RESEARCH ARTICLE

Polyscias scutellaria Aqueous Leaves Extract Increases Insulin Levels and Improves Mammary Gland Histology in Lactating Rats

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Abstract

BACKGROUND: Exclusive breastfeeding could be a protective step to avoid infant stunting. During lactation, several hormones are involved in milk secretion, including insulin. *Polyscias scutellaria* is a perennial plant which has traditionally been used to increase breast milk production. This study was conducted to examine the impact of *P. scutellaria* leaf extract on the mammary gland histology, expression of insulin in the mammary gland, as well as plasma insulin and the insulin receptor levels.

METHODS: Five female unmated rats (UR) and twenty female lactating rats were divided into five groups, namely UR, lactating rats only (LRO), lactating rats treated with Asifit (LRA), lactating rats treated with PSAE at a dosage of 250 and 500 mg/kg body weight (BW) (LRPSAE 250 and 500). Treatments were given orally for 14 days. The dams were sacrificed after the weaning stage (third week after parturition), and the serum, mammary gland, liver, and muscle were collected for further analysis.

RESULTS: The histoarchitecture of mammary gland between the LRA and LRPSAE groups were similar. The LRPSAE 250 group had higher plasma level and immunofluorescent expression of insulin than the LRA group. PSAE did not affect insulin receptor beta subunit (INSR-β) levels in both liver and muscle of lactating rats.

CONCLUSION: PSAE could be used as an herbal treatment to increase breast milk production by improving mammary gland histology and maintaining the mother's insulin levels.

KEYWORDS: *Polyscias scutellaria*, lactating rats, insulin, INSR-β

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Introduction

Breast milk is essential nutrition for a newborn due to containing colostrum, which plays as key role in the immune system. The World Health Organization (WHO) suggests that exclusively breastfeeding infants with only breast milk, without introducing any other foods or liquids, for a duration of six months offers several benefits compared to exclusive breastfeeding for a shorter period of 3-4 months followed by mixed breastfeeding.(1) Lactation is a complex and interconnected process between the mother and the infant, functioning as a two-person organ system and integrating neurobehavioral dynamics. The foundations of these dynamics are established during the mother's adolescence when cyclic stimulation from hormones, such as estrogen and progesterone, plays a crucial role in the development of breast ducts.

During pregnancy, estrogen, progesterone, insulin, cortisol, and thyroid hormones work together to facilitate

the growth and development of glandular tissue within the breasts. This process prepares the breasts for milk production, ensuring they are equipped to produce and supply the necessary nutrients for the newborn. The interplay of these hormones is essential in creating a favorable environment for lactation to occur successfully. (2) Insulin, a hormone that is closely linked to the onset of both obesity (3) and diabetes (4), plays a crucial part in the development of animal.(5) Insulin stimulated lipid lactose and protein synthesis during lactation.(6,7) Insulin also plays an essential role in lactation such as milk maturation, as well as secretory differentiation and activation.(8)

Nutrition and supplementation during breastfeeding are essential for the proper maturation and function of various cells and tissues, including the pancreatic islets responsible for insulin production.(5) Studies have demonstrated that maternal malnutrition during pregnancy and lactation can lead to diminished proliferative capacity and reduced vascularization of pancreatic islets in the offspring. Consequently, this can result in decreased insulin secretion and glucose intolerance.(9)

The use of herbal and pharmaceutical galactagogues, substances, or medicines thought to enhance lactation and promote milk production may be an option for therapy for women who have insufficient milk production and do not respond to lactation counseling or for adoptive parents trying to induce lactation. A variety of botanicals have been traditionally used as galactagogues in folk medicine. Some commonly mentioned ones include red raspberry leaf, *Asparagus racemosus*, fenugreek (10), alfalfa, anise, nettle, blessed thistle, and *Polyscias scutellaria*, among others. They can be a supplementary approach to support breastfeeding efforts when natural lactation is challenging. While their effectiveness may vary and scientific evidence is limited, they have been employed as natural remedies to support lactation in different cultures. It is essential to consult with healthcare professionals before using botanical galactagogues to ensure safety and suitability.(11)

The leaf extract of *P. scutellaria* has demonstrated promising evidence as a potent galactagogue due to its high content of phenolic acids, flavonoids, lignan, and terpenoids. These compounds contribute to their galactagenic properties, making them potentially beneficial for promoting lactation. (12) In this research, further investigation was conducted to examine the impact of *P. scutellaria* leaf extract on the mammary glands histology, expression of insulin in the mammary gland, as well as plasma insulin and the insulin receptor levels. This analysis aimed to provide a deeper understanding of how *P. scutellaria* aqueous extract (PSAE) may influence mammary glands histology, insulin production, and insulin signaling pathways, thereby shedding light on its potential mechanisms of action as a galactagogue.

Methods

P. scutellaria Collection and Extraction

Fresh *P. scutellaria* leaves were collected from a local traditional market in Malang, East Java, Indonesia. The number of *P. scutellaria* leaves specimen was 074/273A/102.7/2020. *P. scutellaria* leaves were extracted using a decoction method. Briefly, fresh leaves of *P. scutellaria* were added to distilled water with a ratio of 1:3, then boiled using low heat temperature (approximately 80^{o}C) for 45 minutes until the water turns into brownish-green and the volume decreased to 1/3 of the initiation volume.(12) PSAE was then kept at 4^{o}C for further analysis.

Animal Experimental Design

Twenty-five unmated female Wistar rats and five male Wistar rats (200±20 g, 8±2 weeks old) were used as animal models. The animals were acclimatized for a week, placed in standard cage, and given free food and water access. This experimental study procedures had been approved by Universitas Brawijaya Principles of Animal Laboratory (No. 093-KEP-UB-2021). After acclimatization, 20 nulliparous female rats were fertilized with five female rats. After fertilization, the male rats were removed, and the female rats were put individually. The gestation period took for 21-23 days long.(13) After parturition stage, the rats were divided into five groups (n=5 rats per group). Five unmated female rats were given distilled water (UR group), while lactating rats in the other four groups were treated with distilled water (LRO), Asifit supplement (Kimia Farma, Jakarta, Indonesia) (LRA), or 250 or 500 mg/kg BW PSAE (LRPSAE 250 and LRPSAE 500). All treatments were given orally for 14 days.(12) Asifit was used as a commercially available positive control drug which is commonly used to increase breast milk production. Asifit contains *Sauropus androgynus* leaves extract, vitamins B1, B2, B6, and B12. Asifit dose was determined according to human dose then adjusted proportionally based on the body weight of rats.

The blood serum of mother rats was collected weekly for 3 weeks during the lactation period. The blood was collected intravenously. After the treatment, the mother rats were euthanized using Ket-A-Xyl (Agrovetmarket, Senasa, Peru). Blood was collected from cardiac puncture,

then centrifuged at 5,000 rpm, 4°C for 10 minutes. The supernatant was collected as blood serum and stored at -20°C for further analysis. The mammary gland was collected and used for immunofluorescent analysis. The liver and muscle tissues were collected for the measurement of insulin receptor level.

Histological Analysis of Mammary Glands

The rat mammary glands were fixed with 4% phosphate buffer saline-paraformaldehyde (Santa Cruz Biotechnology, Texas, USA). The mammary glands were dehydrated and embedded in paraffin, then cut into 5 µm of thickness. The sections were stained using hematoxylin-eosin (HE) (14) and observed under a light microscope (CX23, Olympus, Tokyo, Japan). The mammary glands were analyzed descriptively according to the structure and cell constituents.

Measurement Insulin and Insulin Receptor Levels

Insulin and insulin receptor levels were measured by enzyme-linked immunosorbent assay (ELISA) methods. The insulin measurement was performed using Rat Insulin ELISA Kit (Catalog No.: ER1113, FineTest Biotech, Wuhan, China), while insulin receptor measurement was performed using Rat Insulin Receptor Beta Subunit (INSR-β) ELISA Kit (Catalog No.: ER1114, FineTest Biotech, Wuhan, China). Blood serum was used for insulin level measurement, while liver and skeletal muscle were used for INSR-β measurement. All ELISA procedures followed the manufacturer's instructions. The absorbance of the sample was measured at a wavelength of 450 nm.

Immunofluorescence

The expression of insulin in mammary gland tissue was examined using immunofluorescence method. Mouse monoclonal Alexa-Fluor-594-conjugated anti-insulin (2D11-H5) antibody (sc-8033 AF594, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in 2% bovine serum albumin was used for staining process.(15) The stained slides were then observed under a fluorescence microscope (Olympus, Tokyo, Japan). The intensity of immunofluorescence was measured by ImageJ 153 software (NIH, Bethesda, MD, USA) and presented as intensity/mm^2 (int/mm^2).

Statistical Analysis

Data obtained from ELISA and immunofluorescence was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) as post hoc test and presented as mean±standard deviation. The p-value<0.05 was considered significant. The data was analyzed with SPSS 23 (IBM Corporation, Armonk, NY, USA).

Results

Rat Mammary Gland Histology

Rats in the UR and LRO groups had different mammary gland histoarchitecture. Rats in the UR group had adipose tissue along the mammary gland, while rats in the LRO group had alveoli which contain apocrine secretion. Moreover, lactiferous ducts were found in the LRO group. Furthermore, mammary glands of the LRA and LRPSAE groups had tighter lobule area than those of the LRO group. The lobule area contained tubular acinar gland to produce milk. The histoarchitecture of mammary gland between the LRA and LRPSAE groups were similar (Figure 1).

Insulin and INSR-β Levels

The weekly insulin levels decreased during the first until third week of lactation. In the first until third week observation, insulin level of the LRPSAE 250 group was significantly higher compared with the UR group (p<0.05). LRPSAE 250 group also had the highest insulin levels than other groups. This result indicated that lactating rats had higher insulin levels compared with unmated rats. On the third week, the LRPSAE 250 group had slight decrease of the insulin level, while the LRA group had significant decrease of the insulin level. This result suggested that the LRPSAE 250 group had better maintenance of postpartum insulin levels than the LRA group (Figure 2A). However, the INSR-β levels in both liver and muscle did not significantly differ between groups (Figure 2B).

Immunofluorescent analysis showed that the LRO group had slight difference on insulin expression in the mammary gland compared with the UR group. This result suggested that during lactation, the level of insulin increased in mammary gland. Furthermore, the expression of insulin in mammary gland of the LRPSAE 250 group was higher than those of the LRO and LRA groups (p<0.05) (Figure 3).

Discussion

The mammary gland consists of two main tissue compartments, namely the epithelium and the stroma. Epithelium is composed of luminal epithelial cells, which form mainly with a ductal network and milk-secreting

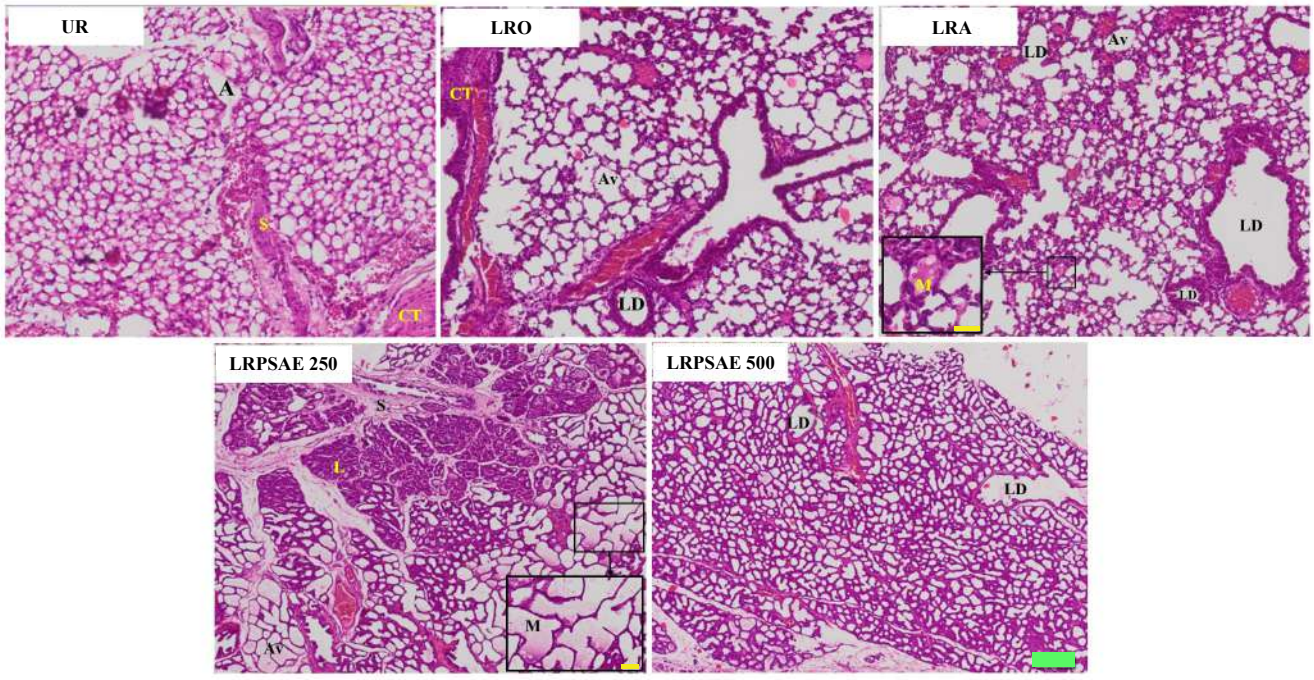

Figure 1. Histology of rat mammary glands. The mammary gland of the unmated rats consisted mostly of adipose tissue, while the mammary gland of the lactating rats showed the development of alveoli and lactiferous ducts. UR: unmated rats; LRO: lactating rats treated with distilled water; LRA: lactating rats treated with Asifit; LRPSAE 250: lactating rats treated with 250 mg/kg BW PSAE; LRPSAE 500: lactating rats treated with 500 mg/kg BW PSAE. A: adipose tissue; CT: connective tissue; S: septa; LD: lactiferous ducts; M: milk; Av: alveoli; L: lobule. Green bar: 200 µm; Yellow bar: 50 µm.

alveoli. Meanwhile, the stroma is composed of adipocytes, endothelial cells, fibroblasts, and immune cells. Adipocytes make up the fat pad in which the extensive system of ducts and alveoli are embedded.(16) Based on the histoarchitecture observation in the current study, mammary gland of unmated rats consisted of mainly adipose tissue. Meanwhile, lactating rats showed the development of lactiferous ducts, lobule, and alveoli.(17) The epithelium compartment of the mammary gland develops during lactation stage. At this stage, the terminal end bud in the mammary gland has the highest proliferation rate, leading to ductal elongation and branching. The mammary glands of the lactating rats also filled with lobule-alveolar structures that play an important role in milk production.(18,19) In this study, the mammary glands of the lactating rats treated with distilled water merely had less lobule-alveolar structures than those of Asifit or PSAE-treated lactating rats. These results suggested that PSAE treatment, especially at a dosage of 250 mg/kg BW,

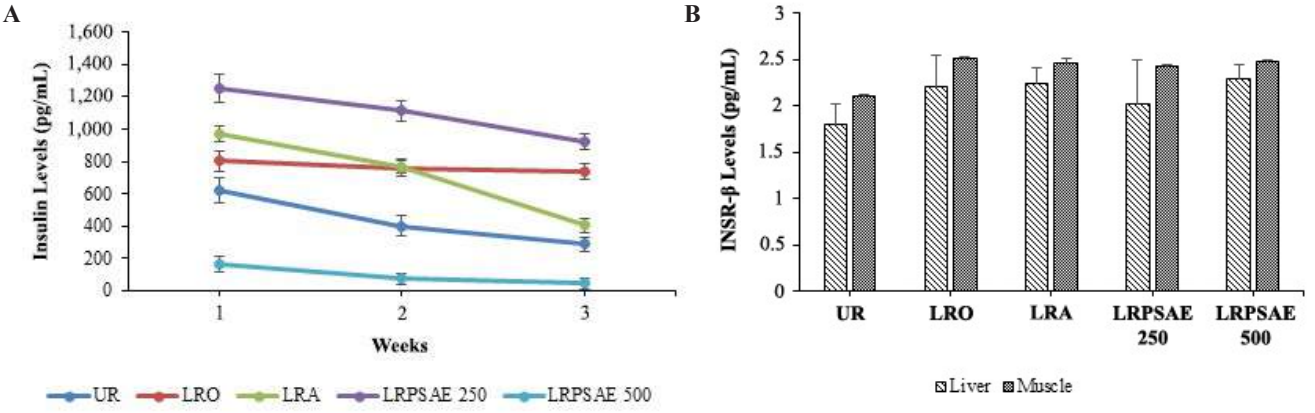

Figure 2. The insulin and INSR-β levels of the unmated and lactating rats. A: Weekly serum insulin levels; B: INSR-β levels in the liver and muscle. UR: unmated rats; LRO: lactating rats treated with distilled water; LRA: lactating rats treated with Asifit; LRPSAE 250: lactating rats treated with 250 mg/kg BW PSAE; LRPSAE 500: lactating rats treated with 500 mg/kg BW PSAE. The measurements were performed in triplicate.

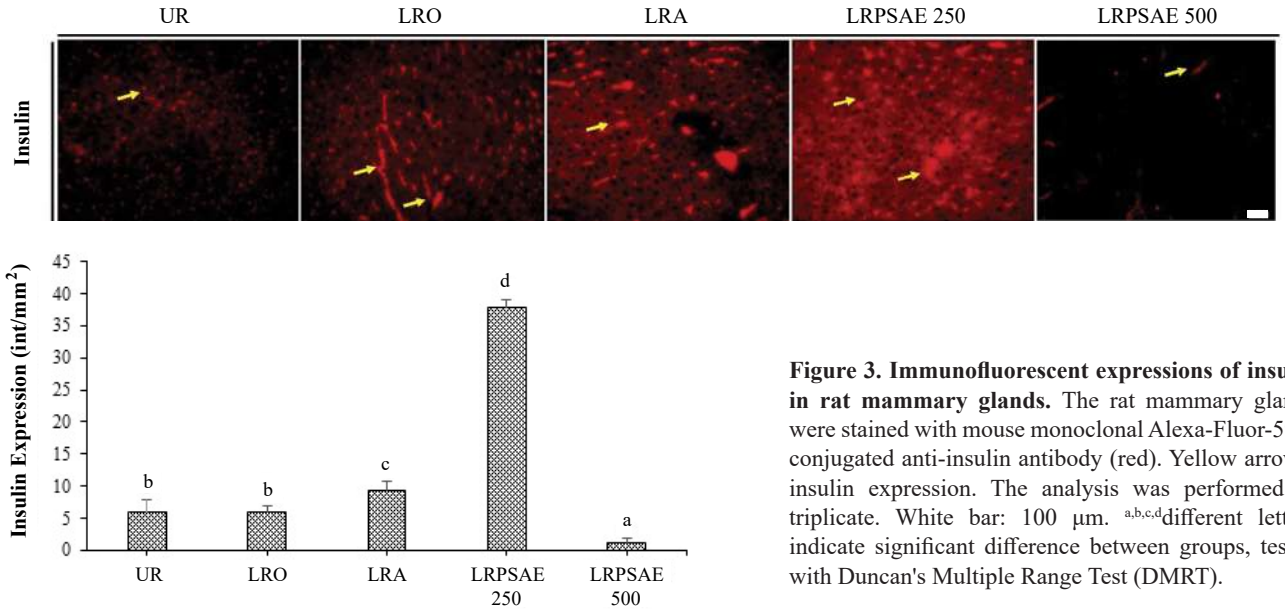

Figure 3. Immunofluorescent expressions of insulin in rat mammary glands. The rat mammary glands were stained with mouse monoclonal Alexa-Fluor-594-conjugated anti-insulin antibody (red). Yellow arrows: insulin expression. The analysis was performed in triplicate. White bar: 100 µm. [a,b,c,d]different letters indicate significant difference between groups, tested with Duncan's Multiple Range Test (DMRT).

had a similar effect on the mammary gland histoarchitecture as Asifit treatment.

In this study, the insulin levels in serum and the expression of insulin in the mammary gland were higher in lactating rats than in unmated rats. Higher serum insulin level and insulin expression level were seen in 250 mg/kg BW PSAE-treated lactating rats. This finding suggested that consumption of PSAE during lactation might be useful to increase breast milk production by increasing the insulin expression. The mechanism of action of *P. scutellaria* in increasing breast milk production may be similar with that of fenugreek.(7) Breast milk ejection reflex is affected by several hormones that regulate mammary gland development, including insulin.(20) Insulin is a polypeptide hormone secreted by pancreatic β cells which plays a key role in regulating glucose levels in the blood.(21) Insulin has been reported to be found in human colostrum at concentrations of 114-306 mU/L, then decrease to similar concentrations to blood insulin levels in day 5 postpartum of healthy mother.(22) Insulin might be involved in *de novo* synthesis of lipids, proteins, and carbohydrates in the milk secreted by the mammary gland. Insulin also has a direct role during lactogenesis, including stimulating the expression of genes involved in milk protein synthesis, including *Fasn*, *Acaca*, *Fabp3*, and *Csn2*.(7,8,23)

The plasma insulin levels of rats decrease with time until reaching the weaning stage. The plasma insulin levels are three times lower at peri-weaning phase compared with the early postpartum phase.(24,25) The current study found that 250 mg/kg BW PSAE could maintain the postpartum insulin levels. However, PSAE did not affect INSR-β levels in liver and muscle of lactating rats. The INSR-β levels has been reported to be increased 2.5-fold during lactation, stimulating transcription and translation of milk protein gene.(26) Insulin receptors have been known to modulate the expression of various genes involved in mammary gland differentiation, including genes involved in milk protein synthesis, milk fat globule formation, milk lactose synthesis, and milk lipid synthesis.(6)

P. scutellaria has been reported to contain various bioactive compounds, which help to increasing breast milk production, including quercetin, kaempferol, rutin, monoterpenes, and triterpenes.(12) Based on an *in silico* study, these bioactive compounds could interact with dopamine receptor and serotonin receptor which involved during lactogenesis process. *P. scutellaria* active compounds have an ability to inhibit dopamine D2 receptor and induce serotonin secretion by binding to serotonergic 5-HT2A receptor.(27,28) PSAE also plays a pivotal role in prolactin and oxytocin secretion in lactating rats.(12) The results of the current study give a new insight into the mechanism of action of *P. scutellaria* in increasing breast milk production via insulin pathway.

Conclusion

The mammary glands of PSAE-treated lactating rats had more lobule-alveolar structures compared with those of untreated lactating rats. Furthermore, the insulin levels in PSAE-treated lactating rats, especially in those treated with 250 mg/kg BW PSAE, were higher than that in untreated lactating rats. PSAE could also maintain the mother's insulin levels. Taken together, PSAE could be used as an herbal

treatment to increase breast milk production by improving mammary gland histology and maintaining the mother's insulin levels.

Authors Contribution

B, SBP, and K were involved in planning, supervised the work, drafted the manuscript, interpreting the results and critical revision of the manuscript, TH and NP performed the measurements and manuscript preparation, SNA and MFA processed the experimental data, performed the analysis, drafted the manuscript, performed the data statistical analysis and designed the figures. All authors discussed the results and commented on the manuscript.

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