The Effects of Water Fraction of Bitter Melon (Momordica charantia) Leaf Extract in Mammary Gland Development of Balb/c Mice (Mus musculus) with Histological and Molecular Biological Analysis of Protein Approaches

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Abstract—Recently many breast-feeding mothers use chemical drugs to ease milk secretion, while the said drug could cause side effects if consumed in a long time. The problem could be solved by using natural medicine like bitter melon leaf because it contains charantin which is characteristically identical to phytosterol [6]. This study aimed to discover the potention of water fraction of bitter melon leaf extract to stimulate mammary gland development in Balb/c mice. The development of mammary gland was observed through histological approach by calculating the number of cells in mammary gland, and through molecular biological analysis of protein approach by using SDS-PAGE. The histology of mammary gland showed that the administration of the extract with concentration of 11,200 ppm had the highest number of cells, followed by 5,600 ppm; 22,400 ppm and 16,800 ppm. The result of SDS-PAGE on isolated mammary gland protein showed that the molecular weight of the proteins in the controlled treatment were 2, 5, 8, 10, 12, 19, 34, 40 and 49 kDa. In treatment with concentration of 5,600 ppm there were only 10, 12, 34, 40 and 49 kDa. In treatment with concentration of 11,200 ppm there were similar kinds of proteins with the controlled treatment, only without the 8 kDa protein. In treatment with concentration of 16,800 and 22,400 ppm, there were only 2, 5, 10, 12 kDa. The conclusion of this study was water fraction of bitter melon leaf extract could affect the development of mammary gland in Balb/c mice.

Keywords—bitter melon leaf, histology, mammary gland, mice, sds-page

INTRODUCTION

Breast milk is the best natural nutrition for babies because it contains energy and substances required during the first six months of a baby's life [1]. Breast milk is good for babies because it can be obtained naturally, easily digested by the baby, and it contains a complete nutrition for baby's growth and free of contaminants from environment because it is given directly from mother to baby [2]. Breast milk can increase endurance and intelligence, protect children against allergies, improve vision and eloquence, promote good jaw development, reduce the risk of diabetes and cancer in children and support child's motor development [3]. Thus, breast milk is very important since it has many benefits. Unfortunately, although the vast majority of new mothers are able to breastfeed, some are unable to breastfeed because of various factors. The main factor is the inability of the mammary gland to produce enough milk.

Various efforts have been done to ease the milk secretion. Drugs such as reseprin and chlorpromazin are often used to stimulate lactation. Some foods and beverages are made of plants known for their ability to increase milk production [4]. The use of natural materials from various kinds of plants as medicine is more preferred by the public because it generally causes fewer side effects than that of chemicals [5].

One of the plants which can be used to increase milk production is bitter melon. Its leaf contains charantin, a steroid saponin. Charantin has characteristics similar to phytosterols [6]. Phytosterols have the ability to enrich the milk through prolactin reflex controlled by the anterior pituitary, thus stimulating the alveoli to produce milk, and it also acts as a precursor to the formation of steroid hormones, like estrogen [7]. Estrogen plays a role in the development of female reproductive organs in most species and increases the number of estrogen receptors in those organs. Estrogen also stimulates the proliferation of mammary gland alveoli [8]. If the proliferation of alveoli increased, the amount of milk product would likely to increase. The growth of mammary gland can be observed through histological section and molecular biological analysis of membrane protein of its cells.

Reference [9] reported that bitter melon fruit juice has a lactogenic activity by increasing the number, diameter and function of alveoli. However, scientific studies on lactogenic properties of bitter melon leaf are still lacking. Therefore, the purpose of this study is to evaluate the effects of water fraction of bitter melon leaf in mammary gland development by determining histological changes and molecular weight of membrane proteins of mammary gland cells.

MATERIALS AND METHODS

1. Materials

a. Extract preparation

Bitter melon (*Momordica charantia*) leaves were collected from the farming region of Kunir Village, Lumajang, East Java. The leaves were washed in running tap water and shade dried for a week until dry. 2.24; 4.48; 6.72 and 8.96 g dry leaves were weighed precisely using analytical balance and poured into 40 mL of boiling water for 15 minutes. The boiled mixture was allowed to cool down before it was filtrated using tea strainer. The filtrated liquid was the extract of bitter melon leaves. The concentrations of resulted extract were 5,600; 11,200; 16,800 and 22,400 ppm.

b. Animals

The study was performed on 25 healthy adult female Balb/c mice (*Mus musculus*) at the age of 10 weeks weighing 20-22 g. The mice were obtained from Malang Murine Farm, Malang, East Java. They were kept under controlled laboratory conditions with a 12 h dark and 12 h light cycle, at 25^{o}C and with free access to tap water and mouse pellets.

2. Methods

a. Experimental design

The mice were divided into control group (K) treated with aquadest and treatment groups (T1, T2, T3 and T4) treated with different extract concentrations: 5,600; 11,200; 16,800 and 22,400 ppm, respectively. Both aquadest and extract were adminitered orally using stomach tube at standard volume (0,5 mL/day/mouse). Following 14 days of treatment, the mice were humanely sacrificed at day 15 to excise the mammary glands.

b. Histological study

The mammary glands were obtained and fixed in 10% formalin solution, dehydrated in ascending series of ethanol, cleared in xylene and embedded in paraffin. Sections were cut out at 6µm thickness with microtome and stained with hematoxylin and eosin stain. The sections were mounted and examined with light microscope. Image acquisition was done using cameroscope connected to a computer interface and mounted on the Olympus binocular research microscope.

c. Proteomic study

1. Protein isolation

Mammary gland cell membrane proteins were extracted by the method of [10]. The excised mammary glands were crushed in PBS using mortal and pestle. The crushed tissue sample was transferred into Falcon tube, added with 3x the sample volume of PBS and vortexed until homogenous. The suspension was centrifuged at 3,000 rpm for 10 min; the supernatant was discarded, PBS was added in 3:1 ratio to the pellet volume and the suspension was then vortexed until homogenous and centrifuged at 3,000 rpm for 10 min. The supernatant was discarded, PBS-T was added in 5:1 ratio to the pellet volume and the suspension was then subjected to one round of vortexing (10 min), two rounds of sonication (10 min each) and two rounds of centrifugation (10 min each) at 12,000 rpm at 4°C. The pellet containing organels and disrupted cell membranes was now discarded; whereas the resulting supernatant containing soluble proteins was transferred into Eppendorf tube, added with an equal volume of cold absolute ethanol and put into refrigerator over night to precipitate the proteins. The sample was then centrifuged at 10,000 rpm for 10 min at 4°C; the ethanol was discarded and the remaining pellet was dried by keeping the tube open over night and then resuspended by adding an equal volume of Tris-Cl. The isolated proteins were kept in freezer at -20°C.

2. Protein separation

Protein samples were separated by SDS-PAGE. The samples were mixed with an equal volume of SDS sample buffer and heated to 100°C for 5 min. Separation of membrane proteins was carried out in Bio-Rad vertical electrophoresis system, using 4% stacking and 12% separating polyacrylamide gels and Tris-glycine-SDS pH 8.8 as electrode buffer. The volume of sample injected to each well was ± 20 µL. The electrophoresis was run at 130 V and 60 mA for 90 min (until the bromophenol blue tracking dye reached the bottom of the gel). The protein lanes were visualized using Coomassie Brilliant Blue R-250 staining for 30 min, then the gels were shaken in acetic acid destaining over night.

d. *Statistical analysis*

Statistical analysis was performed using one-way analysis of variance (ANOVA). Defferences were considered to be statistically significant when P value was <0.05. All statistical analyses were performed with SPSS software.

RESULTS AND DISCUSSION

Effects of water fraction of bitter melon leaf extract on histological changes of mammary gland tissue was presented in Fig. 1. The mammary tissue of the group receiving 11,200 ppm extract exhibited the most number of mammary gland cells (Fig. 1C). The calculation of cell numbers of mammary tissue presented in Table 1. The statictical analysis presented in Table 2 showed that the number of cells in mammary gland in group receiving 11,200 ppm extract (T2) was significantly increased ($p < 0.05$) as compared to the control group and other treatment groups.

Table 1. Effect of Momordica charantia on Number of Cells of Mammary Gland in Balb/c Mice

Groups N = 5	Number of mammary tissue cells ($\bar{X} \pm SD$)
Control	202,40 ± 14,93[a]
T1 (5,600 ppm)	219,40 ± 10,71[b]
T2 (11,200 ppm)	249,00 ± 40,76[c]
T3 (16,800 ppm)	190,20 ± 43,99[a]
T4 (22,400 ppm)	191,00 ± 18,88[a]

N = number of animals per group; same letter within column means non-significant difference ($p > 0.05$), different letter within column means significant difference ($p < .05$)

Table 2. One Way Anova

	Sum of squares	df	Mean square	F	Sig.
Between groups	12096.800	4	3024.200	3.524	0.025
Within groups	17163.200	20	858.160		
Total	29260.000	24			

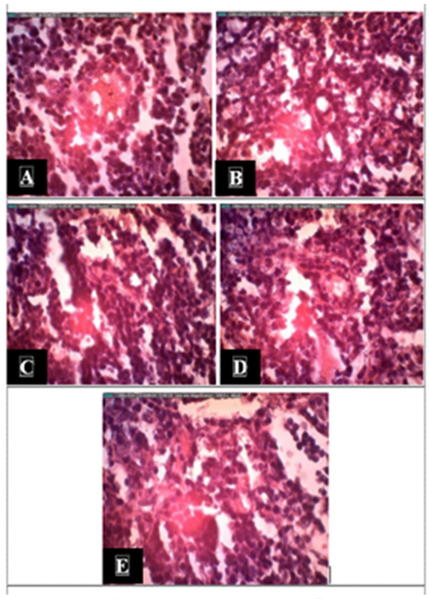
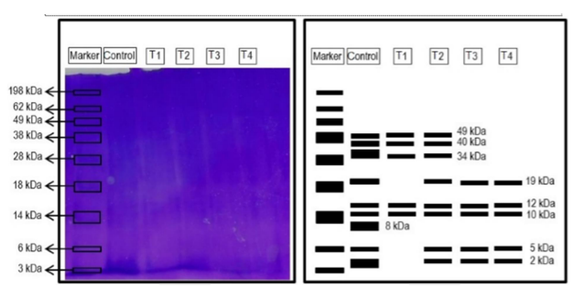


Fig. 1. Sections of mammary tissue of Balb/c mice in (A) control group; (B) group receiving 5,600 ppm extract; (C) 11,200 ppm extract, (D) 16,800 ppm extract and (E) 22,400 ppm extract.

Effects of water fraction of bitter melon leaf extract on the diversity of membrane proteins isolated from mammary gland presented in Fig. 2. The result of SDS-PAGE in control group showed nine protein bands with molecular weight of 49, 34, 19, 12, 8, 5 and 2 kDa. There were only five bands of 49, 40, 34, 12 and 10 kDa in group receiving 5,600 ppm extract (T1). The bands in group receiving 11,200 ppm extract (T2) were similar to those of control group, only missing a band of 8 kDa. There were only five bands of 19, 12, 10, 5 and 2 kDa in groups receiving 16,800 and 22,400 ppm extracts (T3 and T4).

Those results showed that the group receiving 11,200 ppm extract (T2) had the most diverse protein bands among all treatment groups, which were almost similar to the bands in control group, indicating that the concentration of 11,200 ppm could generate the highest effest in increasing the number of mammary glannd membrane proteins. The lack of bands in group receiving 5,600 ppm extract (T1) might be because the given concentration was too low, therefore it could not influence the addition of the mammary gland membrane proteins. On the contrary, the lack of bands in groups receiving 16,800 and 22,400 ppm extract (T3 and T4) might be because the given concentration was too high. The decrease of protein bands in those groups might be because bitter melon leaf contains not only charantin but also trichosanthin. Trichosanthin could cause apoptosis of mammary gland cells [11].



A b

Fig. 2. SDS-PAGE of membrane proteins isolated from mammary gland of Balb/c mice shown in polyacrilamide gel (A) and zimogram (B).

CONCLUSIONS

It can be concluded that the water fraction of bitter melon leaf extract affected the development of mammary gland in Balb/c mice, concentration of 11,200 ppm delivered the highest and significantly different results from other treatments.

ACKNOWLEDGEMENTS

This study was supported by State University of Malang under Student Creativity Program (PKM) that was held every year and funded by Indonesian Ministry of Research, Technology and Higher Education (Ristekdikti).

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