mainly in the prostate, bladder, colon, and immune system.[5] ERα and ERβ have been detected by immunohistochemistry in some bone cells such as osteoblasts, osteocytes, and osteoclasts. ERβ is expressed at higher levels in trabecular bone. Interestingly, in this same study, ERα was detected higher in cortical bone.[6] Symptoms after menopause such as osteoporosis can be overcome with the use of hormone replacement therapy (HRT). Hormone replacement therapy works as non-selective and agonist to all estrogen receptors, thus increasing the proliferation of all estrogen receptor cells. With the technological advances, that was found a class of drugs that work tissue-selective is known as the selective estrogen receptor modulators (SERMs). This class works in some organs as agonists and in other organs estrogen antagonist.

Dayak onion bulbs (*Eleutherine bulbosa* (Mill.) Urb.) extract could increase significantly bone calcium level, bone weight, and bone length of hypoestrogen

rat.[7] It contains eleutherinol compounds that were in the silico study have been shown to bind to estrogen alpha receptors (RE-α).[8] This extract also contains liquiritigenin compound that has an affinity to bind with estrogen beta receptors selectively and can promote osteoblast differentiation and inhibit of osteoclast differentiation.[9] Cowpea (*Vigna unguiculata* (L.) Walp.) extract can be used as antiosteoporosis agent, that was caused by the content of daidzein. Daidzein has been shown to selectively enhance nuclear ER-β levels, which enhances expression of both ER-α and ER-β.[10] These compounds are expected selective and agonist in estrogen receptors in bone, so it can be used as an alternative therapy to treat postmenopausal syndrome such as osteoporosis. Therefore, further research is needed to prove the effects of Dayak onion bulbs and cowpea extract combination scientifically on bone seen from the levels of calcium, bone weight, and bone marrow fat.

MATERIALS AND METHODS

Plant materials

Eleutherine bulbosa (Mill.) Urb. were found and collected from Palu, Central Sulawesi, Indonesia Determined by the Research Center for Natural resources of Tadulako University (Certificate of Determination No. 65/UN.28.UPT-SDHS/LK/2016). *Vigna unguiculata* (L.) Walp. were obtained and identified by Center for Plant Conservation Botanic Gardens and extracted by the laboratory of Indonesian Center for Spices and Medicinal Plants Research, Bogor (Certificate of Determination No. B-/355/IPH.3./KS/IV/2018).

Preparation of extract

The dried powder of Dayak onion bulbs and cowpea separately were extracted by maceration method using ethanol. Then the extract was concentrated by a rotary evaporator. The extractive value of ethanol from dried powder was calculated as % w/w yield. The extractive value of Dayak onion bulbs was found at 3.77% and the cowpea was found 10.58%.

Identification chemical content of extract By LC-MS/MS

The identification of the extracts was conducted in the laboratory of the Center Chemical Research, Serpong. The identification of the compounds in both extracts was performed using LC-MS/MS. 3 ml of each extract was centrifuged at 3000 rpm for 5 min. the supernatant was filtered through a membrane filter (0.45 μm). Mass spectrometry was performed on QTOF. For positive electrospray mode, the capillary and cone voltage was set at 1.5 kV and 30V, respectively. The desolvation gas was set to 500 L/h at a temperature 120ºC. Chromatographic separation was carried out on a C18 column (2.1×1000 mm, 1.8μm particle size) at a column temperature of 40ºC. The mobile phase consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (methanol + 0.1% formic acid). The optimized UPLC elution conditions were as follows: 0-3 min, 95% A; 4-5 min, 60% A; 6-11 min, 20% A; 12-14 min, 100% B; at 15 min, 95% A. The flow rate was set at 300 μL/min. The injection volume was 5 μL.

Animals

White female Sprague–Dawley rats, aged 42 days, were obtained from the Bogor Agricultural Institute (IPB), weighing less than 100 g, as much as 32 individuals. The rats were acclimatized for 1 week in standard animal cages before the commencement of the experiment. At 50 days of age, bilateral ovariectomy was performed via a dorsal midline incision under ketamine HCl injection. This research had been certified by ethical certification of Faculty of Medicine, University of Indonesia (Approval No. 0037/UN2.F1/Ethics/2018).

Animals were divided into 8 groups: The SHAM and negative groups, both given 0.5% carboxymethylcellulose (CMC), the positive group received raloxifene 1.08 mg/200 g BW, cowpea group was given cowpea extract 100 mg/200 BW. The combination of extract groups was given Dayak onion bulbs and cowpea in 4 kinds of dose that were given respectively combination Dose 1:36 mg/200 g Dayak onion bulbs; 100 mg/200 g BW cowpea, Dose 2:18 mg/200 g Dayak onion bulbs; 100 mg/200 g BW cowpea, Dose 3:9 mg/200 g Dayak onion bulbs; 100 mg/ 200 g BW cowpea, and Dose 4:4.5 mg/200 g Dayak onion bulbs; 100 mg/200 g BW cowpea. Ovariectomy was performed on all groups except the SHAM to obtain the condition of hypoestrogen. Four weeks after the surgery, all rats checked for ovariectomy success on day 28, followed by the rats were treated with an oral administration of extract daily for 28 days. Determination of the levels of tibia bone calcium, tibia bone weight, and the scoring of tibia bone marrow fat counted on day 29.

Evaluation of parameters

Rat tibia bone calcium

At the end of the experiment, all experimental animals were sacrificed with ether to remove the right tibia bone. Surgery is performed using a surgical tool to cleans bone from tissue and fat attached. Then, cut from the knee joints to the rat ankle joints. The isolated bone was dried in an oven at 100ºC for 3 h. After that, the bone is weighed. Then the bone is crushed in a furnace with a temperature of 700°C for 4 h until bone changes to white ash. Bones that have become white ash, crushed until smooth. The ash powder is then fed into the erlenmeyer flask and dissolved in 20 ml of 20% HCl, then the solution is heated over the hotplate until the 20% HCL volume is reduced to ¼ gourd. After that, put into a 50 ml measuring flask. The aquadest is added to 50 ml on the measuring flask, then filtered. Pipette 1 ml of the main solution, put into a 100 ml measuring flask, sufficient with aquadest to 100 ml. Subsequently, the test solution measured its calcium content with an atomic absorption spectrophotometer at a wavelength of 422.7 nm.

The weight of rat tibia bone

The rat tibia bone is cleaned from tissue that attached and dried by the oven at 100ºC for 3 h. The bone is weighed using an analytical scale (Ohaus, USA).

Histological analysis

The left tibia bone was fixed in 10% buffered formalin for 48 h. All samples were embedded in paraffin and 3 μm thick sections were cut, mounted and stained with Hematoxylin and Eosin (H&E) for microscopic analysis. Determination of scoring bone marrow fat was carried out using an optical microscope that was connected to a computer. The focus distance of the microscope is arranged so that a clear picture is obtained. Then seen the bone marrow fat conditions and bone structure with scoring value criteria that is "0" (bone marrow and bone structure is normal), "1" (Bone marrow begins to fatty in a few categories, "2" (Bone marrow is moderately fatty and the haversal canal begins to widen), "3" (The bone marrow is heavily fatty and the haversal canal is dilated) (Figure 1).

Statistical analysis

Data from the animal experiments were analyzed statistically using SPSS. Analysis conducted was homogeneity (Levene test) and the test of the normal distribution (Shapiro-Wilk test). To see the relationship between the treatment groups, one-way analysis of variance (ANOVA) was conducted followed by an analysis of Significant Difference test (LSD). p values of less than 0.05 were considered to indicate significant differences.

RESULTS

By phytochemistry screening, Dayak onion extract contains flavonoids, tannins, alkaloids, saponins, triterpenoids, phenolic, and glycosides. Whereas Cowpea extract contains flavonoids, tannins, alkaloids, saponins, phenolic, and glycosides as shown in Table 1. We also identified the content of the extracts by LC-MS/MS as shown in Tables 2 and 3.

Based on Table 2, the five most compounds contained in Dayak onion bulb extracts are 2,4,7-Trihydroxy-9,10-dihydrophenanthrene, cuspidatumin A, dendromoniliside E, liquiritigenin, and natsudaidain. Several studies have reported that liquiritigenin has been reported to be a plant-derived, selective estrogen receptor β agonist, indicating that it is probably useful for the treatment of breast cancer and osteoporosis.[9]

Based on Table 3, the five most compounds contained in the Cowpea extract include 2-Monolinolein, Glycerol-β-stearate, Momorcerebroside 1, Trigonelline, and daidzein. Daidzein is known to bind to alpha and beta estrogen receptors, but this compound is known to bind more strongly to ER beta than to ER alpha.[10] Based on previous research, isoflavones are natural SERMs candidates that have a high affinity with β estrogen receptors.[11]

The level of calcium bone

According to the Table 4, the average of calcium content in the SHAM group was higher than OVX group. Statistical analysis showed that there was a significant difference between SHAM groups OVX group (p < 0.05). The average of Raloxifene group was much higher than OVX group, single cowpea extract and SHAM but lower than dose 2 and dose 3 of combination groups.

Bone weight determination

Table 4 also showed that the average of bone weight in the SHAM group was higher than OVX group. Statistical analysis showed that there was a significant difference between SHAM groups OVX group (p < 0.05). The average of Raloxifene group was much higher than OVX group, single cowpea extract group and SHAM but lower than dose 2 and dose 3 of combination groups.

Scoring bone marrow fat

Based on Table 4 above, the average of scoring of bone marrow fat of the SHAM and dose 3 group were lowest than other groups. The average of Raloxifene group was much largest than other groups. The average of cowpea extract and dose 1 had the scoring of bone marrow fat were lower than Raloxifene and negative control groups. The average of dose 2 and dose 4 were lower than the positive control, negative control, cowpea extract and dose 1 groups, but were greater than the SHAM and dose 3. The dose group 3 had an average of scoring of bone marrow fat was significantly lower compared to OVX group and positive control (p < 0.05), and having an average of scoring of bone marrow fat was same with group SHAM (p = 1.000).

DISCUSSION

There was a significant difference in the level of bone calcium and bone weight between SHAM and OVX groups (p < 0.05), it could be seen that the ovariectomy can decrease the level of bone calcium and bone weight. Hypoestrogen condition in the OVX group can decrease the level of bone calcium and bone weight because osteoclast activity on this condition increases resulting in bone matrix and the calcium bone
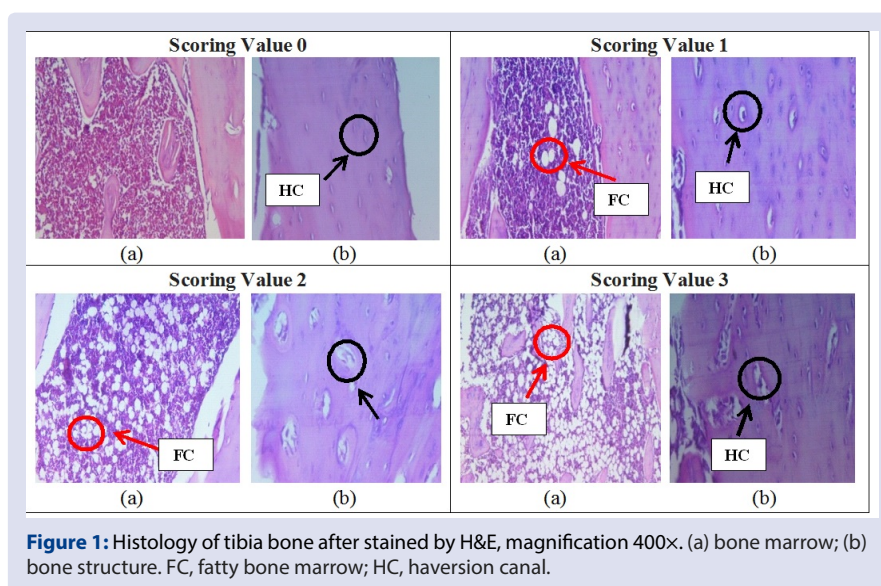

Figure 1: Histology of tibia bone after stained by H&E, magnification 400×. (a) bone marrow; (b) bone structure. FC, fatty bone marrow; HC, haversion canal.

Table 1: Qualitative analysis of dayak onion bulbs extract and cowpea extract.

Dayak onion bulbs extract		Cowpea extract	
Content	Result	Content	Result
Alkaloids	+	Alkaloids	+
Saponins	+	Saponins	+
Tannins	+	Tannins	+
Phenolic	+	Phenolic	+
Flavonoids	+	Flavonoids	+
Triterpenoids	+	Triterpenoids	-
Steroids	-	Steroids	-
glycosides	+	glycosides	+

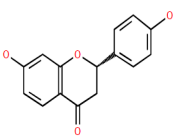**Table 2: Identification of the dayak onion bulbs extracts by LC-MS/MS.**
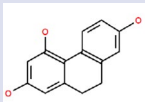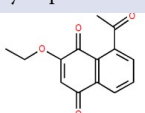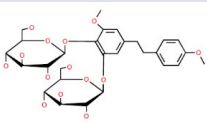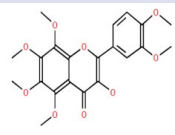
No	Component name	Observed m/z	Group compounds	Effects
1	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	229.0851	Phenanthrene	antioxidants (Guo, Wang, Wang, Kitanaka, & Yao, 2007), Cytotoxic (Zhao et al., 2018)
2	Cuspidatumin A	267.0621	Naftokuinon	The naphthoquinones group has a potential as an α-receptor ligand (Amelia et al., 2014)
3	Dendromoniliside E	621.2144	Glycosides	The bibenzyl group of its core structure is potential as the RE-β ligand (Waibel et al., 2009)
4	Liquiritigenin	257.0801	Flavonoids	Antiosteoporosis, Antiinflammatory, anticancer (Kim et al., 2008; Zhang et al., 2009)
5	Natsudaidain	450051	Flavonoids	Antiinflammatory (Matsui et al., 2009), cardioprotective (Benavente-García & Castillo, 2008) Anticancer (Moenes et al., 2017)

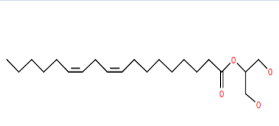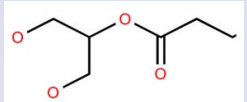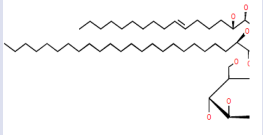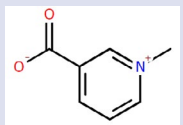**Table 3: Identification of the cowpea extract by LC-MS/MS.**
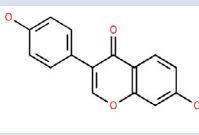
No	Component name	Observed m/z	Group compounds	Effects
1	2-Monolinolein	377.2655	Monoacylglycerides	Antifungal agents (Stoessl, Fisch, & Arditti, 1980)
2	Glycerol-β-steariate	381.2965	Monoacylglycerides	The common oil in plants (Xin, Wang, & Aisa, 2012).
3	Momor-cerebroside 1	866.6699	Glycolipids	important components of brain cells and nerves (Drijfhout & Morgan, 2010)
4	Trigonelline	138.0545	Alkaloids	Neuroprotective, antibacterial, and antiviral (Zhou J, Chan L, 2012)
5	Daidzein	255.0646	Isoflavone	Antiosteoporosis, anticancer (Sun et al., 2016)

Table 4: Tibia bone analysis.

Treatment	Average ± SD							
	Sham	Negative control	Positive control	Cowpea extract	Dose 1	Dose 2	Dose 3	Dose 4
Bone calcium concentration (g/ 100 g)	54.02* ± 0.65	52.04# ± 0.48	57.39#* ± 0.63	54.36* ± 2.37	57.06#* ± 1.63	58.75#* ± 0.87	59.73#* ± 1.98	57.25#* ± 0.64
Tibia bone weight (mg)	249.1* ± 3.00	244.1# ± 1.68	256.3#* ± 1.87	249.3* ± 2.76	254.5#* ± 2.78	257.0#* ± 2.04	260.5#* ± 3.36	256.9#* ± 1.44
Scoring bone marrow fat	0.25* ± 0.50	1.75# ± 0.50	2.75#* ± 0.50	0.75* ± 0.50	0.75* ± 0.50	0.50* ± 0.58	0.25* ± 0.50	0.50* ± 0.58

Note: Sham and control negative = CMC 0.5%, Positive control = ralokxifene 1.08 mg/200 g BW, Cowpea extract= 100 mg/200 g BW, Dose 1 = 36 mg/200 g BW; 100 mg/200 g BW, Dose 2 = 18 mg/200 g BW; 100 mg/200 g BW, Dose 3 = 8 mg/200g BW; 100 mg/200 g BW, Dose 4 = 4.5 mg/200 g BW; 100 mg/200 g BW *significant different to Sham ($p < 0.05$); #significantly different to negative control.

deposit decreased.[12] Estrogens deficiency may induce calcium loss by reducing intestinal calcium absorption and calcium reabsorption in the kidney.[13] The normal condition such as in the SHAM group, estrogen can stimulate calcium absorption in the small intestine, which can lead to increased the level of bone calcium.[14] The normal condition, estrogen can inhibit osteoclast activity through increased apoptosis of osteoclasts and stimulate bone formation so that the bone weight elevated. Giving raloxifene in rats showed that raloxifene can give significant effect to increased the level of bone calcium and bone weight. Raloxifene administration in Raloxifene group increased calcium absorption in the intestine and reabsorption of calcium in the kidney so that the level of bone calcium increased. Raloxifene can increase the bone weight because it can inhibit osteoclast activity resulting in a rapid reduction in bone resorption.[15] Administration of Dayak onion bulbs and cowpea extract combination at various doses could increase the level of bone calcium and bone weight in OVX rats. This was due to the compounds contained in the Dayak onion bulbs and cowpea extract combination. The content of isoflavone compounds especially daidzein in cowpea extract is an important compound that can increase the level of bone calcium and bone weight. Based on previous research, isoflavone compounds can increase the absorption of calcium in the small intestine through direct interaction with estrogen receptors in the epithelial cells of the intestine and decreased calcium excretion in the kidney.[16] Daidzein has an estrogen-like effect on the inhibition of bone resorption or stimulation of bone formation.[17] In addition, the liquiritigenin compounds contained in the Dayak onion bulbs extract has dual effects on the proliferation of bone cells, regarding the promotion of osteoblast differentiation and the inhibition of osteoclast differentiation in order to increase bone weight.[9]

The average of the scoring of bone marrow fat the SHAM group showed lower and was statistically differences with OVX group ($p < 0.05$). This suggests that the condition of hypoestrogen can increase because the estrogen-deficient condition may improve bone marrow fatness. The high bone marrow fatty condition can cause osteoblast proliferation and function to be disrupted so that bone formation can also be impaired.[3] Raloxifene group was given raloxifene had the highest scoring of bone marrow fat and was significantly different compared to all other groups. This is because based on previous research, raloxifene is not able to reduce bone marrow fatty, but can cause an increase in the number of adipocytes in the bone marrow postmenopausal women who have osteoporosis so that it can aggravate the condition of bone marrow fatty.[18] The group was given Dayak onion bulbs and cowpea extract combination have a tendency to decrease the average of scoring of bone marrow fat. This is due to the influence of daidzein compounds contained in the extract. Daidzein compound was known to inhibit bone marrow fatty by inhibition of adipogenic differentiation in bone marrow.[19-31]

CONCLUSION

Administration of Dayak onion bulbs and cowpea extract combination could increase significantly the level of bone calcium and bone weight of ovariectomy rats compared with the single dose of cowpea extract administration. In addition, Administration of Dayak onion bulbs and cowpea extract combination could reduce significantly the scoring of bone marrow fat of ovariectomy rats in dose 2, dose 3, and dose 4 groups compared with the single dose of cowpea extract administration.

ACKNOWLEDGEMENT

CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

ABBREVIATIONS

OVX: Ovariectomy; CMC: Carboxymethylcellulose; BW: Body Weight; IL: Interleukin; TNF: Tumor Necrosis Factor; ER: Estrogen Receptor; HRT: Hormone Replacement Therapy; SERM: Selective Estrogen Receptor; LC-MS/MS: Liquid Chromatography Mass Spectrometry.

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## GRAPHICAL ABSTRACT
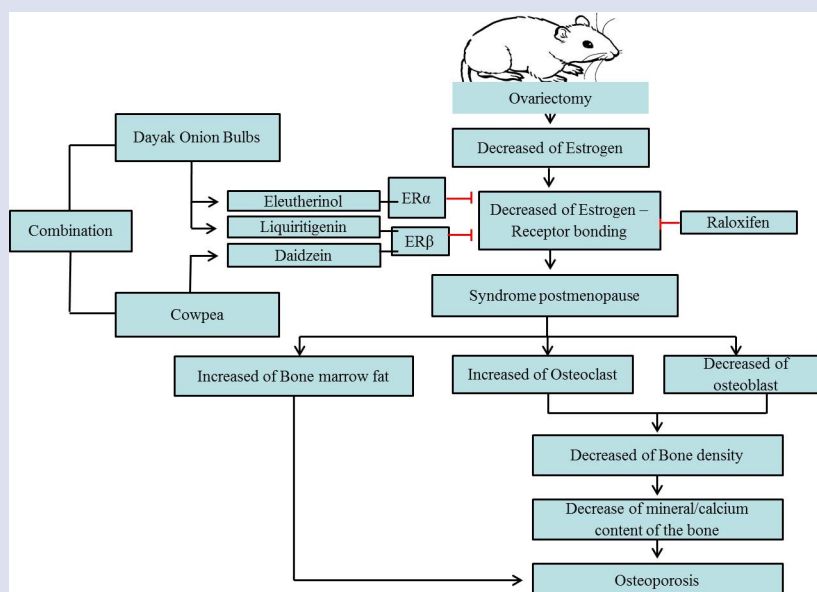
SUMMARY

The combination of Dayak Onion Bulbs and Cowpea extracts have been done in osteoporosis model rat. Rats were overiectomized and established postmenopause syndrome after 21 days post ovariectomy. Then the rats were treated by various doses of combination extracts and also raloxifes as positive control. 21 days after treatment by combination of extracts then the rats were sacrificed and tibia bone was isolated then proceed for the Atomic Absorption Spectrophotometer of calcium content evaluation and bone marrow fat determination. We found that ovariectomy could reduced bone calcium content and increased bone marrow fat, and the combination of extracts could recover the condition and have better result compared with single extract or positive control. As conclusion, The combination could be use as candidate for anti-osteoporotic drug and proceed to the next step in drug development process.

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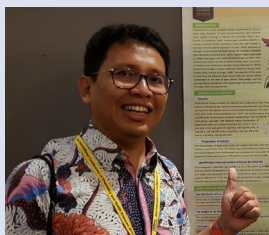
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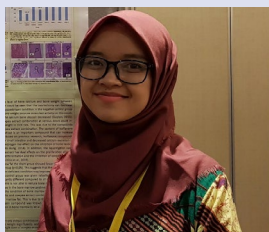
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