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Ethanol Extract of *Carica papaya* Leaf Can Increase Breast Milk in Lactating Rat

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Abstract

BACKGROUND: *Carica papaya* leaves (*C. papaya L*) have been used empirically and traditionally as a galactogogue, but their mechanism as galactogogue is still unknown.

AIM: This study aimed to analyze the effect of ethanol extract from papaya leaves on blood prolactin levels, prolactin receptor (prlr) gene expression, the number of breast alveoli and lobes of lactating rats.

METHODS: This *in vivo* true experimental study with a post-test control group design was conducted on 24 rats with the same lactating period. They were divided into four groups consisting of six rats each. The control group was given daily standard food, whereas the three treatment groups were, respectively, given additionally ethanol extract of 0.95 mg, 1.9 mg, and 3.8 mg/200 g BW/day from day 1 to day 14 of lactation. On day 14, all of the rats were sacrificed, blood prolactin levels were measured by ELISA, prlr gene expressions were measured using reverse transcription polymerase chain reaction, and numbers of breast alveoli and lobes were microscopically observed through staining histological specimens. A statistical analysis was carried out using one-way analysis of variance, Tukey's test, Games–Howell test, and path analysis at 95% confidence level.

RESULTS: Levels of blood prolactin levels, prlr gene expression, the number of breast alveoli, and lobes of all treatment rat groups were significantly above those of the control group ($p < 0.05$). The increases of all parameters were consistent; the most effective dose was 1.9 mg/200 g BW.

CONCLUSIONS: The *C. papaya* leaf ethanol extract had a galactogogue effect on lactating rats by increasing blood prolactin levels, prlr gene expression, and numbers number of breast alveoli and lobes.

Introduction

Breast milk has been commonly known as the best source of nutrition required for the growth and development of babies and children [1], [2], [3]. Breast milk is also a source of energy to increase immunity and intelligence; prevent babies from risk of allergies, reduce the risk of obesity, gastroenteritis, infection, asthma, and several types of cancer in children; and foster a psychological bond between mothers and babies [4], [5], [6]. For mothers, breastfeeding can reduce the risk of breast cancer and ovarian and endometrial cancer and accelerate the return of maternal weight to the state before pregnancy [7].

Prolactin is a polypeptide hormone that is responsible for lactation, breast development, and hundreds of other mechanisms required to maintain the lactation process, particularly in the growth and development of the mammary glands (mammogenesis), milk synthesis (lactogenesis) and maintenance of breast milk production (galactopoesis) [8], [9], [10]. During the lactation process, prolactin is needed to meet the nutritional components of breast milk. Prolactin can stimulate glucocorticoids in breast milk [11] proteins in mammary epithelial cells. It can also trigger the growth and development of alveolar cells [9]. Previous studies have indicated that alveolar cell culture added with prolactin, epidermal growth factor, and glucocorticoid can trigger the secretion of a β-casein signal. In addition, prolactin can also increase the absorption of several amino acids, glucose, and immunoglobulin A from the intestinal tissue [12].

Prolactin activity begins when it binds to the prolactin receptor (prlr) located on the surface of the mammary epithelial cell membrane. It possibly induces prlr dimerization, thereby activating the Janus Kinase 2 (Jak2) signaling pathway. Jak2 is a transcription factor that can activate the expression of signal transducer and activator of transcription 5 (STAT5), which plays a role in controlling the differentiation process of the mammary gland ducts [13], [14].

Prlrs are glycoproteins consisting of 598 amino acid residues encoded by genes located on chromosome 5. Prlr is located on the surface of the mammary epithelial cell membrane, pituitary gland, ovary, uterus, liver, and kidneys [15] Apart from Jak2, prlr can be activated by ligand mediation through tyrosine kinase signaling pathways such as Fyn, Raf2, STAT, mitogen activated protein kinases, triphosphate inositol, and VAV [16].

Increased levels of prolactin can be influenced by several factors, such as breastfeeding frequency, experience, breast milk production, drug use, and galactogogue [8]. Delayed onset of lactogenesis II is defined as the increase in lactation during the first 72 h postpartum. Risk factors for this case include primiparity, cesarean section, high body mass index, and stress during labor or prolonged labor. Nursing mothers with low milk production can be given galactogogue therapy in herbal and pharmacological forms [17], given that the herbal galactogogue at the age of 1–2 weeks postpartum is effective in increasing prolactin serum and oxytocin serum levels in nursing mothers [18], [19]. Currently, herbal galactogogue is more widely used as alternative therapy to increase breast milk production.

Galactogogues are synthetic materials or plant molecules or substances that are used to induce, maintain, and increase breast milk production [20], [21]. Papaya leaves have been used by the community to increase breast milk production. Papaya leaves contain alkaloids including carpaine and pseudocarpaine, enzymes (papain, chymopapain, and cystatin), tocopherol, ascorbic acid, tannins, nicotinic acid, saponins, peonidin, chlorogenic acids, coumarin compounds, phenolic compounds (caffeic acid, p-coumaric acid and protocatechuic acid) as the main phytochemicals and flavonoids [22], [23]. Flavonoid level in *Carica papaya* leaves is 126 mg/100 g, with quercetin and kaempferol as the main compounds [24]. Although, papaya leaves have been used to increase breast milk production, scientific evidence and an explanation for this increase have not been studied yet. This study aims to analyze the effect of papaya leaves ethanol extract on blood prolactin levels, prlr gene expression in mammary tissue, and an overview of the number of alveoli and the number of lobes of mammary tissue of lactating rats.

Methods

Research method and subject

This *in vivo* true experimental laboratory study with the post-test-only control group design was conducted using female Wistar rats obtained from the Laboratory of Animal Breeding Division, PT Biofarma, Bandung, Indonesia. The research was carried out from September 2019 to September 2020 in several laboratories, namely the School of Life Sciences and Technology ITB, Anatomical Pathology Laboratory of Universitas Brawijaya, and Animal Physiology Laboratory and Central Laboratory of Universitas Padjadjaran. This research obtained research permission and was approved by the Research Ethics Commission of Universitas Padjadjaran Bandung (no. 1340/UN6.KEP/EC/2019).

Experimental animals

The samples of this study were 24 female Wistar rats (*Rattus norvegicus*) aged 12–14 weeks, with a body weight of 200–225 g. The rats were simultaneously paired mated within one night, after synchronization of the estrous cycle utilizing the natural phenomena, namely Leeboth pheromone and Whitten effect, so that they had the same period of lactation [25]. The lactating rats were then divided into four groups consisting of six rats each, the control group and groups D1, D2, and D3 as treatment groups. The control group was given daily ordinary food, while groups D1, D2, and D3 were, respectively, given the ethanol extract of papaya leaves with dose 1 (0.95 mg/200 g BW/day), dose 2 (1.9 mg/200 g BW/day), and dose 3 (3.8 mg/200 g BW/day), from day 1 to day 14 post delivery. The rats were then anesthetized, by taking blood through cardiac puncture for measurement of the blood prolactin level.

C. papaya ethanol extract

Papaya leaves *(C. papaya L.)* were obtained from Manoko (Lembang), West Java plantation. The extraction process of papaya leaves for the test material was determined at the School of Life Sciences and Technology, ITB, Bandung, West Java, according a previous study [26].

Measurement of the prolactin level

Serum was obtained from 3 mL of rat blood which was put into a tube and allowed to clot for 2 h at room temperature or overnight at 4°C and then centrifuged for 15 min at 1.000 × g. Measurement of blood prolactin levels was carried out using ELISA, ELISA KIT Cusabio (# CatCSB-E06881r).

Measurement of prlr gene expression (prlr)

Prlr gene expression was measured using Real Time polymerase chain reaction (PCR), which measures the prlr gene expression calculated using relative quantification by comparing the prlr mRNA expression with the GAPDH housekeeping gene. The primary sequence of prlr gene used is forward

5'-TGCCAGCCTCGTCTCATAG-3' and reverse 5'-GGCTTAACACCTTGACCTGGATACTC-3'. The primary sequence GAPDH used is forward GAPDH: 5'-TGCCAGCCTCGTCTCATAG-3' and reverse GAPDH: 5'-ACTGTGCCGTTGAACTTGC-3'. The examination procedure is according to Reagent (Onestep quantitative reverse transcription PCR (qRT-PCR), Mytaq Bioline) and real-time PCR machine (PikoReal, Thermo Fisher Scientific). The number of cycles, temperature, and time are according to the previous optimization for each gene being examined. Reverse transcription was carried out at 45°C for 10 min. Initial activation (holding stage) was carried out at 95°C for 2 min and 40 PCR cycles: (cycling stage) denaturation at 95°C for 5 s. Annealing of prolactin was done at 59°C for 20 s, GAPDH at 57°C for 20 s, followed by a melt curve at 60°C–95°C. The level of gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method.

Histological examination of mammary tissue

The numbers of alveoli and lobes of mammary tissue were examined and calculated through histological examination with hematoxylin-eosin (HE) staining; then the numbers of alveoli glands and lobes at 400× magnification in five visual fields were read by two experts without prior knowledge of the group sample. Mammae samples were taken from the thoracic section.

Data analysis

The statistical methods used in this study include the Shapiro–Wilk test, one-way analysis of variance (ANOVA), Tukey's test, Games–Howell test, and path analysis at a 95% confidence level ($p \leq 0.05$).

Results

The effect of papaya leaves ethanol extract on prolactin levels

After confirming that the data of blood prolactin levels were normally distributed ($p > 0.05$ – Shapiro–Wilk suitability test), we used one-way ANOVA to analyze their mean differences. The result indicated that levels of blood prolactin in all treatment groups were significantly higher than those in the control group, especially groups D2 and D3 ($p < 0.05$), and the group with the highest levels was group D2, which had received a papaya leaf extract at a dose of 1.9 mg/200 g BW/day (Figure 1).

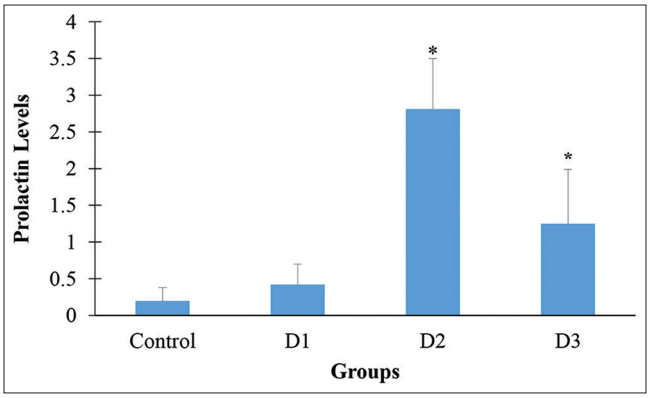

*Figure 1: The Effect of Papaya Leaves Ethanol Extract on Prolactin Levels. D1 = Dose of 0.95 mg/200 g BW/day, D2 = Dose of 1.9 mg/200 g BW/day, and D3 = Dose of 3.8 mg/200 g BW/day. The data are presented as means ± standard errors of means. *p < 0.05 compared with the control group*

The effect of papaya leaves ethanol extract on prlr gene expression (prlr)

The results of the one-way ANOVA test indicated addition of papaya leaf ethanol extract had an effect on the level of prlr gene expression in the four groups ($p = 0.000 < \propto$). Meanwhile, a post hoc analysis using the Games–Howell test indicated that the three treatment groups were significantly different from the control group, and the results indicated that the dose of papaya leaf ethanol extract that produced the highest prlr gene expression to increase the expression of the prlr gene in lactating rats was 1.9 mg/200 g BW/day. The analysis of prlr gene expression for the four groups is presented in Figure 2.

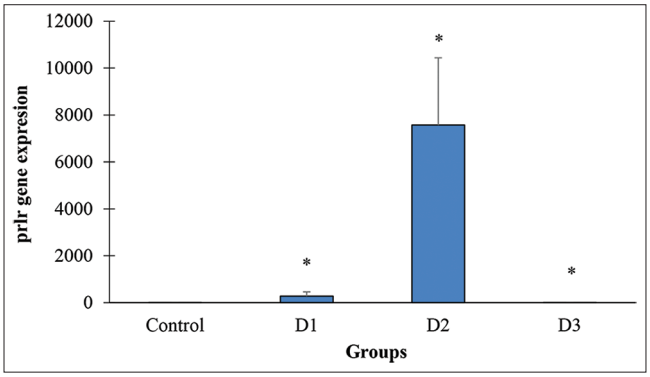

*Figure 2: The Effect of Papaya Leaves Ethanol Extract on Prolactin Receptor Gene Expression. D1 = Dose of 0.95 mg/200 g BW/day, D2 = Dose of 1.9 mg/200 g BW/day, and D3 = Dose of 3.8 mg/200 g BW/day. The data are presented as means ± standard errors of means. *p < 0.05 compared with the control group*

The effect of papaya leaves ethanol extract on the number of breast alveoli

The normality test was carried out using the Shapiro–Wilk test, and all data were normally distributed ($p > 0.05$). The one-way ANOVA test results

indicated that there was a significant difference in the mean number of alveoli in the four groups ($p = 0.000 < \propto$). Tukey's test indicated that the dose of papaya leaf ethanol extract that produced the highest prlr gene expression to increase the expression of the prlr gene in lactating rats was 1.9 mg/200 g BW/day to increase the number of alveoli in lactating rats. HE stain for breast alveoli is presented in Figure 3a. Analysis of the number of breast alveoli in the four groups is presented in Figure 3b.

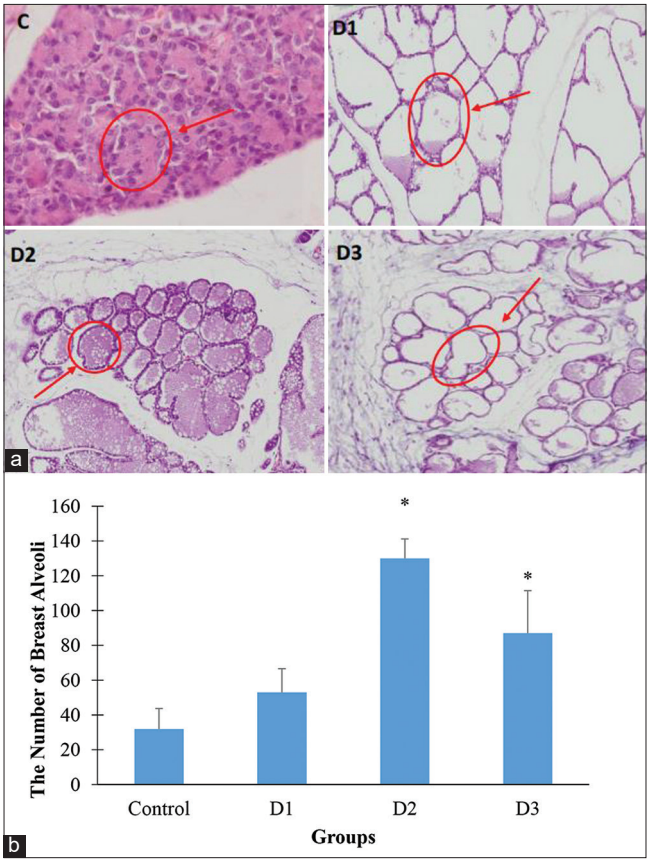

*Figure 3: The Effect of Papaya Leaves Ethanol Extract on the number of breast alveoli. D1 = Dose of 0.95 mg/200 g BW/day, D2 = Dose of 1.9 mg/200 g BW/day, and D3 = Dose of 3.8 mg/200 g BW/day. (a) A number of breast alveoli of mammary tissue were stained with hematoxylin-eosin staining, with 400× magnification. (b) The data are presented as means ± standard errors of means. *p < 0.05 compared with the control group*

The effect of papaya leaves ethanol extract on the number of lobes

The one-way ANOVA test results indicate that there is evidence that the mean number of lobes of the four observation samples ($p < 0.000$) in this test was carried out after the normality test using the Shapiro–Wilk test ($p > 0.05$). Tukey's test indicated that a dose of 1.9 mg/200 g BW/h was the highest dose that increased total protein levels in lactating rats. An HE stain for mammary lobes is presented in Figure 4a. Analysis of the number of lobes is presented in Figure 4b.

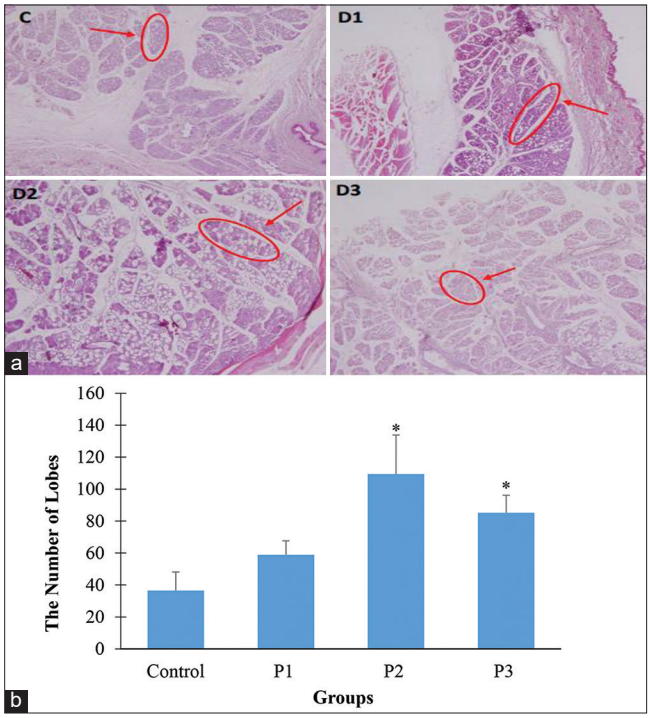

*Figure 4: The Effect of Papaya Leaves Ethanol Extract on the number of breast lobes. D1 = Dose of 0.95 mg/200 g BW/day; D2 = Dose of 1.9 mg/200 g BW/day; D3 = Dose of 3.8 mg/200 d BW/day. (a) A number of breast lobes of mammary tissue were stained with hematoxylin-eosin staining, with 400× magnification. (b) The data are presented as means ± standard errors of means. *p < 0.05 compared with the control group*

Path analysis of prolactin levels, prlr gene expression (prlr), number of breast alveoli and number of lobes

The path analysis indicated that there was an effect between an ethanol extract from the papaya leaf on prolactin levels of 0.182 ($p = 0.038$), with a percentage of effect of 18.2%. The effect between prolactin levels on prlr gene expression of 0.752 ($p = 0.000 < \propto$), with a percentage of effect of 75.2%. The effect of prlr gene expression on the number of breast alveoli is 0.554 ($p = 0.000 < \propto$), with the percentage of effect of 55.4%. The effect of the number of breast alveoli on the number of lobes is 0.809 ($p = 0.000 < \propto$), with the percentage of effect of 80.9%. The effect of prolactin levels on the number of breast alveoli was 0.903 ($p = 0.000 < \propto$), with the percentage of effect of 81.5%. The effect of ethanol extract from the papaya leaf on the number of breast alveoli was 0.315 ($p = 0.004$), with a percentage of effect of 31.5%, and the effect of ethanol extract from the papaya leaf on the number of lobes was 0.372 ($p = 0.002$), with a percentage of effect of 37.2%. Path analysis of prolactin levels, prlr gene expression, the number of mammary alveoli, and the number of lobes is presented in the Figure 5.

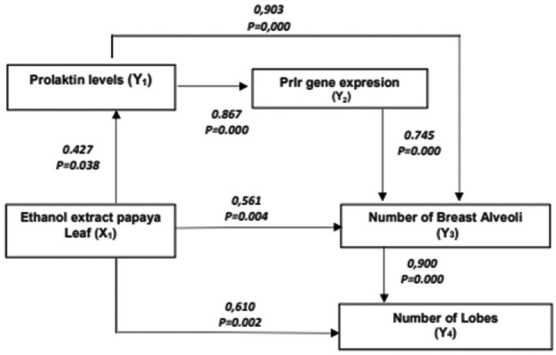

Figure 5: Path Analysis. The relationship model between ethanol extract from the papaya leaf (X_1), prolactin levels (Y1), prolactin receptor gene expression (Prlr) (Y2), alveoli in mammae (Y3), and mammae lobes (Y4)

Discussion

Research on papaya leaves as a galactogogue has not been conducted much; however, the papaya leaf extract has been used empirically as a nutraceutical because it contains nutritional components that can function as medicine. The flavonoids, glycosides, and vitamins in papaya leaves function as antioxidants; the minerals contained in these leaves can increase the mobility of the membrane transport system, and amino acids can function as protein precursors that can protect cells from free radicals [27].

The results of this study indicate that the ethanol extract of papaya leaves has a positive effect on increasing prolactin levels with the best concentration of 1.9 mg/200 g BW/day. Based on previous research, certain phytochemical components can have a galactopoietic effect. Alkaloids can increase breast milk excretion; isoflavones can increase the excretion of breast milk protein, fat, and lactose; polyphenols can increase milk protein production; and tannins play a role in the protein digestion process [28]. A group of alkaloid compounds, flavonoids, and saponins can increase prolactin levels in plasma by inhibiting the estrogen-progesterone [29].

In this study, the D3 ethanol extract did not cause an increase in response to prolactin levels, presumably because D2 is the maximum compensatory response to the control group, also known as chemical conditioning hormesis, and can activate the target cell signaling pathway at the low dose [30]. In addition, each metabolite has a different bioavailability in the target organs, especially phytochemical compounds that have low bioavailability, so that even low doses can affect the metabolism [31].

A flavonoid is a secondary metabolite that is synthesized in almost every part of the plant in response to the environment. If it is consumed, the flavonoid acts as an immunomodulator, regulating gene expression, which affects the bioactivity and bioavailability of the metabolite and plays an important role in cellular interactions [32]. A flavonoid is a type of phytoestrogen, which is a derivative compound from natural (plant) materials that have the same structure and function as the estrogen in the body [33].

Papaya leaves contain alkaloid compounds such as carpaine and pseudocarpaine, enzymes (papain, chymopapain, and cystatin), tocopherol, ascorbic acid, tannins, nicotinic acid, saponins, peonidins, chlorogenic acid, the coumarin compound, and phenolic compounds as the main phytochemicals and flavonoids [34], [35], [36]. Quercetin as a phytoestrogen can stimulate prolactin production [37]. The content of metabolite compounds in papaya leaves is the same as that in three types of plants that are widely used as a galactogogue, namely *Trigonella foenum-graecum* (fenugreek), *Asparagus racemosus* (shatavari), and *Moringa oleifera* (malunggay) [38]. *Asparagus* contains large amounts of saponins [39]. Whereas *Trigonella* and *Moringa* contain flavonoids [40], [41].

The path analysis indicated that ethanol extract from a *C. papaya* leaf had an effect on prolactin levels, and 18.2% of prolactin levels were affected by extract ethanol from the *C. papaya* leaf. Prolactin levels had a strong effect on prlr gene expression, and 75.2% of prlr gene expression was affected by prolactin levels. Prolactin levels have a strong effect on the number of breast alveoli, and 81.5% of the number of breast alveoli are influenced by the level of prolactin. Prlr gene expression had a strong effect on the number of breast alveoli, and 55.4% of the alveoli were influenced by the expression of the prlr gene. The number of breast alveoli has a strong effect on the number of lobes, and as much as 80.9% of the number of lobes is influenced by the number of breast alveoli. Extract ethanol *C. papaya* leaf has an effect on the number of breast alveoli, and as much at 31.5% of the number of breast alveoli were influenced by extract ethanol *C. papaya* leaf. Extract ethanol *C. papaya* leaf has an effect on the number of lobes, and as much at 37.2% of the lobes were influenced by extract ethanol *C. papaya* leaf. These results are in accordance with the mechanism of the action of prolactin and prlr.

Prlr expression is strongly influenced by the level of prolactin in the blood [42], so that when the prolactin level increases, it will trigger an increase in prlr gene expression. At the same time, it increases the activity of proliferation and differentiation of mammary epithelial cells so that the number of alveoli and lobe cells increases and, in turn, leads to an increase in milk production [43], [44]. Overall, this study still has limitations because it does not carry out the isolation and analysis of active secondary metabolites so that the mechanism of the active substance cannot be certainly explained.

Conclusions

The ethanol extract from papaya leaves has an effect as a galactogogue on the mechanism of increasing prolactin levels, prlr gene expression, the number of alveoli cells, and the number of lobes in mammary epithelial cells in lactating rats. Thus, this plant can be used as an alternative to a galactogogue herb to increase breast milk production.

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