

The Ethyl Acetate Fraction of *Torbangun* (*Coleus amboinicus* L.) Leaves Increasing Milk Production with Up-Regulated Genes Expression of Prolactin Receptor

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ABSTRACT

This study is aimed towards determining the lactagogue effect of *torbangun* leaves on lactogenic hormone plasma levels and on the expression of their receptors in mammary glands of lactating rats. Lactagogue activity was evaluated by volume of milk was produced by the rats treated with commercial milk booster containing 'katuk' leaves extract (AF), ethyl acetate fraction of *torbangun* leaves (EA), water extraction of *torbangun* leaves (AQ) and kaempferol (KP). Lactating rats (n=5) of Sprague dawley with six pups were fed with AF, EA, AQ, and KP in the amount of 50 mg/kg, 30 mg/kg, 80 mg/kg and 60 mg/kg body weight, respectively. The volume of milk was estimated by the increment in pup weight after breastfed. The levels of serum lactogenic hormones were determined by ELISA methods. To access the expression level of lactogenic hormone's receptors in the mammary gland real time – polymerase chain reaction method was employed. The results showed that ethyl acetate fraction of *torbangun* leaves (EA) (a) was not significantly stimulating the synthesis of serum prolactin and estradiol at day 14 and day 28 lactation period, (b) down-regulated the gene expression of estradiol receptor (ER α) at day 28, and (c) up-regulated the gene expression of prolactin receptor (PRLR) in mammary gland at day 14 and day 28. This study suggests that ethyl acetate fraction of *torbangun* leaves was induced milk production, by up-regulating the gene expression of prolactin receptor (PRLR) in the mammary gland of lactating rats.

Keywords: *Coleus amboinicus* L., lactogenic hormones, genes expression, lactagogue, milk booster, *torbangun*

Introduction

The mothers have many reasons that were not given exclusive breastfeeding (0-6 month) to the baby; one of them is limited of breast milk production. Milk production may be affected by nutritional factors and endocrine factors [1]. Lactagogue herbs such as *Coleus amboinicus* Lour have been suggested as an alternative to solve the limitation of milk production [2, 3].

Torbangun leaves (*C. amboinicus* L.), Lamiaceae family, an indigenous Indonesian herbal medicine, has been reported to have potential of

increasing breast milk production [4]. The extracts of *torbangun* leaves and its fraction contained phytochemical content, viz., terpenoids, phenolic, flavonoids and ester groups [5, 6, 7]. Some of the flavonoids can bind to estrogen receptors (ER) can affect ER regulation, metabolism and estrogen synthesis, and apoptosis. Flavonoids that have estrogen-like activity are known as "phytoestrogens" [8]. Phytoestrogens had similarities in chemical structure with estradiol, which can bind to alpha and beta estrogen receptors [9, 10].

Prolactin has an important role in the develop-

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ment of mammary glands during early gestation until postpartum [11]. The interaction of prolactin and its receptor on the membrane epithelial cell (MEC) of the mammary gland determine the amount of milk production so that the presence of prolactin receptor (PRLR) was required for the expression of milk protein during lactation [12]. PRLR plays a role in activating the Janus kinase pathway (Jak2), by activating STAT-5 which is required for lactogenesis processes during pregnancy and lactation to occur [13]. Estradiol plays an important role in the regulation of prolactin. As published by [14], estradiol may increase the concentration of prolactin mRNA, by increasing the transcription of the prolactin gene, in the hypothalamus. Estradiol receptors (ER) play a role in the regulation of tryptophan hydroxylase enzymes, where it is known that tryptophan is a neurotransmitter precursor of serotonin [15, 16]. Moreover, the other plausible mechanisms involved in increasing milk production can be by up-regulation of oxytocin receptor gene and protein in the anterior pituitary [17].

Torbangun leaves and their interaction with lactagogue have been widely demonstrated; although there is lack of evidence explaining the mechanisms by which lactagogue compounds act against lactogenic hormones and their receptors. In our previous studies, ethyl acetate fraction of *torbangun* leaves showed potent antioxidant activities and increasing milk production in lactating rats [6,7]. Hence, the present work is aimed to determine the lactagogue effect of *torbangun* leaves to plasma levels of lactogenic hormones and gene expression of their receptors in mammary glands of lactating rats.

Material and Methods

Reagents and materials

Torbangun leaves (*C. amboinicus* L.) were collected from the Sukabirus garden, Megamendung Bogor, Indonesia ("S 06°39'58.3"; "106°52'28.7"; 558 mdpl). The cutting interval of *torbangun* leaves were 60 days, each cutting was cultivated with 25 cm radius from the other, and taken 15 cm from the end of each branch [18,19]. Botanical authentication was performed by the botanist from "Herbarium Bogorienses", Research Center for Biology, Indonesian Institute of Sciences, where the voucher specimen has been deposited (No.145/IPH.1.01/II.8/II/2015).

The materials of research were ethanol, ethyl acetate, n-hexane, phosphate buffer saline (PBS), distilled water and agarose gel. Experimental animals used pregnancy female rats (Sprague dawley strain), is approximately four months old, which obtained from PT. Indoanilab, Bogor-Indonesia.

The levels of prolactin and estradiol serum

On 14th day and 28th day of the lactation period, lactation rats were adjusted to blood sampling procedure through a lateral tail vein. The blood samples were centrifuged (14,000 rpm, 15 minutes) and serum was stored at -20°C. Serum prolactin and estradiol were estimated by enzyme-linked immunosorbent assay (ELISA) method. The concentrations of estradiol and prolactin hormones were measured by BT-Laboratory Rat Estrogen ELISA Kit® (No. E0176Ra) and BT-Laboratory Rat Prolactin ELISA Kit® (No. E0190Ra).

Extraction of RNA

The RNA isolation of the mammary gland used RNeasy® Lipid Tissue Mini Kit (Qiagen, Germany), cDNA was obtained by using Transcriptor Synthesis First Stand DNA Kit (Thermo Scientific, Lithuania, EU), and the expression gene receptor used the SYBR Green Select Master Kit (Applied Biosystem, USA). All RNA samples from the mammary glands were extracted using the RNeasy® Lipid Tissue Mini Kit method (Qiagen, Germany). Samples (70 mg) were added into nitrogen (N₂) liquid, inserted 1 mL of lysis buffer (QIAzol Lysis Reagent), then crushed with a micropestle and incubated at 25°C, for 5 minutes. Chloroform (200 µL) was added to the sample, mixed for 15 seconds, incubated at 25°C, for 3 minutes, then centrifuges 12000 rpm, at temperature 4°C, for 15 minutes. Water-soluble phase (500 µL) was taken, then added ethanol solvent (500µL, 70%). A total of samples (700 µL) were inserted into 2 mL RNeasy mini spin column, centrifuge 10000 rpm, at 25°C, for 15 seconds. The solution was discarded, then added RW1 buffer (700 µL) into the RNeasy column, centrifuge 10000 rpm, at 25°C, for 15 seconds, the solution was discarded, then added 500µL RPE Buffer into RNeasy column, centrifuge 10000 rpm, temperature 25°C, for 15 seconds. The solution was discarded, 500 µL RPE Buffer was added into RNeasy column, closed, centrifuge 10000 rpm, at 25°C, for 2

minutes, then placed in 2mL RNeasy column tube, centrifuge 14000 rpm, temperature 25 ° C, for 1 minute. RNeasy column was transferred into tube 1.5 mL, then added RNase free water (40 µL), centrifuge 10000 rpm, for 1 minute. RNA template obtained is stored at -80oC until ready to be analyzed. The quality and quantities of RNA were measured using a spectrophotometer with an OD260/OD280 ratio is 1.9 to 2.1.

cDNA synthesis of isolated RNA

The RNA isolated was reverse transcribed into complementary DNA (cDNA) using Transcription Synthesis First Stand DNA Kit method (Thermo Scientific, Lithuania, EU). The solution consists of 2 µL RNA template, 1 µL oligo (dT) and 9 µL water, was incubated at 65°C for 5 minutes. Then added 4 µL 5×RB (buffer), 1 µL riboblock, 2 µL dNTP and 1 µL reverse transcriptase, and incubated using polymerase chain reaction (PCR) at 42°C and 78°C, respectively, for 5 minutes. The quantification of cDNA was analyzed using a spectrophotometer (Agilent 8453, USA) with an OD260 / OD280 ratio is 1.8.

The expression analysis of estradiol and prolactin receptors genes using real-time PCR

The cDNA obtained used for the expression of prolactin (PRLR) and estradiol (ERα) receptor genes using real time - polymerase chain reaction (RT-PCR) (Analytic Jena, AG qTower 4 channel, Germany). Reaction of real time - PCR using SYBR Green Select Master Kit (Applied Biosystem, USA), i.e.: 10 µL mixed reaction used to contain 5 µL master mix SybGreen; 0.5 µL each forward and reverse primers (20 µM); 1 µL cDNA (70 ug/µL) of the sample; 3 µL nuclease-free water. PCR condition was run at the following: 95°C for 5 minutes, 40 cycles at 95°C for 10 seconds, followed with 60°C for 1 minutes, and 72°C for 30 seconds.

The expression of prolactin (PRLR) and estradiol (ERα) receptor gene were calculated based on the relative quantities of mRNA target genes (PRLR and ERα) with control genes (β-actin) by the CT comparison method (Δ CT). The expression between target genes and control genes can be compared with equation $2^{-\Delta\Delta CT}$, with delta CT (Δ CT) = CT target gene - CT control gene (housekeeping gene) [22].

Primers were used for housekeeping gene (β-

actin) consist of: Forward- 5'GTA AAG ACC TCT ATG CCA ACA'3, Reverse- 5'GGA CTC ATC GTA CTC CTG CT'3; and to amplify the mRNA of PRLR and ERα consists of PPLR: Forward-5'CAA CAT CAG CCT CCT GAA GG'3, Reverse- 5'GTA GTC TGG ACA TTC GTA GG'3; and ERα: Forward- 5'CAG AGA GAT GG TGG GA GG'3, Reverse- 5'GGT TGG TCA ATA AGC CCA TC'3.

Statistical analysis

Data were presented in mean ± standard deviation and then tested for normality. All statistical analysis was performed using Microsoft Excel 2013. The differences between treatments were analyzed using ANOVA. Significant differences between mean values were determined using Duncan's Multiple Range Test ($\alpha = 5\%$).

Results and Discussion

The characteristics of rats

The characteristics of rat samples used in postpartum interventions are presented in Table 1. Table 1 showed that there were no significant differences between groups of rats sampled from the duration of pregnancy, the number of pups in one birth and birth weight of pups ($P > 0.05$). This is indicated that the sample of rats used in postpartum intervention was homogeneous.

The levels of estradiol and prolactin hormones

The hormonal system plays an important role in milk production. Based on our previous study, the ethyl acetate fraction of *torbangun* leaves,

Table 1. The average of length of pregnancy, number of pups in once gave birth, and birth weight of pups.

Parameter	Group				
	KO	AF	EA	AQ	KP
Length (days)	22.0	21.7	21.7	22.0	21.7
Number of pups (pups)	8.6	9.2	8.8	8.3	9.2
Birth weight of pups (g)	8.2	8.1	7.8	7.7	7.8

Data are expressed as mean (n = 5). KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group. Data are expressed as mean±SE. *shows significant differences ($P < 0.05$). flavonoid kaempferol and commercial milk booster increased milk production 17%, 51%, and

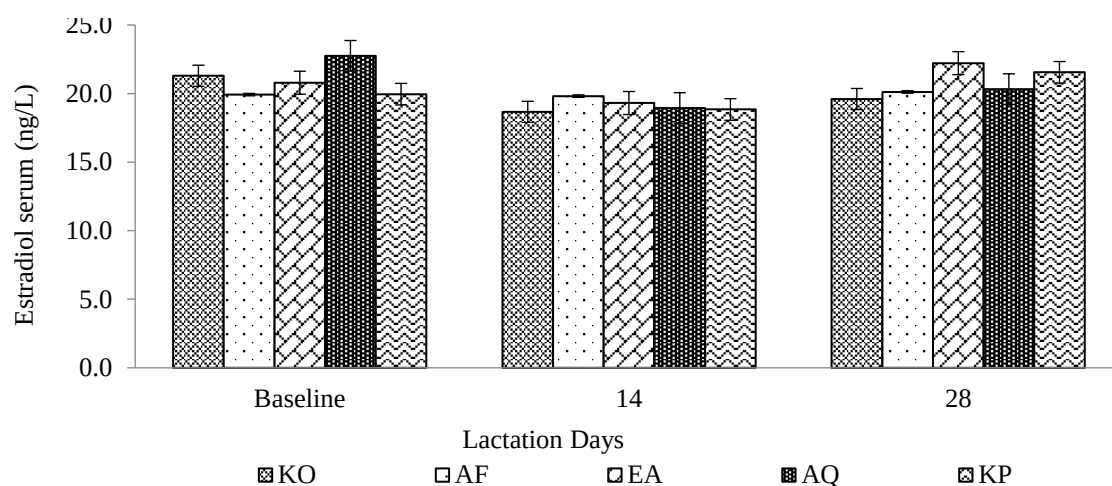


Figure 1. The levels of estradiol of lactating rats during days 14 and days 28. Baseline (days 2 after postpartum before getting intervention). KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group. Data are expressed as mean \pm SE. *shows significant differences ($P < 0.05$).

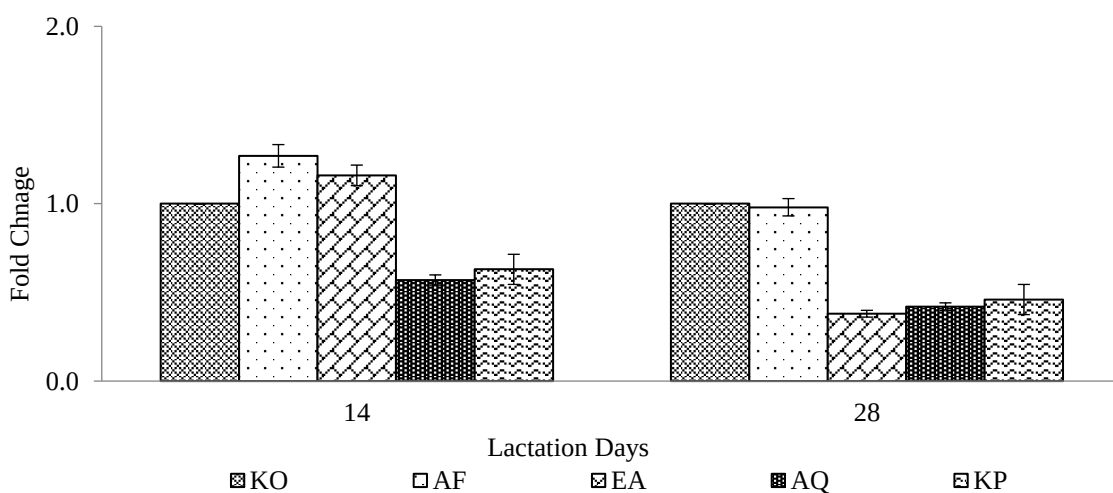


Figure 2. The Expression of the estradiol receptor gene ($ER\alpha$) of the mammary gland in lactating rats. These are real-time PCR results and fold change is with respect to the housekeeping gene. KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group. Data are expressed as mean \pm SE. * shows significant differences ($P < 0.05$).

22%, respectively, compared to the control [6, 23]. According to a study estrogen plays an important role in the growth, differentiation, and function of various types of tissues, especially the mammary glands [24]. The levels of estradiol (E2) hormone serum in lactating rats are shown in Figure 1.

The levels of E2 hormone serum on Day 14 and Day 28 of lactation period were not significantly affected by the administration of the *torbangun* leaves extract and its fraction ($P > 0.05$).

The results indicated that the *torbangun* leaves extract and its fraction, especially bioactive compounds, did not affect the levels of estradiol (E2) hormone in the serum of lactating rats. This can be caused by several factors, such as inappropriate dosage of treatment, it may be that the dosage was used just as limited as antioxidants and were not able to affect estradiol receptors. The concentration levels of flavonoid compounds mainly phytoestrogens, can affect the activity of estradiol receptors.

The water extract and ethyl acetate fraction of *torbangun* leaves contained phenolics, flavonoids, and esters [6, 23]. The *torbangun* leaves extract and its fraction contained quercetin, rutin and kaempferol [5].

Prolactin (Prl) is a polypeptide hormone secreted by the hypothalamus (anterior pituitary). Prolactin has a role in water and electrolyte balance, growth, and development, reproduction, regulation of the immune system and actions related to pathophysiological diseases [26]. According to [14], the regulation of the prolactin hormone in the hypothalamus was affected by estradiol and the administration of estradiol (exogenous) may increase gene transcription and the levels of prolactin mRNA.

Our previous studies [6] shows that the administration of the *torbangun* leaves extract and its fraction has not affected the levels of prolactin hormone during lactating at Day 14 and Day 28. These results are in disagreement with [27] that reported; the feed fenugreek seeds can increase milk production followed by increased levels of serum prolactin. The difference in these results might be in the extracts, and their fractions of *torbangun* leaves do not give effect to Estradiol Receptor (ER), so it can not affect the expression of prolactin gene in the hypothalamus. However, the tendency of the prolactin levels decreased with increasing days of lactation.

The increased milk production observed in this study may be due to the ability of bioactive compounds of *torbangun* leaves to induce expression of prolactin receptor gene, by up-regulation of casein production and lactose synthetase enzyme activity in epithelial cells of mammary glands [28].

The expression of estradiol and prolactin receptor genes

The estrogen receptor is the core receptor of the fat-soluble estradiol (E2) receptor, it caused receptor transformation into a dimmer form and initiate gene transcription where the E2 molecule enters into the cell freely, through the cell wall and binds to the ligand binding domain of the intracellular of estrogen receptor [29].

In this study, the effect of administration of *torbangun* leaves on the expression of the estradiol receptor gene (ER α) in the mammary gland of lac-

tating rats is presented in Figure 2. The results showed that the expression of the estradiol receptor gene (ER α) on the mammary gland of lactating rats on day 14 was not influenced by the bioactive compounds that contained in water extract or ethyl acetate fraction of *torbangun* leaves ($P > 0.05$). However, on day 28 of the lactation period, the administration of kaempferol, water extract and ethyl acetate fraction of *torbangun* leaves influenced the expression response of estradiol receptor gene (ER α) ($P < 0.05$). The expression of the estradiol receptor gene down-regulated by an ethyl acetate fraction (0.62-fold), water extract of *torbangun* (0.58-fold), and kaempferol (0.53-fold) compared to control (Figure 2).

These results indicated that at day 28 during the lactation period, the levels of estradiol hormone differ significantly, and the bioactive compounds (phytoestrogens) of *torbangun* leaves had estrogenic activity in the mammary glands of lactating rats. According to [30], the estrogenic potential of a component is influenced by several factors, such as the differential effect on the transactivation functionality of the receptor, the involved co-activator, and the promoter of the gene and the target cell. Kaempferol had a lower potency and transactivation activity than the 17- β -estradiol (E2) of ER α (0.35) and ER β (0.53) [25].

The prolactin receptor (PRLR) is present in the plasma membrane of each target tissue. In the lactation period, the prolactin and its receptors bind to the mammary glands and can determine the quantity of milk to be produced [12]. According to [31], when the prolactin hormone and its receptors were binding, transcription of the active target genes and biological effects occurs. The average expression of prolactin receptor gene (PRLR) in the mammary gland of lactating rats after administration extract of *torbangun* leaves and its fraction was shown in Figure 3.

Figure 3 showed that the expression of prolactin receptor gene (PRLR) in the mammary gland on day 14 of lactating rats was influenced by the bioactive compounds that contain in extracts and its fraction of *torbangun* leaves ($P < 0.05$). The kaempferol group (KP) showed highest expression of the prolactin receptor gene with 3-fold up-regulation by rats during lactation period followed by AF(2-fold) > AQ and EA (1.5-fold) compared to the control group. Similarly, the commercial milk booster group (AF) showed the highest up-

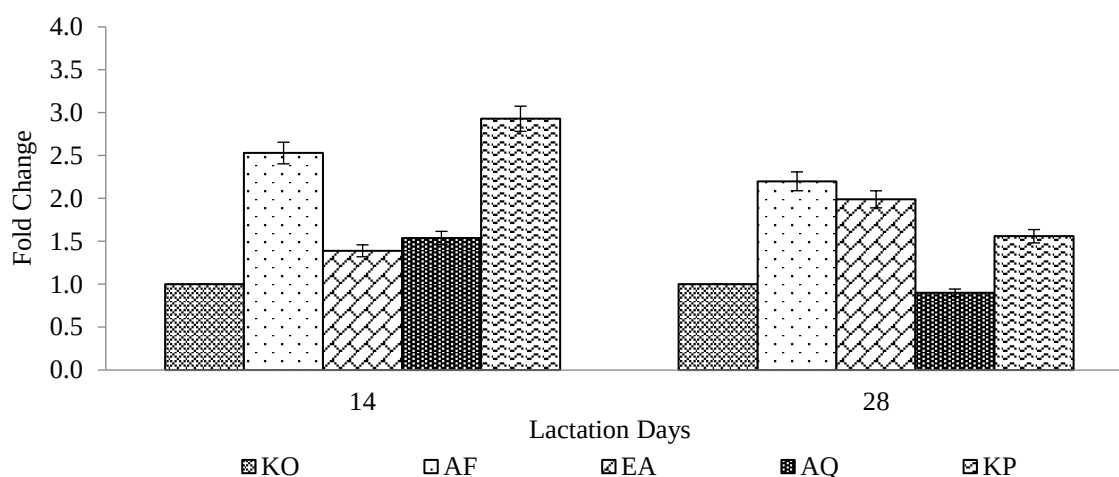


Figure 3. The Expression of the prolactin receptor gene (PRLR) of the mammary gland in lactating rats. These are real-time PCR results and fold change is with respect to the housekeeping gene. KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group. Data are expressed as mean \pm SE. * shows significant differences ($P < 0.05$).

Table 2. The summarized results of the expression of the estradiol receptor gene ($ER\alpha$) and prolactin receptor gene (PRLR) of the mammary gland in lactating rats (Different alphabet shows significant differences ($P < 0.05$))

Treatments	$ER\alpha/\beta$ -actin (C_t)	Fold change	PRLR/ β -actin (C_t)	Fold change
<i>Day-14:</i>				
KO	5.99 \pm 0.65	1.00 ^c	8.36 \pm 0.55	1.00 ^e
AF	5.65 \pm 0.47	1.27 ^a	7.02 \pm 0.44	2.53 ^b
EA	5.77 \pm 0.18	1.17 ^b	7.88 \pm 0.11	1.39 ^d
AQ	6.79 \pm 0.35	0.57 ^e	7.74 \pm 0.08	1.54 ^c
KP	6.66 \pm 0.44	0.63 ^d	6.81 \pm 0.23	2.93 ^a
<i>Day-28:</i>				
KO	6.95 \pm 0.63	1.00 ^a	8.69 \pm 0.18	1.00 ^d
AF	5.98 \pm 0.29	0.98 ^b	7.60 \pm 0.38	2.13 ^a
EA	8.33 \pm 0.02	0.38 ^e	7.70 \pm 0.70	1.99 ^b
AQ	8.21 \pm 0.15	0.41 ^d	8.84 \pm 0.03	0.90 ^e
KP	8.06 \pm 0.22	0.46 ^c	8.09 \pm 0.09	1.52 ^c

regulation of prolactin receptor gene (2.7-fold) followed by EA (2.4-fold) > KP (1.5-fold) > control at day 29 during the lactation period. The results were in agreements with [32], which reported administration of flavonoid “rutin” increased the levels of prolactin hormone and up-regulated the expression of prolactin receptor genes in the mammary gland of vasectomy rats. Moreover, the administration of methanolic extract of *torbangun* leaves reported increased expression of prolactin receptor gene in MCF-12A cell lines [33].

Conclusion

Ethyl acetate fraction of *torbangun* leaves dos-

age 30 mg/kg BW (EA) and kaempferol dosage of 60 mg/kg BW (KP) did not significantly affect the stimulation of serum prolactin synthesis and estradiol at day 14 and day 28 of the lactation period. On day 28 of the lactation period, bioactive compounds of EA, KP, and AF significantly affected the expression of estradiol receptor genes by down-regulating their expression. Meanwhile, bioactive compounds of EA, KP, and AF affected the expression of prolactin receptor gene (PRLR) by up-regulating their expression on the mammary glands at day 14 and day 28. This study indicates that ethyl acetate fraction of *torbangun* leaves induced milk production, by down-regulating the

gene expression of estradiol receptor (ER α), and up-regulating the gene expression of prolactin receptor (PRLR) in the mammary gland of lactating rats.

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