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THE EFFECTS OF ADDING MORINGA OLEIFERA LEAVES EXTRACT ON RABBIT DOES' MILK PRODUCTION AND MAMMARY GLAND HISTOLOGY

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ABSTRACT

The objective of this study was to examine the effects of adding Moringa leaves extracts on rabbit does' milk production and histological changes of mammary glands during their lactation phase. Three different solvents, which are n-hexane, ethanol and mixed of n-hexane and ethanol (50: 50), were used to extract Moringa leaves powder (MLP). Moringa extract was given to the does' feed by seven different treatments: 0% dosage (without extract), 0.13% dosage of HeEF, 0.26% dosage of HeEF, 0.37% dosage of EtEF, 0.74 % dosage of EtEF, 0.54% dosage of EtCE and 1.08 % dosage of EtCE. Each treatment was replicated at four does. The variables were dry matter consumption, average does' daily milk production, bunnies' daily weight, and the does' mammary gland histology. The milk production was measured twice per day in the morning (7 a.m.) and the evening (4 p.m.), obtained from the bunnies' weight gap before and after suckling. The results showed that there was a significant difference ($P < 5\%$) in treatments' effect on dry matter consumption, daily milk production and bunnies' average daily gain (ADG). From the results, treatments with the highest daily milk production were 0.54% EtCE (134.70 ± 5.77 g / day) and 0.26% EtEF (128.83 ± 10.11 g/day). The potential effect of Moringa leaves extract on the milk production was shown by an overview of mammary gland alveoli, which indicates the increasing number of the epithelial cells as well as the alveoli number and size.

KEY WORDS

Rabbit, Moringa leaves extract, milk production, mammary gland histology, galactagogue.

Nutrients sufficiency and hormones affect milk synthesis and production. Some people are using a compound called 'galactagogue' to optimize the trigger hormones. Galactagogues are drugs or substances believed to assist in the initiation, maintenance or augmentation of milk production (Rajagopal *et.al*, 2016; Wadha *et.al*, 2013). Some drugs serve as galactagogues, such as metoclopramide, domperidone, chlorpromazine, sulphiride, oxytocin, growth hormone, thyrotropin-releasing hormone, and medroxyprogesterone (Zuppa *et.al*, 2010). A few researchers reported that some plants function as galactagogue herbs such as *Moringa oleifera* (Estrella *et.al*, 2000), *Sauropusandrogynus* (Akbar *et.al*, 2013), Fenugreek (*Trigonellafoenumgraecum*) and Silymarin (*Silybummarianum*) (Zuppa, *et.al*, 2010). Antonet *et.al* (2003) reported that moringa leaves effects to accelerate milk secretion are similar to domperidone and metoclopramide. Most people prefer galactagogue herbs as they have fewer side effects.

Moringa oleifera leaves potentially increase milk production of nursing mothers in the Philippines (Estrella *et.al*, 2000; Almirante and Lim, 1996), of cows (Sanchez *et.al*, 2005; Mendiata-Aracia, 2011), of goats (Soetanto *et.al*, 2010), of Wistar rats (Pearl *et.al*, 2013; Mutiara *et.al*, 2012), and of rabbits (Alemede *et.al*, 2014). This potential is associated with the leaves' having plenty of nutrients content such as amino acids complete, vitamins and others that are used as a synthesis of milk; therefore, dairy farming highly recommends using *Moringa oleifera* leaves as feedstuff (Mendiata-Aracia *et.al*, 2010).

In addition, bioactive compounds contained in *Moringa oleifera* are considered to function as a galactagogue. According to Almirante and Lim (1996), milk production is

affected by several processes: (1) mammogenesis, which is the growth of the mammary gland namely ducts and alveoli with estrogen, progesterone, prolactin and growth hormone (2) lactogenesis, which is the initiation of milk secretion influenced by the development of mammary glandular secretion and distribution of estrogen and progesterone to the fetus and placenta (3) galactocentric, which is influenced by the oxytocin hormone promoting breast contraction to produce milk from the myoepithelial cell to the milk ducts, and (4) galactopoiesis, which is milk production maintenance, whose role is played by the prolactin hormone.

Galactagogue compound is related to the hormones; so, it is assumed that phytohormone in *Moringa oleifera* leaves plays a part in increasing milk production. The leaves contain phytosterol and isoflavone as the phytoestrogen compound (Leone *et.al*, 2015) which has estrogenic activity due to its similar chemical structure to estradiol (Ryokkynen, 2006). Phytosterols are steroids dissolved in non-polar solvents such as n-hexane; while isoflavones are a group of flavonoids optimally extracted with ethanol (Kudou *et.al*, 1991).

The objectives of this study were to examine the galactagogue effects of MLP extracts with different solvents on the milk production, ADG of bunnies and does, as well as rabbits' mammary gland histology.

MATERIALS AND METHODS OF RESEARCH

A hexane extract fraction (HeEF), an ethanolic extract fraction (EtEF) and an ethanolic crude extract (EtCE) of MLP were used in this study. The MLP was produced by *Moringa* Indonesia in Blora, Central Java, Indonesia. The particle size of the MLP was 80 mesh.

EtCE was produced by extraction of MLP using 96% ethanol (1: 8; w/v). Maceration was conducted for 24 hours and was stirred by the shaker; after that, the filtrate was evaporated and freeze-dried for 30 hours. Meanwhile, HeEF and EtEF were produced by extraction using a mixed solvent of 96% ethanol and n-hexane (50: 50; v: v). The ratio of MLP and the solvent was 1: 8 (w: v). Then, the filtrate was separated by separation funnel. The n-hexane solution was at the top layer and the ethanol solution was at the bottom layer. Next, each fraction solution was evaporated: the n-hexane was evaporated by 72 mbar and the ethanol was evaporated by 175 mbar. After that, the filtrates were freeze-dried: HeFE for 24 hours and EtFE for 30 hours.

The types of phytosterol and isoflavone compounds in MLP extracts were identified qualitatively through Liquid Chromatography-Mass Spectrometry (LCMS) in Instrumental Laboratory of *Politeknik Negeri Malang*. The identification of isoflavone was based on Konar *et.al*. (2012), while phytosterol was based on Miller and Schnute (2014).

The research involved twenty-eight heads of New Zealand White lactation rabbits on second to the third parity, whose average body weight was 2.1 kg (block-I), 2.35 kg (Block-II), 2.64 kg (block-III) and 2.85 kg (block-IV). The does were divided into four groups based on their initial body weight and allotted into a randomized complete block (RCB) design (Steel and Torrie, 1980), using seven treatments and four replications.

The treatments were E0 (pellet without MLP extract), 0.13% HeEF0 (feed containing HeEF equal 10% MLP in pellet), 0.26% HeEF (feed containing HeEF equal 20% MLP in pellet), 0.37% EtEF (feed containing EtEF equal 10% of MLP in pellet), 0.74% EtEF (feed containing EtEF equal 20% MLP in pellet), 0.54% EtCE (feed containing EtCE equal 10% MLP in pellet) and 1.08% EtCE (feed containing EtCE equal 20% MLP in pellet). Table 1 presents the composition of each treatment's feedstuff and Table 2 presents the nutrient content of pellet and forage.

The does were given treatment feed immediately after parturition. Preliminary feeding was done before mating. The ration consisted of 40% forage and 60% pellet. The rabbits were fed twice: with pellets at 7: 00 a.m and with forage at 3.00 pm.

The variables were milk production, weaning weight, bunnies' daily body weight gain, does' weight gain during lactation, mammary gland histology, the number of epithelial cells, as well as the number and size of alveoli. The milk production measurement was conducted

by weighing the bunnies before and after feeding on day-1 to day-21 after birth because at that time the bunnies were still exclusively suckling the doe's milk. The rabbits were housed in individual cages and bunnies were separated from its doe until they were 21-days old. Milk production was calculated by the difference in the total weight of the bunnies after and before feeding. Bunnies were suckling milk twice a day, which was at 7: 30 a.m and at 4: 30 pm. The weaning weight of bunnies was measured when they were 21-days old. The bunnies' ADG was calculated by the difference of the weaning weight at 21-days and the birth weight divided by 21.

Table 1 – The Feedstuff Composition of Each Treatment

Feedstuff	E0	HeEF 0.13%	HeEF 0.26%	EtEF 0.37%	EtEF 0.74%	ECE 0.54%	ECE 1.08%
Forage (%)	40	40	40	40	40	40	40
Broccoli leaves (%)	100	100	100	100	100	100	100
Total (%)	100	100	100	100	100	100	100
Pellet (%)	60	60	60	60	60	60	60
Polished rice bran (%)	70	69.87	69.74	69.63	69.26	69.46	68.93
Fish meal (%)	5	5	5	5	5	5	5
Coconut cake (%)	20	20	20	20	20	20	20
Top mix (%)*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Salt (%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Maize meal (%)	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Tapioca flour (%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
HeEF (%)	0	0.13	0.26	0	0	0	0
EtEF (%)	0	0	0	0.37	0.74	0	0
EeCE (%)	0	0	0	0	0	0.54	1.07
Total (%)	100	100	100	100	100	100	100

*Every 10 kg of Top mix contains 12.000.000 IU of Vitamin A, 2.000.000 IU of Vitamin D3, 8.000 IU of Vitamin E , 32.000 mg of Vitamin K, 2000 mg of Vitamin B1, 5000 mg of Vitamin B2, 500 mg of Vitamin B6, 12000 mg of Vitamin B12, 25000 mg of Vitamin C, 6000 mg of Ca-D-Pantotenate, 40.000 mg of Niacin, 10.000 mg of Cholin Chloride, 30.000 mg of Methionin, 30.000 mg of Lysine, 120.000 mg of Manganese, 20.000 mg of Iron, 200 mg of Iodine, 100.000 mg of Zinc, 200 mg of Cobalt, 4.000 mg of Copper, 10.000 mg of Santoquin, 21.000 mg of Zinc basitracin , and supporting materials up to 10 kg.

Table 2 – The Nutrient Content of Pellet and Forage*

Nutrient	Pellet (all treatments combined)	Forage (Broccoli leaves)
Dry matter (%)	88.75	18
Crude Protein (%)	18.22	27.63
Extract Ether (%)	9.2	1.06
N-free Extract (%)	54.41	48.31
Crude Fiber (%)	8.79	8.28
Ash (%)	9.38	15.16
Gross energy (kcal/g)	3188.25	2955

*The results of the proximate analysis at Animal Nutrition and Feed Laboratory, Faculty of Animal Husbandry, University of Brawijaya.

The number of epithelial cell and alveoli, alveoli diameter and width were calculated in five microscope field-of-view through 400x magnification using Image Raster 3 Program. Each field-of-view was measured on 10 alveoli. The data were analyzed by ANOVA RCB design using SPSS version-23 (IBM, Harvard University). Provided there was a significant difference, it proceeded to be analyzed by LSD (Least Significant Difference).

The data about milk production and reproductive performance were analyzed using SPSS version-23 (IBM, Harvard University) with covariance analysis of randomized complete block (RCB) design using the number of bunnies as the covariance. Provided there was a significant difference, it proceeded to be analyzed by LSD.

RESULTS AND DISCUSSION

Table 3 present the result of identification phytosterol and isoflavone compounds in MLP extracts by LCMS. Both EtCE and EtFE contain isoflavone compounds which positive polarity namely daidzein, biochanin A, and glycitein, while formononetin was contained by

EtFE only. The MPL extracts do not contain genistein, the type of isoflavone commonly found in soybeans. Whereas, both EtCE and HeFE contain phytosterol types consist of lathosterol, campesterol, stigmasterol, and β -sitosterol.

Table 3 – The Identification of Phytosterol and Isoflavone Compounds in MLP Extracts

Compounds	Precursor ion	Product ion	Moringa Leaves Extract		
			EtCE	EtFE	HeFE
Isoflavone					
Daidzein	252.6	223.1; 207.7	+	+	Nid
Formononetin	266.6	251.6; 222.7	-	+	Nid
Genistein	268.6	158.6; 132.8	-	-	Nid
Biochanin A	282.6	267.5; 238.6	+	+	Nid
Glycitein	282.6	267.5; 238.6	+	+	Nid
Daidzin	417.3	255.1; 199.0	-	-	Nid
Ononin	431.3	270.3; 269.1	-	-	Nid
Genistin	433.3	271.1	-	-	Nid
Glycitin	447.3	284.9; 269.8	-	-	Nid
Sisotrin	447.3	285.1	-	-	Nid
Phytosterol					
Lathosterol	369.4	95.1; 81.1	+	Nid	+
Campesterol	383.4	161.2; 95.0	+	Nid	+
Stigmasterol*)	395.4	83.1; 83.1	+	Nid	+
β -Sitosterol	397.4	161.0; 135.2	+	Nid	+

Note: *nid* = not identified.

Table 4 presents the significant effect of different types and levels of MPL extract administration on feed intake. According to the table, the treatment of 1.08% EtCE showed the highest dose of ethanol crude extract consumption, which was 375.57 mg/kg WB/day. This led to a decrease in feed intake. The treatment of 1.08% EtCE revealed the lowest feed intake, which was 138.45 ± 16.59 g/head/day. It was only 1.08% EtCE treatment that showed different feed consumption. Therefore, it is safe to say that the effects of treatment were caused by extract consumption. The highest content of flavonoids was found in 1.08% EtCE treatment diet. Flavonoids taste bitter thus reducing feed palatability (Roland et al, 2014).

Table 4 – The Dry Matter Intake and Extract Consumption

Treatment	Extract Consumption (mg/kg BW/day)	Feed Intake (g DM/day)
E0	0	144.37 \pm 15.55 ^b
HeEF0.13%	45.63	144.05 \pm 13.1 ^{9b}
HeEF0.26%	91.26	145.82 \pm 14.57 ^b
EtEF0.37%	129.87	145.95 \pm 14.64 ^b
EtEF0.74%	259.74	142.20 \pm 12.68 ^b
EtCE0.54 %	189.54	145.95 \pm 12.68 ^b
EtCE1.08%	375.57	138.45 \pm 16.59 ^a

* *Superscript differences in the same column indicate significant differences (P < 0.5).*

Table 5 reveals MLP extract administration in the feed had a significantly different effect ($P < 0.5$) on the does' milk production and reproductive performances. The table demonstrates that the treatments have a significant effect ($P < 0.05$) on does' daily milk production and bunnies' daily weight gain. The 0.54% EtCE treatment showed the highest milk production, which was 134.70 ± 5.77 g/day. The does' milk production affected bunnies' average daily gain. The 0.26% HeFE treatment resulted in the highest bunnies' ADG, which was 12.20 ± 3.86 g/day; following this, the 0.54 % EtCE treatment result was 10.04 ± 0.96 g/day.

Figure 1 indicates the does' average daily milk production in each treatment. It presents the graphic of daily milk production in the treatments. Accordingly, the 0.26% HeEF and 0.54% EtCE treatments showed higher daily milk production than the others. The peak of milk production varied in each treatment, occurring between 9-15 days after parturition.

The highest peak milk production, which was more than 160 g/day, was obtained through 0.26% HeEF and 0.54% EtCE treatments at 12 days after the does' parturition.

Table 5 – Milk Production and Reproduction Parameters

Treatment	Bunny Size	Bunny Weight (g)	Birth Weight (g)	Daily milk production (g)	ADG of the bunny (g)	Changes of BW Does during suckling
E0	6.75±2.98	325.4±105.8	51.14±15.54	103.69±14.137 ^a	7.78±3.47 ^a	-180.0±89.07 ^{ab}
HeEF0.13%	5.25±1.50	255.29±54.75	50.18±7.63	98.45±11.67 ^a	7.10±2.94 ^a	-61.25 ±117.64 ^b
HeEF0.26%	6.25±2.36	286.32±84.74	47.45±6.82	128.83±10.11 ^b	12.20±3.86 ^b	-50.0±135.40 ^b
EtEF0.37%	5.5±2.38	282.01±92.40	53.53±8.18	108.55±11.78 ^a	8.13±3.81 ^a	0.0±35.36 ^b
EtEF0.74%	5.5±2.08	268.58±73.60	50.59±6.40	96.46±12.35 ^a	7.35±4.15 ^a	6.25±31.46 ^b
EtCE0.54%	6.0±0.81	298.26±48.84	49.73±5.34	134.70±5.77 ^b	10.04±0.99 ^{ab}	-125.75±65.25 ^b
EtCE1.08%	6.75±2.87	332.74±64.28	52.57±12.11	104.04± 6.62 ^a	6.295±0.96 ^a	-286.25±116.25 ^a

* Superscript differences in the same column indicate significant differences ($P < 0.05$).

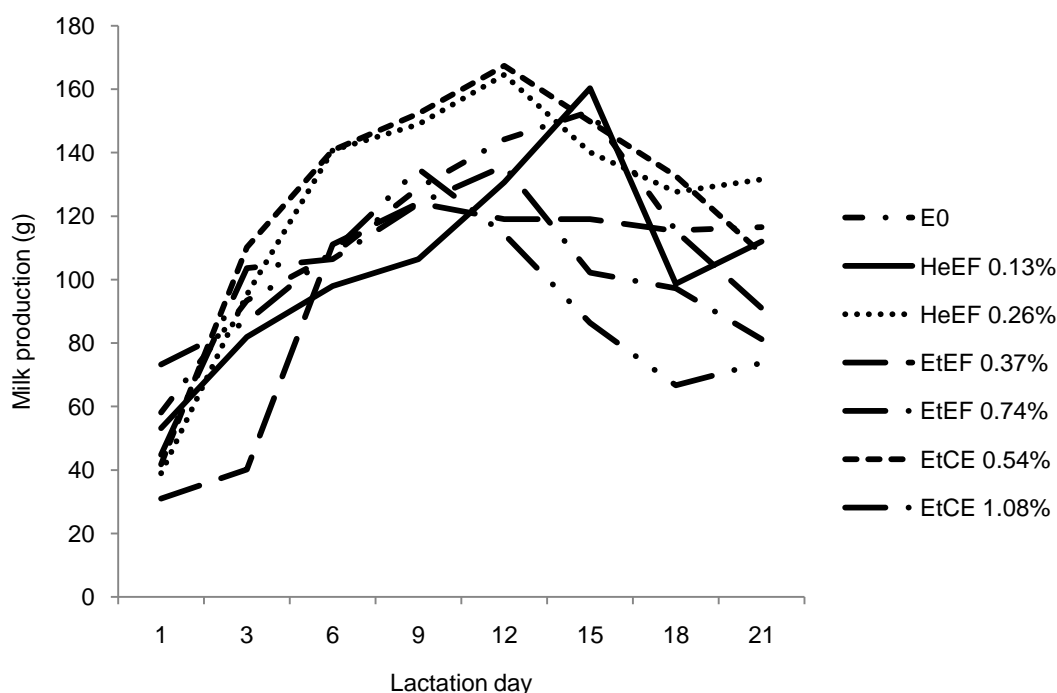


Figure 1 – The average daily milk production (g) up to 21st day of lactation.

Differences in milk production are possible due to the effect of the bioactive compounds contained in MPL extract (Mutiarra et al, 2013). It can be assumed that the compounds in hexane fraction extract had more effects on milk production than those in ethanol fraction. Based on the polarity of the solvents, hexane extract fraction contains phytosterol compounds while the ethanol crude extract compounds contain phytosterols and flavonoids in particular isoflavones (Anjani et al, 2003; Kudou et.al, 1991). Both of these compound groups have estrogenic activity (Ryokkynen, 2006). The 0.54% EtCE treatment containing phytosterol and isoflavone caused a significant increase in milk production at lower doses (which equal 10% MPL in the feed), whereas 0.26% HeFE treatment increased milk production (which equal 20% MLP in the feed).

Phytosterols are compounds whose chemical structure is similar to cholesterol, which is a precursor of steroid hormones such as estradiol and progesterone, while isoflavones are compounds whose chemical structure is similar to estradiol. Suprayogi *et.al* (2009) reported that the hexane fraction of *Sauropus androgynous* leaves has higher galactagogue potency than the fraction of the water, acetyl acetate, and ethanol crude extracts. According to Xinmei, et al. (2015), the supplementation of phytosterol at cow early lactation increased milk production, milk composition, milk fat, lactoprotein, and total solids- non-fat.

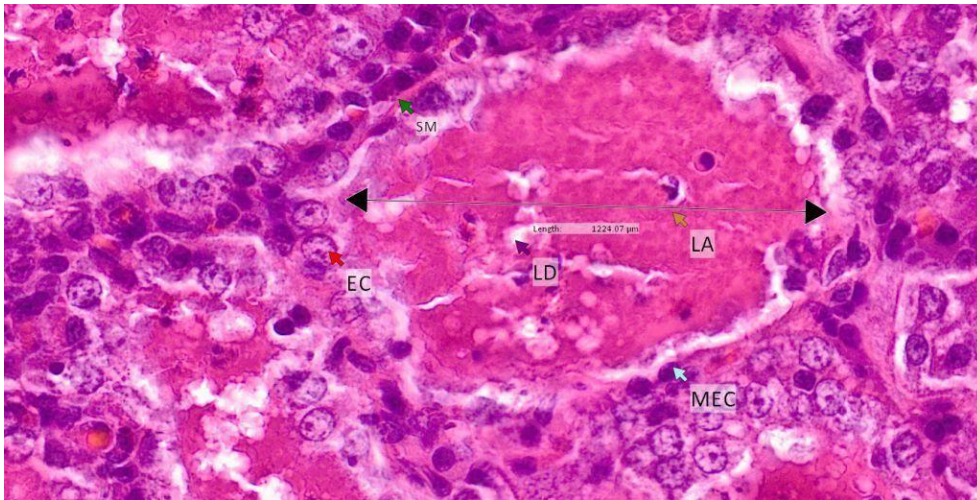


Figure 2 – The Histology of Lactating Doe's Mammary Gland, E0 (Control Treatment), 400x Enlargement (LA = lumen of the alveolus. EC= epithelial Cell. MEC =MioEpithelialCell.LD = Lipid droplets. SM = Smooth Muscle. S=Stroma)

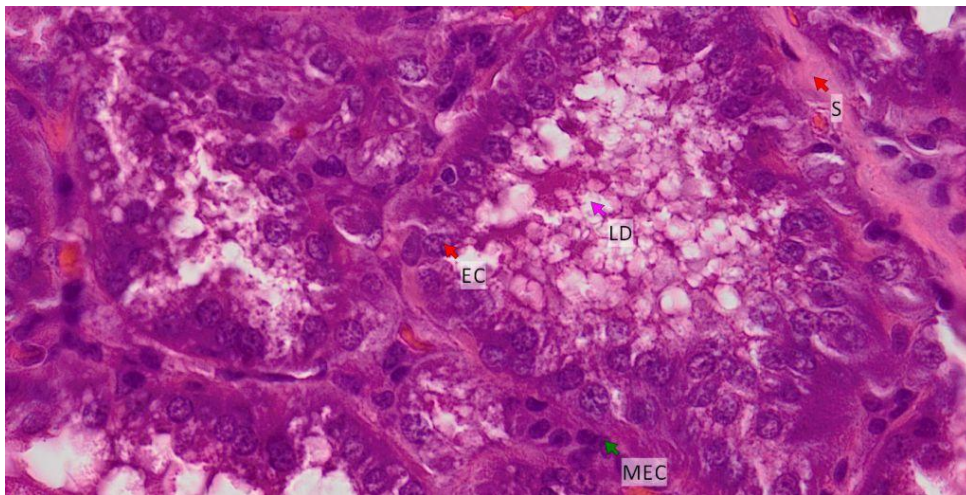


Figure 3 – The Histology of Lactating Doe's Mammary Gland, 0.26% of HeEF Treatment, 400x Enlargement (LA = lumen of alveolus. EC= epithelial Cell. MEC =MioEpithelialCell.LD = Lipid droplets. SM = Smooth Muscle. S=Stroma)

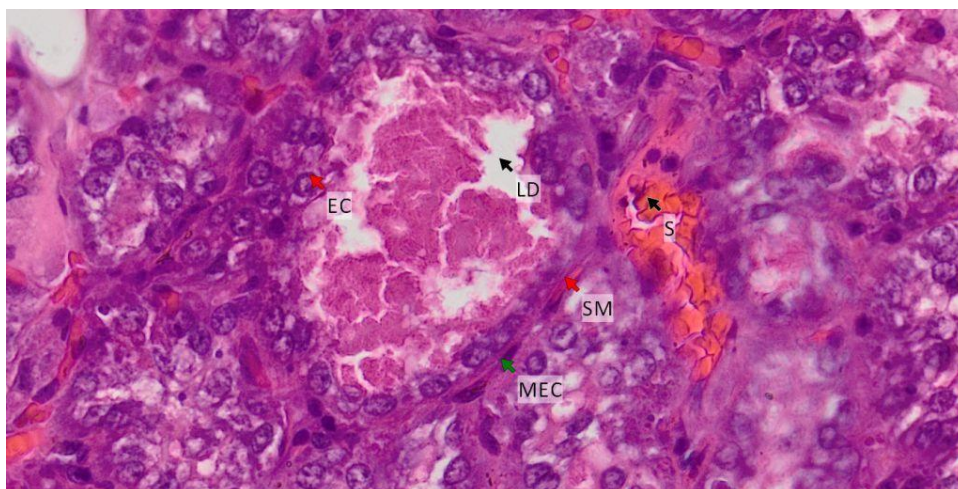


Figure 4 – The Histology of Lactating Doe's Mammary Gland, 0.54% of EtCE Treatment, 400x Enlargement (LA = lumen of alveolus. EC= epithelial Cell. MEC =MioEpithelialCell.LD = Lipid droplets. SM = Smooth Muscle. S=Stroma)

Figure 2 to 4 show the mammary histology of some treatments. The histology can calculate the number of epithelial cells and alveoli, alveoli containing milk, and alveoli size using the extensive alveolar diameter of each treatment. Table 6 presents milk production, the number of epithelial cells, alveoli diameter and width per microscope field-of-view.

The 0.54% EtCE and 0.26% HeFE treatments causing higher milk production indicated several epithelial cells and active alveoli. The total alveoli at 0.26% HeFE were 55.4 ± 8.68 , with 89.53% active alveoli and the total alveoli at 0.54% EtCE were 59.2 ± 6.94 , 95.27%, which were actively producing milk. If 0.54% EtCE is compared with E0 (control) treatment, the former increased the number of active alveoli producing milk by 62.9%, alveoli diameter by 49.75%, and epithelial cells by 300%.

Table 6 – The Number of Epithelial Cells, Diameter, and Width of the Alveoli per Microscope Field-of-view

Treatment	Milk Production (g/day)	Number of epithelial cells	Number of the alveoli			Average of alveolar lumen diameter (μm)
			Total	With milk (%)	Without milk (%)	
E0	103.69 \pm 14.137 ^a	12.39 \pm 4.8 ^a	47.6 \pm 8.96	68.49	31.51	64.02 \pm 29.59
HeEF0.13%	98.45 \pm 11.67 ^a	15.06 \pm 2.6 ^{ab}	55.4 \pm 8.68	57.14	42.86	71.51 \pm 8.77
HeEF 0.26%	128.83 \pm 10.11 ^b	35.18 \pm 1.7 ^c	46.2 \pm 12.56	89.53	10.47	85.76 \pm 7.77
EtEF 0.37%	108.55 \pm 11.78 ^a	19.96 \pm 2.09 ^{ab}	45.4 \pm 8.73	53.74	46.26	78.62 \pm 11.02
EtEF0.74%	96.46 \pm 12.35 ^a	23.33 \pm 4.68 ^b	35.2 \pm 4.97	77.84	22.16	96.51 \pm 4.59
EtCE0.54 %	134.70 \pm 5.77 ^b	37.22 \pm 2.69 ^c	59.2 \pm 6.94	95.27	4.73	95.87 \pm 27.56
EtCE1.08%	104.04 \pm 6.62 ^a	22.25 \pm 4.48 ^b	41.8 \pm 9.28	66.51	33.49	68.56 \pm 8.84

*Superscript differences in the same column indicate significant differences ($P < 0.05$).

The difference of mammary gland figure in the treatments suggested the correlation between types and doses of phytosterols and isoflavones in each MLP extract. Phytosterols are compounds whose chemical structure is similar to cholesterol, which is a precursor of steroid hormones such as estradiol and progesterone; while isoflavones are compounds whose chemical structure is similar to estradiol and have estrogenic activity (Ryokkynen, 2006). The increasing milk production was possible due to the direct or indirect effect of compounds contained in MLP extract. The direct effect of estradiol formed from phytosterol stimulates the proliferation of myoepithelial cell and induce the maturation of mammary acini for milk production (Yart, *et.al.* 2013). The indirect effect was phytosterol stimulating anterior and posterior hypophysis to secrete oxytocin and prolactin. According to Santell *et.al* (1997), the isoflavones genistein and hormone estradiol administration in ovariectomized rats increased the level of prolactin in the blood. It is evident that there is a relationship between phytoestrogens and estradiol; it affects the secretion of the prolactin hormone, which brings an impact on milk production. Kleden *et.al* (2017) asserted that adding MLP in feed can increase prolactin in lactating does' blood.

Milk production is the result of a mechanism in the epithelial cells (endocytosis) and the release mechanism (exocytosis) (Trucket and Olliver, 2009) related to the prolactin and oxytocin hormones. The hypothalamus releases oxytocin hormone, encouraging epithelial cells contraction in the cell which allows milk secretion, while prolactin serves to maintain milk production (lactogenesis) (Lollivier *et.al*, 2006).

The estrogenic activity of phytosterol compounds can increase the estradiol and progesterone hormones. Progesterone stimulates the secretory formation in mammary gland while estradiol stimulates the posterior pituitary to secrete oxytocin that serves to release the milk and stimulates the anterior pituitary to secrete prolactin and growth hormone that serves to stimulate the growth of mammary glands and to stimulate milk production (Mutiarra *et.al*, 2013).

Rabbits' mammary gland epithelial cells express oxytocin receptors and bind these receptors. There are two important effects on the mammary gland oxytocin: lactation is to accelerate the transfer of intracellular casein and to push myoepithelial cell contraction (Lollivier *et.al*, 2006).

CONCLUSION

The ethanol crude extract of MLP containing phytosterol and isoflavone has greater potential as galactagogue than the ethanol and n-hexane fraction extracts. The milk production was increased by increasing the number of epithelial cells by 300%, the alveoli producing milk by 63%, and the diameter of alveoli by 50%.

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