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RESEARCH ARTICLE

Pharmacokinetic Profile of Equol in the Combination of 70% Ethanolic Extract Dayak Onion Bulbs (*Eleutherine bulbosa* (mill.) Urb) and Cowpea (*Vigna unguiculata* (l.) Walp.) and their Effect on Postmenopause Syndrome

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Abstract

Alternative therapies for postmenopausal conditions are needed because of the high risks that outweigh the benefits of using hormone replacement therapy. Equol is metabolized from daidzein and is well-known as a supplement for postmenopausal conditions. Lately, liquiritigenin is also known to have a good effect on postmenopausal conditions. The aim of this study was to note the effect of the combination of *Eleutherina bulbosa* 70% Ethanol Extract (EEB) containing daidzein and *Vigna unguiculata* (EVU) containing liquiritigenin to obtain a better effect for uterine morphology, vaginal histopathology, and hot flushes. The other aim was to analyze the pharmacokinetic interaction that can affect the level of equol from EVU. Thirty-six female Sprague-Dawley rats were used in this study, 32 rats were ovariectomized (OVX) and 4 rats were Sham. Rats were divided into 9 groups: Sham group (CMC), negative group (CMC), positive group (raloxifene), EVU (100 mg/200g BW), EEB (18 mg/200g BW), The four various doses of combination EEB and EVU (D1-D4) were, EVU dosage was 100 mg/200 g BW and EEB were 36 mg, 18 mg, 9 mg, 4.5 mg/200 g BW respectively. Equol in rat serum was analyzed using HPLC/UV-V is in an isocratic condition. Observation of hot flushes was carried out every week, uterus weight wet and vaginal epithelium at the end of the treatment. The D3 and D4 combination decreased the average tail skin temperature 1.86±0.31°C and 1.83± 0.20°C respectively and the rectal temperature 0.58±0.49°C and 0.71±0.28°C ($P<0.05$). All groups except Sham did not increase the weight of the uterus. The D3 group increased vaginal epithelial thickness to 38.24 ± 6.47 µm ($P<0.05$). The combination did not significantly change equol pharmacokinetic parameters. The combination of *Eleutherina bulbosa* 70% ethanol extract (EEB) and *Vigna unguiculata* is better than EVU and EEB alone in reduced post-menopause syndrome in hypoestrogen rats without changing equol pharmacokinetic parameters.

Keywords: *Eleutherine bulbosa*, Equol, Hot Flashes, Postmenopause, *Vigna unguiculata*

1. Introduction

Menopause occurs mostly in a woman around 40-50 years[1]. Typical complaints that often occur in menopausal conditions are vasomotor symptoms such as hot flashes and night sweats, vulvovaginal atrophy (on an average experienced by 50% of postmenopausal women with dry vaginal symptoms, itching, pain during intercourse, and irritation)[2], osteopenia and osteoporosis, psychiatric disorders, sexual dysfunction,

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skin lesions, cardiovascular diseases, as well as metabolic disorders and obesity[3]. These conditions have an impact on home life, social (36%), and employment (36%) which will also have an impact on the quality of life of the woman[4].

The therapy commonly used in menopausal condition is the administration of estrogen therapy or hormone replacement therapy (HRT). The mechanism of HRT is like, endogenous estrogen which binds non-specifically to the estrogen receptor-α (ER-α) (mainly activates) or with estrogen receptor-β (ER-β) (mainly inhibits). Because of HRT not specifically binding to ER, it can cause the risk of endometrial cancer. Hence, the current menopause therapy approach uses Selective Estrogen Receptor Modulators (SERMs). The selectivity of SERMs to ER is that it can be an agonist in some tissues and it can be antagonistic in other tissues[5]. The ideal effect of SERMs has a therapeutic effect on vaginal atrophy, cardiovascular, lipid metabolism, hot flushes, and bone but has an antagonistic effect or prevents uterus and breast gland cancer[6].

Isoflavone compounds and their metabolites are known to have benefits in bone and postmenopausal heart health7. Equol is a metabolite of daidzein which is one of the non-steroidal isoflavones present in cowpea (*Vigna unguiculata* (L.) Walp.)8. Equol, especially the S-equol isomer is known to be a strong ligand for RE-β9. Other compounds that are also known to be selective to estrogen receptors are eleuterinol. Eleuterinol is a group of naphthoroquinone compounds that are one of the main components in Dayak onion (*Eleutherina bulbosa* (Mill.) Urb.) which are known to be selective towards RE-α10.

The synergism expected from the combination of equol in cowpea extract with selective RE-β activity and liquiritigenin in Dayak onion with RE-β agonist activity, induced the purpose for the current study. The aim of the combination study (*Vigna unguiculata* and *Eleutherina bulbosa*) was to obtain a better effect in uterine morphology, vaginal histopathology, and hot flushes. It is known that eleuterinol has an anti-bacterial activity, which affects the flora normal in the intestine and metabolises Daidzein to equol. Hence, pharmacokinetic equol levels and pharmacodynamic effects of the combination of 70% ethanol extract of *Eleutherina bulbosa* (Mill.) Urb. and *Vigna unguiculata* (L.) Walp. fruit was carried out.

2. Materials and Methods

2.1 Plant Materials and Chemical

Vigna unguiculata (L.) Walp. were collected from Bogor, Jawa Barat, Indonesia. Authenticated by the Center for Plant Conservation Botanic Gardens of Indonesian Institute of Sciences (Certificate of Determination no. B/355/IPH.3./KS/IV/2018). *Eleutherine bulbosa* (Mill.) Urb. bulb was collected from Palu, Sulawesi Tengah, Indonesia and it was authenticated by the Research Center for Natural resources of Tadulako University (Certificate of Determination No. 206/IPH.1.01/If.07/I/2016). The materials were extracted with 79% ethanol by maceration method in the laboratory of Indonesian Center for Spices and Medicinal Plants Research, Bogor. Equol ≥98% (Sigma-Aldrich), Raloxifene HCl (Ralista®, Cipla LTD, India), Aquadest (Brataco Chemical, Indonesia) and Ketamine HCl (Hameln®, German) were procured. All extracts used were 70% ethanol.

2.2 Animals and Treatments

The procedures were approved by the Ethics Committee of the Faculty of Medicine, the University of Indonesia with approval no. 0031/UN2.F1/ETHIC/2018). Female Sprague–Dawley rats (n=36; 80-100 g), aged 42 days, were purchased from Institut Pertanian Bogor. Animals were housed under standard laboratory conditions (temperature 25±2°C and 12 h natural light/dark cycle) and had free access to commercial pellet diet and water *ad libitum*. The rats were acclimatized to laboratory conditions for 1 week before the commencement of the experiment.

The rats were divided to be two groups before surgery. All rats were anesthetized with Ketamine HCl (120 mg/Kg BW) and received bilateral ovariectomy via a dorsal midline incision under for OVX group (n=32) and another group as a sham group was without surgery without (n=4). After 28 days recovery period, the OVX rats were randomly divided into 8 groups: negative group (CMC 0.5% p.o), positive group (raloxifene

1.08 mg/200 g BW p.o), extract of *Vigna unguiculata* (EVU) (100 mg/200g BW p.o), extract of *Eleutherine bulbosa* (EEB) (18 mg /200 g BW p.o), combination group D1-D4: dose of EVU were 100 mg/200 g BW and EEB were 36 mg, 18 mg, 9 mg, 4.5 mg/200 g BW p.o with four animals per group. Sham group was given orally CMC 0.5%. Treatment period was for 28 days. Blood was collected after one week of treatment for pharmacokinetic studies. On the day after the last dose, the rats were sacrificed under light anesthesia. The uterus and vagina were removed for morphology analysis and histological study.

2.3 Identification of Extracts by LCMS/MS

Ethanol extract (70%) of *Vigna unguiculata* (EVU) and *Eleutherine bulbosa* (EEB) were analyzed with LCMS/MS at the Research Center for Chemistry of Indonesian Institute of Sciences, Serpong.

2.4 Hot Flashes Analysis

Rats were restrained in a holder in a conscious state and the tail skin temperature (TST) and rectal temperature (RT) were measured for 1 h at 0, 30, and 60 minutes. Tail skin temperature was measured at the dorsal surface of the tail with an infrared thermometer. Rectal temperature was measured by inserting the thermometer, 2 cm into the rectum. The environment temperature was 25±2°C and the data were taken at 11.00 a.m- 02.00 p.m. Hot flashes were measured every week.

2.5 Morphology of Uterus Analysis

The uterus was removed, washed with NaCl 0.9% and wet weight was noted. Uterus of every group were compared on mathematic block paper to view the atrophy in uterus wall[11-13].

2.6 Histological Vaginal Epithelium Analysis

The vaginas were fixed in 10% buffered formalin for 48 h. All samples were embedded in paraffin and 5-µm thick sections were cut, mounted, and stained with hematoxylin and eosin (H&E) for microscopic analysis to view the proliferation of vaginal epithelium.

2.7 Pharmacokinetic of Equol Analysis

One week after treatment, blood was collected (0.5mL) via retro-orbital plexus from each rat at 0, 2, 4, 6, 8, 12, 16, 20, 24, 27, and 30 h. Serum was separated by centrifuging the blood for 10 min at 3000 rpm. Deproteinization was performed with methanol (1:2) then the sample was centrifuged for 10 min at 12000 rpm. The supernatant was used for analysis by HPLC under isocratic condition. Column C18 5µm, 4.6x150 mm was used. The eluent was mixture of 60% solvent A (0.05 % formic acid in water, v/v) with 40 % solvent B (Acetonitrile: methanol 20:80 v/v). The flow rate was 2 mL/min, run time was 14 min, and the injection volume was 30 µL. Ultraviolet detection was done at a wavelength of 230[14]. The stock solution was serially diluted to obtain a set of calibration standards. The peak area versus concentration was used to generate the calibration curves and the concentrations of equol in serum were determined from these curves.

2.8 Statistical Analysis

The statistical significance of differences between the groups was assessed with one-way ANOVA, followed by LSD post-hoct test and Kruskal-Wallis followed by Mann-Whitney analysis using SPSS software. p values less than 0.05 were considered as statistical significance.

3. Results

3.1 Identification Extract by LCMS/MS

The data showed that the five major components in EEB, they were 2,4,7-Trihydroxy-9,10-dihydrophenanthrene, cuspidatumin A, dendromoniliside E, liquiritigenin, and natsudaidain (Figure 1). Five major components were identified in EVU, they were 2-monolinolein, glycerol-β-stearate, momor-cerebroside 1, trigonelline, and daidzein (Figure 2).

3.2 Hot Flashes Analysis

The result showed that after 4 weeks of OVX, the rats TST and RT of the OVX group significantly increased (Table 1). This result also showed that after 4 weeks of treatment, EEB group, D1, D2, D3, D4 significantly decreased the TST compared to the negative group

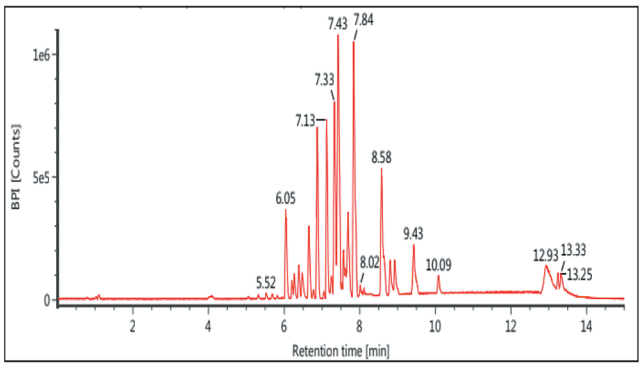

Figure 1. LCMS/MS chromatogram of *Eleutherina bulbosa* (EEB) components. Liquiritigenin with RT 6.88.

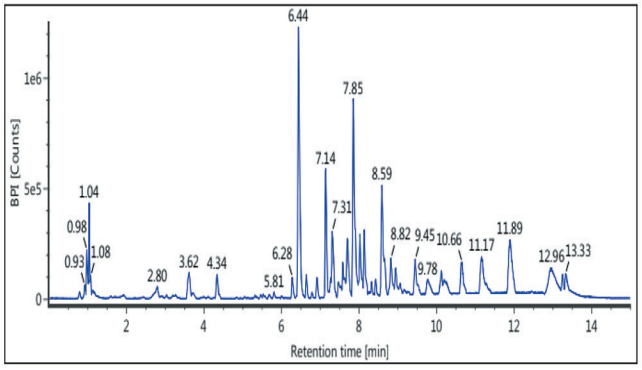

Figure 2. LCMS/MS chromatogram of *Vigna unguiculata* (EVU) components. Daidzein with RT 6.65.

(Table 2). The group that significantly decreased in rectal temperature after 4 weeks of treatment was D3 and D4 groups as compared to the negative control group (Table 3).

Table 1. Average of difference in tail skin surface temperature (TST) and rectal temperature (RT) of rats after 4 weeks OVX

Groups	4 weeks after OVX (°C)	
	TST	RT
Sham	0.22±0.34	0.24±0.26
OVX	1.44±0.11*	0.97±1.13*

Values are presented as the mean ± SD. * = p < 0.05 compared with negative control

Table 2. Average of difference in tail skin surface temperature (TST) of rats after 4 weeks treatment

Groups	1 week of treatment (°C)	2 week of treatment (°C)	3 week of treatment (°C)	4 week of treatment (°C)
Sham	0.23±0.49	0.12±0.32	0.08±0.51*	0.26±0.44*
Negative	0.40±0.45	0.27±0.34	0.50±0.46	0.78±0.53
Positive	0.31±0.45	0.45±0.41	0.44±0.42	0.94±0.56
EVU	0.74±0.39*	0.87±0.43*	0.99±0.33*	0.71±0.42
EEB	0.20±0.27	0.36±0.32	0.55±0.33	1.30±0.29*
D1	0.32±0.26	0.91±0.60*	1.19±0.37*	1.54±0.50*
D2	0.25±0.32	0.48±0.49	0.67±0.54	1.33±0.44*
D3	1.85±0.41*	1.01±0.47*	1.85±0.41*	1.86±0.31*
D4	0.78±0.47	1.28±0.33*	1.58±0.32*	1.83±0.20*

Values are presented as the mean ±SD. *=p< 0.05 compared with negative control, #= p<0.05 compared with sham.

Table 3. Average of difference in RT rectal temperature (RT) of rats after 4 weeks treatment

Groups	1 week of treatment (°C)	2 week of treatment (°C)	3 week of treatment (°C)	4 week of treatment (°C)
Sham	-0.08±0.63	0.07±0.51	0.042±0.60	0.18±0.49
Negative	0.21±0.50	0.07±0.38	-0.05±0.37	-0.02±0.48
Positive	0.17±0.54	0.04±0.30	0.08±0.37	0.18±0.51
EVU	0.23±0.24	0.0.8±0.40	0.05±0.39	0.28±0.34
EEB	0.05±0.21	-0.11±0.19	0.07±0.28	-0.12±0.29
D1	0.05±0.43	-0.47±0.54*	-0.19±0.46	0.17±0.56
D2	-0.09±0.19	-0.56±0.50*	-0.02±0.53	0.12±0.34
D3	0.29±0.51	-0.14±0.41	0.14±0.38	0.58±0.44*
D4	0.35±0.24	0.50±0.55	0.61±0.53*	0.71±0.28*

Values are presented as the mean ±SD. *=p< 0.05 compared with negative control, # = p<0.05 compared with sham.

3.3 Morphological Analysis of Uterus

Table 4 shows that, OVX significantly decreased the weight of uterus and treatment with raloxifene, EVU, EEB, or combination of EVU and EEB (D1, D2, D3, and D4) did not significantly increase the uterus weight as compared to the sham group (Figure 3).

Table 4. Wet uterus weight after 28 days treatment

Groups	uterus weight (mg/g BW)
Sham	1.60±0.45
Negative	0.37±002#
Positive	0.48±0.03#
EVU	0.34±0.07#
EEB	0.23±0.03#
D1	0.34±0.02#
D2	0.46±0.05#
D3	0.37±0.04#
D4	0.47±0.02#

Values are presented as the mean ±SD. #= p<0.05 compared with sham.

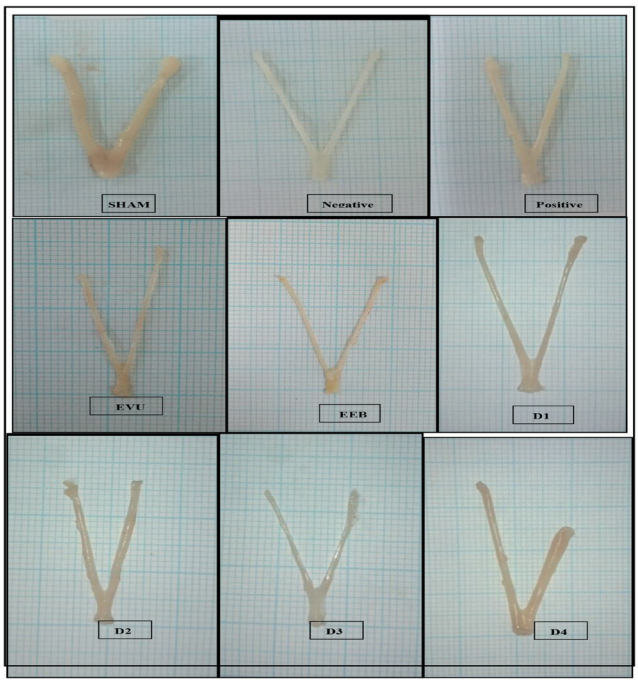

Figure 3. Morphology of uterus.

3.4 Histology of Vaginal Epithelium Analysis

Table 5 shows that OVX decreased the thickness of vaginal epithelium. The treatment with raloxifene increased the vaginal epithelium thickness of rats like the sham group. The result also showed that only D3 group increased the epithelium thickness significantly as compared to the negative control (Figure 4).

Table 5. Vaginal epithelium thickness after 28 days of treatment

Groups	vaginal epithelium thickness (µm)
Sham	43.11±4.09*
Negative	25.79±7.85*
Positive	44.13±8.92#
EVU	27.78±4.40*
EEB	22.18±6.63*
D1	22.53±7.89*
D2	27.28±8.39*
D3	38.24±6.47#
D4	23.99±5.22*

Values are presented as the mean ±SD. *=p< 0.05 compared with negative control, #= p<0.05 compared with sham.

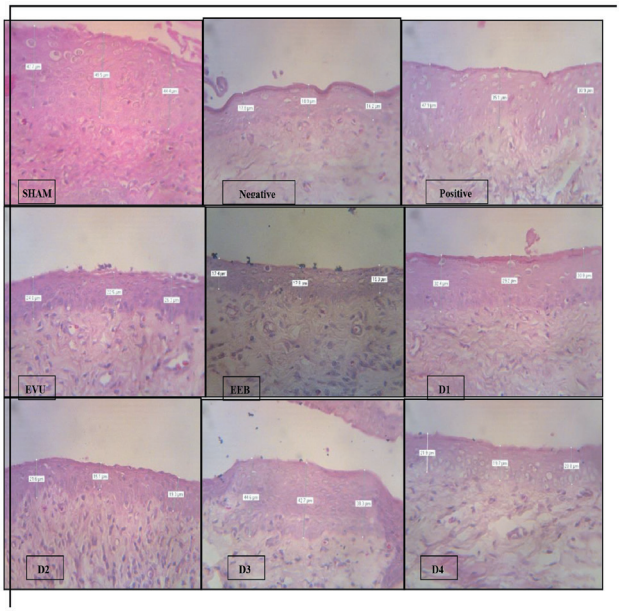

Figure 4. Histology of Vaginal Epithelium thickness (µm).

3.5 Pharmacokinetic of Equol analysis

Equol in serum sample was identified by comparison of the retention time with the respective standard (HPLC, UV-Vis). The retention time of equol is 11.3 min (Figure 5). The calibration curves were linear over the concentration ranges in serum (Figure 6). The regression equations were y=75,513x + 803,89 (r^2=0,9984). The pharmacokinetic study was carried out only for the EVU group and the D3 group.

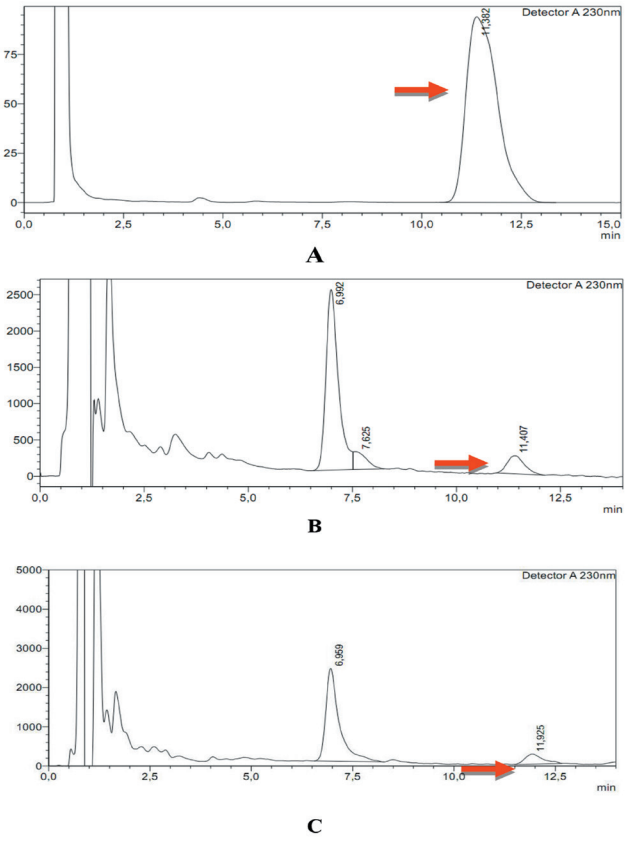

Figure 5. HPLC chromatogram of equol. **(A)** equol standard with RT 11.362 in dimethyl sulfoxide **(B)** Equol with RT 11.407 in rat serum given equol (*in vitro*), **(C)** Equol with RT 11.925 in rat serum given EVU p. o. Analyzed with HPLC/UV-Vis under isocratic condition. The column was C18.5µm, 4.6x150 mm. The eluent were mixture of 60% solvent A (0,05 % formic acid in water, v/v) with 40 % solvent B (Acetonitrile: methanol 20:80 v/v). The flow rate was 2 mL/min, the run time was set to 14 min, and the injection volume was 30 µL. Ultraviolet detection was done at a wavelength of 230 nm.

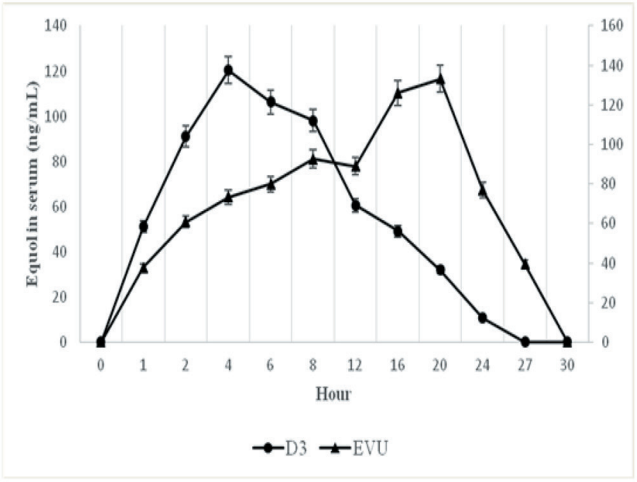

Figure 6. Equol concentration in serum

Table 6. Pharmacokinetic parameters

Pharma cokinetic parameters	Unit	Parameter value	
		D3	EVU
Vd	(mg)/(ng/ml)	0,012±0,006	0,005±0,002
Cl	(mg)/(ng/ml)/h	0,001±0	0,0007± 0,0001
$t_{1/2}$	h	9,381±7,614	4,864±2,165
k	/h	0,150±0,153	0,167±0,071
$AUC_{0-\infty}$	ng/mL*h^2	2069,417± 827,731	2783,052± 520,754
AUMC	ng/mL*h^2	38086,21± 30667,3	47306,93± 14621,95
$Cp_{max\ (ng)}$	ng/mL	173,791± 49,716	174,6201± 68,621
$T_{max\ (h)}$	h	5,2±2,28	16±6,928

EVU= EVU 100 mg/200g BW; D3=EVU 100 mg/200g + EEB 9 mg/200g BW

Results showed that the use of combination, statistically did not change the value of equol pharmacokinetic parameters although there was a tendency for changes in some pharmacokinetic parameter values such as $t_{1/2}$ and T_{max} (Table 6).

4. Discussion

Selective Estrogen Receptor Modulators (SERMs) are more likely to have minimal side effects compared to hormone replacement therapy, but their effectiveness in resolving all the symptoms of menopause is still not ideal. The ideal effect of SERMs is that it has a therapeutic effect on vaginal atrophy, cardiovascular, lipid metabolism, hot flushes, and bone but has an antagonistic effect or prevents uterus and breast gland cancer[6]. The aim of combination extracts in this study was to get a better effect and to find out pharmacokinetic interaction in the level of equol. The results of LCMS/MS showed that in EVU contains daidzein, a compound that will be converted into equol by intestinal bacteria. Equol in a previous study was found to reduce the incidence of hot flushes in humans compared with placebo[15]. The mechanism for decreasing the incidence of hot flushes by equol is through its binding to the β estrogen receptor in the brain.

Liquiritigenin in EEB is an agonist and selective to ERβ[16]. Liquiritigenin is known to be effective and safe in clinical studies, it is also reported to reduce the frequency and severity of hot flushes in postmenopausal women compared to placebo[17,18]. The observation of hot flushes in rats showed that the combination group was better to decrease hot flushes compared to single EVU. The combination group D3 and D4 showed a significant decrease in TST and RT, as equol and liquiritigenin binds to estrogen receptor β (RE-β)[9,16]. The equol mechanism in reducing the incidence of hot flushes is still uncertain, but previous research conducted shows that compounds that have high selectivity in RE-β can reduce the increase in basal temperature of rat skin surface caused by OVX and also as effective as administering estradiol[19,20]. The mechanism of liquiritigenin in reducing hot flushes is associated with its ability to directly bind to estrogen β receptors in neurons associated with thermoregulatory[21]. Liquiritigenin is also known to easily penetrate the blood-brain barrier[22]. The binding with estrogen receptors in neurons associated with thermoregulatory can cause inhibition of norepinephrine release which plays a major role in the incidence of hot flushes[23]. The positive control group using raloxifene did not show a decrease in the average temperature of mice, which complies with other previous studies[24].

Observation of uterus wet weight showed that administration of raloxifene, single or combination EVU in OVX rats did not cause thickening of the uterus. Raloxifene does not increase the risk of the uterus or endometrial cancer because it does not cause an increase in uterus wet weight, which is associated with estrogen antagonist effects in the uterus[25-28]. This study shows that a single or combination administration does not cause an increase in uterus wet weight. This shows that the treatment causes the endometrial proliferation process to stop. Atrophy occurs due to decreased estrogen levels which play a role in endometrial proliferation, and the majority of estrogen receptors found in the uterus are α receptors[29,30]. The content in EEB associated with estrogen receptors is liquiritigenenin and liquiritigenin activity more to the β estrogen receptor, so it does not cause an increase in wet weight[31]. Other studies have shown that EEB does not cause an increase in wet uterus weight[32]. Daidzein which is a phytoestrogen compound in cowpeas will undergo biotransformation to equol. Equol is a compound that is known to also have an activity to β estrogen receptors so it does not cause an increase in uterus wet weight[9].

The expected activity in the uterus is to have acted as an estrogen antagonist which is one of the ideal pharmacological effects of SERMs because it can reduce the risk of endometrial cancer[6]. The results of this study indicate that single or combination administration does not cause an increase in uterus wet weight, so it is estimated that EEB and EVU do not contain agonist compounds at estrogen receptors in the uterus and this is the expected effect in this study because it does not increase the risk of endometrial cancer.

This thinning of the vaginal epithelium is associated with a large number of estrogen receptors found in the vagina. Estrogen receptors (RE-α) are widely distributed in the vaginal epithelium and muscularis, and a decrease in the amount of estrogen can cause vaginal atrophy and the thinning of the vaginal epithelium[33-35]. Vaginal atrophy is a part of the menopause process that is known to have an impact

on the quality of life because it can cause vaginal dryness, pain, itching, and other such changes[35,36]. Estrogen therapy is known to be effective for treating vulvovaginal atrophy caused by hypoestrogenic conditions or due to increasing age in menopausal women[32]. The condition of decreasing estrogen levels in the blood causes a negative feedback mechanism that explains the occurrence of overexpression of RE-α[37]. The histopathological examination of the vaginal epithelium (Figure 3) showed that only the D3 group significantly increased the vaginal epithelial thickness like the sham group. These results indicate that the combination of extracts especially the D3 group has a positive effect on the vagina.

The results (Table 6) have shown that the use of combination statistically did not change the value of equol pharmacokinetic parameters although there was a tendency for change in some parameters of pharmacokinetic values (Figure 4). Parameters that tend to change are those with the maximum time value (T_{max}) to reach the maximum plasma concentration. The combination tends to shorten the T_{max} value (5.2 ± 2.28 hours) compared to the EVU group (16 ± 6.928 hours). The possible phase is affected to change the T_{max} value is the acceleration of equol absorption because there is no change in the maximum equol (C_{max}) concentration of the two groups. The results of this study also showed that the naphthoquinone content in Dayak onion which was previously thought to affect the bacterial population in the intestine did not affect the bacteria that biotransforms daidzein to equol. The complexity of the compound content in the extract makes it difficult to estimate which compounds are responsible for accelerating the absorption process and changes the T_{max} equol value. Further research needs to be carried out to observe the compounds responsible for increasing the absorption of equol.

5. Conclusion

Based on this study, the combination of EVU and EEB produces a better effect than the administration of a single EVU, especially the D3 group (EVU 100 mg/200g BW+ EEB 9 mg/200g BW) which can reduce the incidence of hot flushes, increase the thickness of the vaginal epithelium and does not cause thickening of the uterus. The combination also did not affect the equol pharmacokinetic parameters in hypoestrogen rats.

6. Acknowledgement

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7. Conflict of Interest Statement

We declare that there are no conflicts of interest that correlated with this publication.

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