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Ethanolic Extract of Papaya (*Carica papaya*) Leaf Exhibits Estrogenic Effects *In Vivo* and *In Silico*

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Abstract

The menopause women have the low level of estrogen in the body. The lack of estrogen changes physiological function in women's body that affects in health condition. *Carica papaya* L. leaf contains flavonoid quercetin which exhibits estrogenic effect. The aim of this study is to determine the estrogenic effect of papaya leaves extract (PLE) *in vivo,* and *in silico*. Papaya leaves were extracted by ethanol 70% maceration. The *in silico* study were done by molecular docking between quersetin and Estrogen Receptor (ERα and ERβ) to obtain the docking score. Based on this study, docking score of quercetin was almost similar to the native ligand of ER. The *in vivo* study was done as follow: 36 female rats Sprague Dawley divided into six groups. The groups are shame-ovariectomized (S-OVX), control ovariectomized (OVX), CMC-Na control (OVX+CMC-Na), positive control (OVX+Estradiol), and the PLE treatment groups dose 750 mg/kgBW (OVX+750mg/kgBW) and dose 1000 mg/kgBW (OVX+1000 mg/kgBW). Administrations of PLE were done in three weeks orally, while estradiol was administrated intraperitonially. The mammae and uterine were sliced for analysis. Based on the study, the treatment of PLE increased the number of mammae lobules and uterine weight as well as estrogen does. In summary, PLE can be developed as a source of phytoestrogens.

Keywords: *Carica papaya* L., phytoestrogen, estrogen receptor, mammae lobule, uterine

INTRODUCTION

Estrogen is one of the important hormones in female that regulates several physiological functions. Estrogen facilitates some actions, such as the development of uterus and vagina, regulates the reproduction and menstruation cycle, and stimulate the proliferation of ductal and mammae glands. The production of estrogen hormones are decreased because of menopause condition and causes some symptoms such as heart disease, osteoporosis, hot flashes, insomnia, sweating in the night, sexual disturbance, the vaginal dryness, and atherosclerosis (Mitchell, 2007)

The common therapy given to supply the needs of estrogen is by the Hormon Replacement Therapy (HRT). The hormone used in HRT facilitates the physiological regulation that the natural estrogen does.But, based on the several researches, HRT gives the risk in the stimulation of carcinogenesis process, lead to the enhancement of breast cancer, stroke, and blood coagulation cases (Jordan, 2004). Based on the data, 500 menopauses women had a cancer after consuming the HRT in England (Beral, 2003). Therefore, the alternative therapy for HRT that is safer and affordable for the society should be developed.

Phytoestrogens are the compounds found in plants that have a similar structure and function of estrogen. Phytoestrogen quercetin can be isolated from plants such as onions, apples, and grapes (Price, 1997). Quercetin also reduces LDL oxidation and decreases the synthesis of TAG (triacylglycerol) so that the formation of VLDL-TAG (Very Low Density Lipoprotein-triacylglycerol) was reduced (Gnoni *et al*., 2009).

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According to Canini *et al.* (2007), methanolic extract of papaya leaves contain quercetin 0.04 mg/g of 0.25 mg/g of dry leaves. Thus the development of papaya leaves as phytoestrogens through various studies *in vivo* and *in silico* are needed. In order to determine the affinity of the bond between quercetin and estrogen receptor, the *in silico* research by molecular docking was done. While the *in vivo* research was conducted to determine the effects of papaya extract on the uterus development and the mammary cells proliferation by calculating the mammary lobules.

MATERIALS AND METHODS

Materials

Papaya leaves were obtained from Bantul Yogyakarta, ethanol 70% *(E.Merck)*, CMC Na *(E. Merck)*, aquadest, formaldehyde (Asia lab), NaCl 0,9 % (PT Otsuka, Jakarta), β-estradiol*(Sigma)*, corn oil, cholesterol kit standard (DSI, Jerman), *Plain catgut sutures* 3/0 Meiyi® (HuaianMeiyi Medical Instruments Co., Ltd, Jiangsu), antibiotic Enbatic® (PT Erela, Semarang), Hematoksilinmeyer (Merck), Eosin (Merck), 3,3-*diaminobenzidine* (DAB) (Lab Vision), Phosphate Buffer Saline (PBS) (Sigma), PC, software PLANTS 1.1 manual, Co-Pendrivelinux-KDE, YASARA dan MarvinSketch.

In Silico Molecular Docking

Molecular docking was done to know the interaction between the estradiol as the reference ligand, and quercetin as the major component in PLE to the protein targets ERα (PDB ID: 3UUD) and Erβ (PDB ID: 3OLL). The structure of 17β-estradiol and quercetin were drawn by Marvin Sketch Sofware version 6.0.5. The structure of protein complex was downloaded from Protein Data Bank (PDB) sites (http://www.pdb.org/pdb/home/home.do), and protonized with MOE (Molecular Operating Environment) 2010. Molecular Docking was performed with PLANTS (Protein-Ligand ANT System) version 1.2. Chemplp was used as scoring function, and reference ligand was used for binding site definition. Native ligand of 3UUD and 3OLL (estradiol) were used for docking estradiol in each respective protein. Genistein from PDB 1X7R and 1X7J were used as reference ligand for docking quercetin.

The validation of binding was done by the calculation of RMSD (*Root Mean Square Distances*) of *heavy atom* (ligand) and ligand copy by showing RMSD value less than 2 Å. The analysis was done to get the docking score to show the interaction affinity between the ligand and the protein receptor. The affinity increase as the score docking value reduces. The visualization where done by MOE 2010.10 software to see the interaction between ligands and residues of amino acid residues on each protein.

Collection and Preparation of Papaya Leaves Extract (PLE)

Papaya leaves were taken from Bantul and was determined in Pharmacognocy laboratory of Biological Pharmacy Universitas Gadjah Mada. The powder of dried papaya leaves was extracted by maceration of ethanol 70% in 5 days then was concentrated by rotary vaccum evaporator.

Animals and Dosing

As many as 36 Female Sprague Dawley rats aged 6-7 weeks with body weight 86-118 grams were used in the study. The animals divided in to 6 groups of treatment. The groups are shame-ovariectomized (S-OVX), control ovariectomy (OVX), CMC-Nacontrol (OVX+CMC-Na), positive control (OVX+Estradiol), and the PLE treatment groups dose 750 mg/kgBW. (OVX+750mg/kgBW) and dose 1000 mg/kgBW (OVX+1000 mg/kgBW). Administrations of PLE were done in three weeks orally, while estradiol administrated intraperitonially, and necropted for mammae and uterine.

Histopathology Observation of Mammae

Animal test mammaes were taken during necropsy and stored in buffered formaldehyde for the making of paraffin preparate. Hematoxylin-eosin staining (HE) were used and histopathological observation was done under a binocular microscope (OLYMPUS® DP12 Microscope Digital Camera System) for the number of lobules of the mammary preparations.

Measurement of Uterine Weight

The uterine of each animal test was taken and weighed three times for replication. Uterine

weight percent is calculated based on mean of weight/100gBW.

RESULTS AND DISCUSSION

In Silico Molecular Docking

In Silico molecular docking aimed to predict the ability of quercetin interaction with estrogen receptors (ERα and ERβ). The interaction of estrogen-ER will give estrogenic effect that is important in women health (Rollerova and Urbancikova, 2000). The parameters used are docking score that represents the energy required to bind. The smaller the docking scores, the stronger bonding of protein with ligand. Docking score of ligands and ERα can be seen in Table I while ligands and ERβ can be seen in Table II.

Table I. Docking *score* between quercetin with Erα (3UUD)

Ligand	Score
Native Ligand (Estradiol)	-93.7913
Quercetin	-61.2598
	RMSD: 0.4193 Å

Table II. Docking *score* between quercetin with Erβ (3OLL)

Ligand	Score
Native Ligand (Estradiol)	-83.4502
Quercetin	-60.1127
	RMSD:0.7295 Å

Table III. Residues of amino acis interacts with ligands

Protein target	Ligand	Amino Acids	Interaction	Distance
Erα (3UUD)	Native Ligand (Estradiol)	Glutamate 353	H-donor	1.76
		Histidine 524	H-acceptor	1.84
		Arginine 394	H-acceptor	2.10
	Quercetin	Glutamate 353	H-donor	1.57
		Histidin 354	H-acceptor	2.19
		Arginine 394	H-acceptor	1.93
Erβ (3OLL)	Native Ligand (Estradiol)	Glutamate 305	H-donor	2.10
		Histidine 475	H-acceptor	2.10
	Quercetin	Glutamate 305	H-donor	1.38 and 2.12
		Histidine 475	H-acceptor	1.74

ER distributed in various tissues thus estrogen plays a role in regulating the survival of various body functions. ERα was found in the endometrium, the cells of the breast, ovary, and hypothalamus, whereas ERβ was found in the kidney, tissue endothelium, lung, and intestine (Hess, 2003). Mechanism of estrogen binding to its receptor in regulating various body functions is the occurrence of receptor dimerization. Furthermore, the binding of ER-ERE complex (Estrogen Receptor Element) occurs in the DNA of cells that are sensitive to estrogens such as MCF-7 cells (Bishop *et al*., 1997). The complex bond regulate gene transcription regulators such as the proliferation of ERα+, so it can stimulate cell proliferation and is able to cope with menopausal symptoms in women (Liu *et al*., 2001).

Based on the results of molecular docking, quersetin have almost the same score, which mean a similar affinity to native ligands on ERα and ERβ. This is shown on the score approaching quercetin against ERα and ERβ score against native ligand estradiol. The interaction between ligands and residues of amino acids on ERα and ERβ can be seen in Table III and Fig. 1.

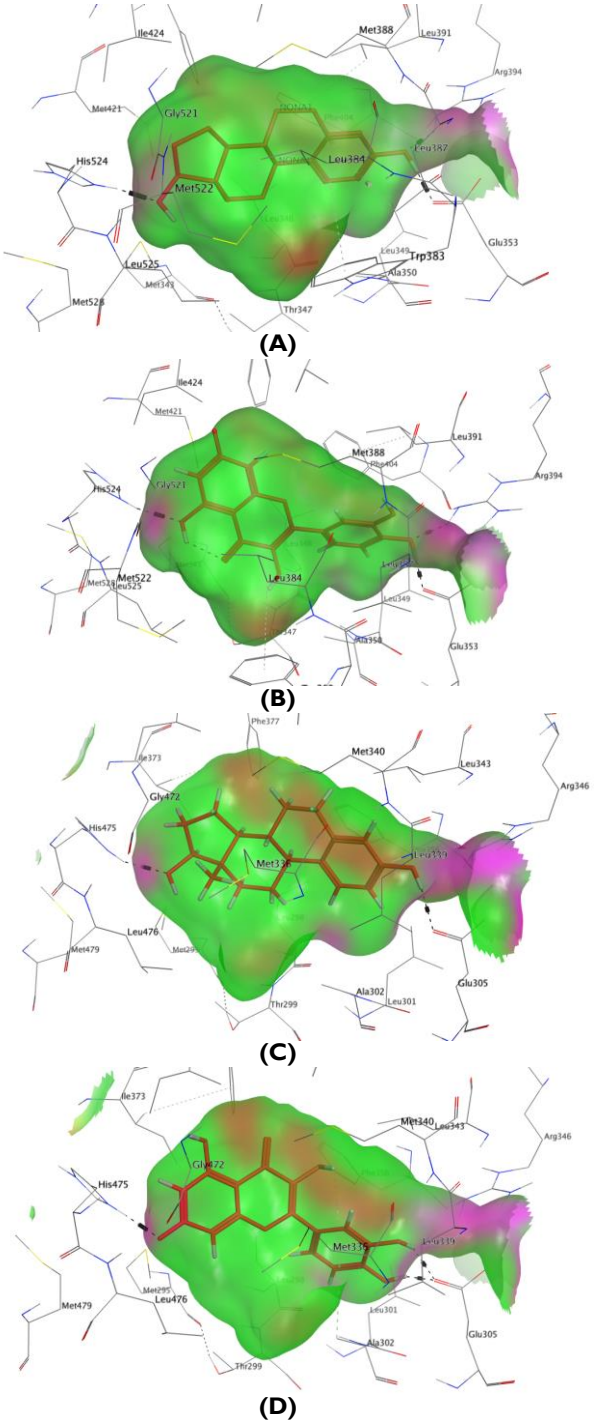

Figure 1. Visualization of Ligand Interaction between native ligand (estradiol) and quercetin with residue of amino acids on ERα and ERβ. Interaction between ligands and protein were visualized with MOE. The ligand is shown with red chain. Ligand interaction between estradiol with residue of amino acids on ERα (A), quercetin with residue of amino acids on ERα (B), estradiol with residue of amino acids on ERβ (C), quercetin with residue of amino acids on ERβ (D).

Histopathology observation of mammae

Histopathology profile of mammae was observed using Haematoxylin Eosin (HE) staining. In this study, the effects of PLE were observed by calculated the number of mammary lobules. Based on this study, number of mammary lobules was reduced after the OVX, due to the decrease of endogenous estrogen level. Administration of estradiol as exogenous estrogen increased the number of mammary lobule. The treatments of PLE also were able to increase the number of mammary lobules (Fig.2). In the development of mammae glands, estrogen stimulates stroma development and the accumulation of lipid (Guyton, 1995). Estrogen hormone plays its function in the regulation of mammary gland proliferation (Ruggiero and Likis, 2002). As the phytoestrogen has a structure mimics the estrogen, it could interact with estrogen receptor (ER) giving the similar effects in increasing the mammary glands proliferation characterized by the development of mammae lobules number.

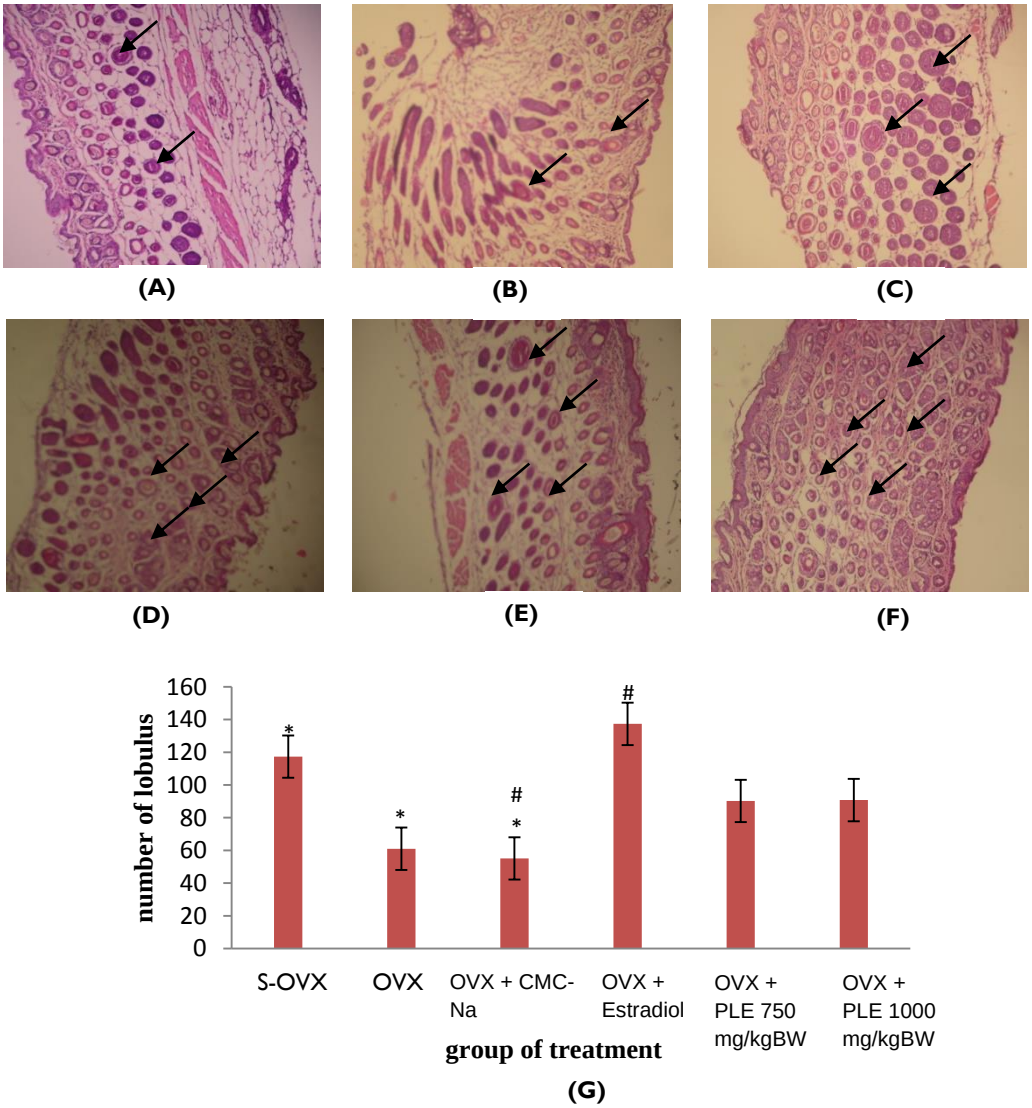

Figure 2. Effects of PLE on the mammary lobule number. The mammae of animal test were sliced during necropsy, and the paraffin preparate of mamae were made to be stained by Hematoxylin Eosin staining as decribed in the methods. Pictures shows the preparate of mammae on Non ovariectomy baseline group (NOVX) (A), the baseline group ovariectomy (OVX) (B), group OVX + CMC-Na (C), OVX + estradiol groups 2µg/days (D), group OVX + PLE 750 mg/kgBW (E), OVX + PLE group 1000 mg/kgBW (F). The arrows indicate the mammary lobule. Lobule is calculated based on the average number of the three field of view and three times replication. Statistical analysis one-way ANOVA

followed post-hoc Tuckey HSD level of 95% indicates that significant changes occurred between NOVX with OVX group, NOVX with OVX + CMC-Na, and OVX + CMC-Na with OVX + estradiol (G).

Measurement of uterine weight

Estrogen plays role in the proliferation of uterine. Uterine, a major target tissue for ovarian hormones, is composed of heterogeneous cell types (stroma, luminal epithelial, glandular epithelial, and smooth muscle), that undergo continuous synchronized changes of proliferation and differentiation in response to changes in levels of circulating estrogen (Martin, 1973). Estrogen stimulates uterine epithelial proliferation *in vivo* and is obligatory for normal uterine epithelial morphogenesis, cytodifferentiation, and secretory activity (Vallet *et al.*, 2004).

Estrogen elicits its effects via ER. These receptors are expressed in both epithelial and stromal cells of juvenile and adult uterus, suggesting that the effects of estrogen on epithelium and stroma of uterine are mediated directly through ER in these tissue compartments (Stumpf, 1976). The ovariectomy change concentration of circulating estrogen in the body so that uterine development will be inhibited. Thus, the uterine weight percent would reduce after ovariectomy. The average weights of the uterus per 100 grams of body weight of rats (gr/100grBW) were calculated (Fig.3). Based on this study, the treatment of PLE increased the uterine weight as well as estrogen does.

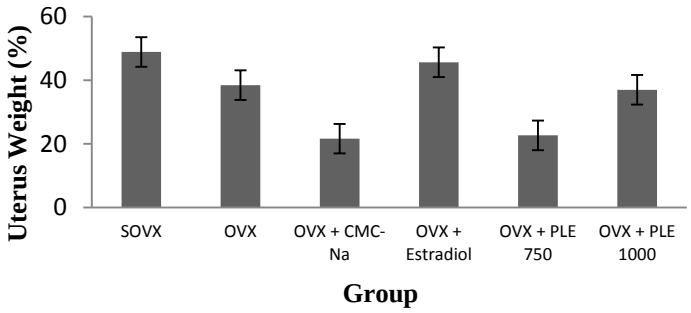

Figure 3. Graph of uterine weight. The uterine of animal test were sliced during necropsy and weighed. Uterine weight percent is calculated based on mean of weight/100gBW.

Based on *in silico* and *in vivo* done in this study, it is concluded that PLE can be a source of phytoestrogens. Further development needs to be done is extract standardization and PLE dose optimization which give the optimum estrogenic effect.

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