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Torbangun (*Plectranthus amboinicus* (Lour.) Spreng) Leaf Extract Upregulates Expression of Lactation-Related Genes in Human Mammary Epithelial Cells MCF-12A

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***Abstract*— Torbangun (*Plectranthus amboinicus* (Lour). Spreng) leaf is a herbaceous plant capable of increasing breast milk, although the mechanism of bioactive compounds on the molecular level remains relatively unknown. Therefore, this study aimed to evaluate the effect of torbangun leaf extract on the expression of genes associated with alveologenesis and the formation of milk protein, namely prolactin receptor (PRLR), glucocorticoid receptor (GR), signal transducer, as well as activator of transcription 5A (STAT5A) and β-casein (CSN2). The result showed that the extract and fractions of torbangun leaf had IC_{50} values above 20 ug/ml against MCF-12A cells. Ethyl acetate fraction also enhanced the expression of gene GR, STAT5A, and CSN2. Water fraction of torbangun leaf enhanced expression of gene PRLR, GR, STAT5A, and CSN2. The bioactive compound was found to increase the expression of genes associated with proliferation, alveologenesis, and the formation of milk protein during gestation and lactation.**

***Keywords*— *Plectranthus amboinicus*, lactation, STAT5A, prolactin, glucocorticoid, alveologenesis**

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I. INTRODUCTION

Torbangun (*Plectranthus amboinicus* (Lour). Spreng) is a herbaceous plant in the family of *Lamiaceae*. This plant has been widely consumed in Indonesia, particularly among Bataknese population to enhance breast milk production of nursing mothers and maternal health. The main contents of torbangun leaf include carvacrol, thymol, humelene, terpinene, undecanal, cymene, terpineol, caryophylene oxide, and salinene which can stimulate breast milk [1]. Previous studies in animal models and lactating mothers showed that torbangun leaf administration in food could increase milk production and prolactin hormone level [2], [3]. Furthermore, it can be developed as a galactogogue, a compound with the potential to initiate, maintain, or increase breast milk volume [4].

Breast milk production is a complex process including various hormones that have been initiated since pregnancy. During pregnancy, the mammary gland undergoes major changes in preparation for lactation, which include cell maturation and alveologenesis. The Luminal epithelial cells in the lobules will differentiate into alveolar cells to produce milk [5]. Mammary gland epithelial cells also differentiate into lobuloalveolar under the influence of steroid and peptide hormones. Gland differentiation and breast milk production are mainly influenced by prolactin and glucocorticoid hormones, which bind to their receptors located on mammary gland epithelial

cells and activate the pathway associated with alveologenesis and milk protein formation. Prolactin and glucocorticoid are representative lactogenic hormones that activate the signal transducer and transcription 5 (STAT5) as well as glucocorticoid receptor (GR) pathways [6]. Prolactin receptor (PRLR)/STAT5 path is required for alveologenesis and milk protein gene expression [7]. Prolactin and PRLR play an important role in milk production [8]. During this process, prolactin will bind to PRLR in the mammary gland epithelial cells and activate janus kinase2 (Jak2). Subsequently, Jak2 would phosphorylate transcription 5A (STAT5A) in the cytoplasm followed by STAT5A dimerization and translocation into the nucleus. This allows the activation of gene transcription related to lactation function. PRLR gene expression in the mammary gland increases during pregnancy and early lactation, followed by a decrease at the end of lactation period [9]. Previous studies showed that expression of PRLR and STAT5A in breast milk increased sharply until day-4 postpartum [10]. In the absence of PRLR, lactation would fail [11] due to the initial development of glucocorticoid modulates mammary gland [12]. GR is a transcription factor located in the cytoplasm. When bound to the ligand, the activated GR will translocate to the nucleus and bind to DNA, modulating the target gene. Furthermore, an activated GR can interact with activated STAT5A, forming complex molecules and cooperate to transcribe β-casein (CSN2) gene [13], which is a milk protein widely available in breast milk.The majority of galactogogue functions by enhancing prolactin production from the anterior pituitary gland. This enhancement is performed by suppressing hypothalamus to hinder the prolactin inhibitor factor and stimulate the hypothalamus during lactation [14]. Despite the significant potential, the mechanism of bioactive compounds in torbangun leaf in affecting breast milk production at the cellular level is still unknown. Therefore, this study aimed to evaluate the effect of torbangun leaf extract on the expression of genes associated with alveologenesis and the formation of milk protein, namely PRLR, GR, signal transducer, as well as STAT5A and β-casein (CSN2)

II. MATERIAL AND METHODS

A. *Material*

All reagents used in this study were of analytical grade. Methanol, n-hexane, chloroform, ethyl acetate, H_2SO_4, HCl, ethanol, DMSO, Folin-Ciocalteu reagent, Na_2CO_3, CH_3COOH, NaOH were from Merck (Germany). Dragendorff's, Mayer's, Wagner's reagent, $FeCl_3$, $AlCl_3$, CH_3COOK, gallic acid, quercetin, *3*-(*4*,*5*-dimethylthiazol-*2*-yl)-*2*,*5*-diphenyltetrazolium *bromide* (MTT) were obtained from Sigma-Aldrich (USA). MCF-12A cell line (ATCC CRL-10782) was purchased from ATCC (USA). Mammary epithelial cell growth medium (MEGM CC-3150) was obtained from Lonza (USA). *P*hosphate buffered saline (PBS) and MTT were obtained from Sigma (USA). The RNeasy mini kit was purchased from Qiagen (Germany) and PCR master mix used was KAPA SYBR FAST Bio-Rad iCyclerTM One-Step qRT-PCR from Kapa Biosystem (USA).

B. *Extraction and Fractionation of Torbangun Leaf*

Torbangun leaf used in this study originated from Experimental Farm in Leuwikoppo IPB (Bogor Agricultural Institute), Dramaga, Bogor. The samples collected were identified by the Research Center for Biology, Indonesian Institute of Sciences (certificate was shown in supplementary). For the experiment, torbangun leaf was freeze-dried and produced into powder, which was extracted using 80% methanol solvent (1:20) with a sonicator (Branson) for 50 minutes. Filtration was conducted using Whatman 42 filter paper and the residue was re-extracted using 80% methanol. Subsequently, the filtrate obtained was combined and evaporated using a rotary vacuum evaporator (Buchi) at 40-50°C. Methanol extracts obtained were further dried using nitrogen gas. Partial methanol extracts were dissolved in water and fractionated using n-hexane solvent, chloroform, and ethyl acetate using a separating funnel. The fraction obtained was concentrated using a rotary vacuum evaporator and dried with nitrogen gas. Extraction and fractionation processes were performed 3 times.

C. *Qualitative Phytochemical Analysis*

Alkaloid test

NH_3 was added into torbangun leaf extract, dissolved, followed by mixing with 5 ml of $CHCl_3$ and filtering through filter paper. Furthermore, 2 M H_2SO_4 was added into previously obtained filtrate and divided into 3 parts. Dragendroff, Mayer's, and Wagner's reagents were added to each part. Alkaloid presence was shown by color change of the sediments to orange (with Dragendroff), white (with Mayer), and brown (with Wagner).

Flavonoid, tannin, and saponin tests

Torbangun leaf extract was dissolved in distilled water, heated for 5 minutes, and filtered using filter paper. The filtrate was divided into 3 parts to test flavonoid, tannin, and saponin. Mg powder was added into the first filtrate, followed by 10 drops of HCl solution:ethanol (1:1) and 10 drops of amyl alcohol. The color change of the alcohol layer into orange showed flavonoid content. This was followed by adding 3 drops of $FeCl_3$ into the second filtrate and tannin presence was indicated by a color change to dark green. The third filtrate was heated for 5 minutes, shaken strongly, and evaluated for the formation of stable foams to show saponin content.

Steroid and triterpenoid tests

Torbangun leaf extract was added to 5 mL of hot ethanol and filtered with filter paper. The filtrate was heated until dry, added with 1 mL diethyl ether, and homogenized. A drop of concentrated H_2SO_4 was added, followed by a drop of anhydrous CH_3COOH. The presence of steroid and triterpenoids was shown by a color change of the solution into green/blue or red/purple, respectively.

Hydroquinone test

Torbangun leaf extract was dissolved in 5 mL of methanol, heated, and filtered using filter paper. Subsequently, 3 drops of 10% NaOH were added to the filtrate. Hydroquinone presence was shown by a red color change in the solution.

D. *Determination of Total Phenol*
Total phenol in torbangun leaf extract was measured with Folin-Ciocalteu colorimetric method according to Majd et al. [15] with slight modifications. A 200 µl extract was inserted into a reaction tube, added with 1 ml of 10% Folin-Ciocalteu solution, set for a minute, followed by 3 ml of Na_2CO_3, and vortexed. After being stored in a dark room at room temperature for 2 hours, the absorbance was measured by UV-Vis spectrophotometer (UV-2450, Shimadzu) at a wavelength of 760 nm. Total phenol was determined based on the gallic acid standard curve and expressed as mg of gallic acid equivalent per gram plant extract (mg GAE/g extract).

E. *Determination of Total Flavonoid*
Total flavonoid in torbangun leaf extract was colorimetrically measured by referring to Shah and Hosain's [16] with slight modification. A 500 µl extract was inserted into a reaction tube, added with 100 µl of 10% $AlCl_3$, 100 µl of 1 M CH_3COOK, 4.3 ml of distilled water, and vortexed. After being stored at room temperature for 30 minutes, the absorbance was measured by UV-Vis spectrophotometer (UV-2450, Shimadzu) at a wavelength of 415 nm. Total flavonoid was determined based on quercetin standard curve and expressed as mg of quercetin equivalent per gram plant extract (mg QE/g extract).

F. *Cell Viability Test*
Viability of MCF-12A cells treated with torbangun leaf extract was conducted using MTT *assay.* The extract was dissolved in mammary epithelial medium MEGM. A series of dilutions were conducted to obtain test solutions. Concentrations of the extract tested were 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1,000 µg/ml. A 100 µL of cell suspension in MEGM medium (with 5 x 10^3 MCF-12A cell density) was inserted into each well of a 96-well plate and incubated in an incubator at 37°C with 5% CO_2 for 24 hours. The medium was removed and replaced with torbangun leaf extract solution, followed by incubation for 48 hours. The medium was removed, washed using PBS, and added with 10 µl of MTT solution with 5.000 µg/ml in each well, and incubated for 4 hours. Viable cells reacted with MTT to form a purple-colored formazan. The existing medium was removed and added with 100 µl of 96% ethanol to dissolve the formazan. Absorbance values were read using a microplate reader (Biorad) at a wavelength of 595 nm. The percentage of viable cells was calculated by (A-C)/(B-C)* 100% formula where A, B, and C represented absorbance of samples (with torbangun leaf extract), control cells (without extract), and medium (without extracts and cells), respectively. Correlations between log concentration and the percentage of viable cells were used to calculate IC_{50} value, which indicated the potential cytotoxicity of the extract.

G. *Quantitative Real-Time PCR*
Approximately 3.9 x 10^6 MCF-12A cells were grown in a 6-well plate, incubated at 37°C with 5% of CO_2 for 24 hours. The medium was removed and immediately supplemented with the testing materials, which were n-hexane, chloroform, ethyl acetate, and water fractions of torbangun leaf with a concentration of 25 µg/ml. Samples were incubated for 4 hours and untreated cells were used as reference. The medium was removed, cells were washed with PBS, harvested using trypsin and further processed into cell pellets.
RNA from MCF-12A cells were extracted using an RNeasy mini kit (QIAGEN) and the concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific). Genes evaluated in this study were PRLR, GR, signal transducer, and activator of STAT5A and CSN2 (primer sequences are listed in Supplementary Table 1). PCR master mix used was KAPA SYBR FAST Bio-Rad iCyclerTM One-Step qRT-PCR. PCR was performed using a qRT-PCR machine (Biorad) in a reaction volume of 20 µl. The amount of RNA used per reaction was 15 ng. The PCR condition was as follows 42°C, 5 minutes for reverse transcriptase activation, 95°C, 5 minutes for reverse transcriptase inactivation, 35 cycles at a temperature of 95°C for 5 seconds (denaturation), and 55°C for 30 seconds (annealing), except for β- casein where annealing temperature was 61°C. Ct values were normalized to housekeeping gene Glyceradehyde 3-Phosphate Dehydrogenase (*GAPDH*). Relative gene expression was calculated using ΔΔCt method and expressed as relative expression (in fold change).

H. *Statistical and Data Analysis*
SPSS was used to perform one-way Analysis of Variance (ANOVA) followed by paired Duncan test on the phenolic and flavonoid content data. The independent t-test was used for the evaluation of gene expression data, with $P<0.05$ considered significant. Pearson's correlation test was performed to evaluate the correlation between phenolic compound in torbangun leaf extract and CSN2 expression.

III. Result and Discussion

A. *Phytochemical*
Phytochemical content of torbangun leaf extract in **Table 1** showed the presence of flavonoid, tannin, saponin, and steroid, but did not contain alkaloid and quinone. These results were in line with Paramasivam *et al.* [17] showing major contents in torbangun leaf were flavonoid and tannin. Steroid contents were mostly found in n-hexane fraction followed by chloroform. High levels of tannin and flavonoid contents were found in ethyl acetate and water fractions. Previous reports showed flavonoid, saponin, and steroid were largely found compounds in galactogogue plants [18], [19], although tannin did not affect the fat and protein content of cow milk [20].

TABLE 1
PHYTOCHEMICAL TORBANGUN LEAF EXTRACT

No	Phytochemical	n-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Water fraction
1	Alkaloid	-	-	-	-
2	Flavonoid	+	++	+++	+++
3	Tannin	-	+	+++	+++
4	Saponin	+	-	+	++
5	Steroid	+++	++	+	+
6	Triterpenoid	-	-	+	-
7	Quinon	-	-	-	-

+++ = High; ++ = middle; + = low; - = nothing

Total phenol and flavonoid in torbangun leaf fraction are presented in **Table 2**. The highest phenolic content was found in ethyl acetate fraction (429.81 mg GAE/g extract) and the lowest was in n-hexane fraction (44.97 mg GAE/g extract). Total phenolic content in each fraction, from highest to lowest was as follows: ethyl acetate> water> methanol> chloroform> n-hexane. Specifically, phenolic contents in ethyl acetate fraction were 8 times higher than n-hexane fraction. This showed that phenolic contents present in torbangun leaf were mostly soluble in semi-polar solvent (ethyl acetate). Phenolic contents in torbangun leaf extract were carvacrol, syringic acid, thymol, and vanilin [21].

TABLE 2.
TOTAL PHENOLIC AND FLAVONOID CONTENT OF TORBANGUN LEAF EXTRACT

No	Fraction	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid Content (mg QE/g extract)
1	n-Hexane fraction	44.97 ± 1.15 a	63.39 ± 5.10 a
2	Chloroform fraction	65.95 ± 9.86 a	56.62 ± 4.19 a
3	Ethyl acetate fraction	429.81 ± 18.94 c	89.85 ± 3.55 c
4	Water fraction	319.62 ± 4.10 b	48.82 ± 1.66 b

The value is the means ±SEM (n=3); a,b, and c show significantly different results by duncan test, $p<0.05$

The highest flavonoid contents were found in ethyl acetate fraction (89.85 ± 3.55 mg QE/g extract), while the lowest was in water fraction (48.82 ± 1.66 mg QE/g extract). Flavonoid contents in each fraction from the highest to the lowest were as follows ethyl acetate> n-hexane> methanol extract> chloroform> water. Total flavonoid in ethyl acetate fraction was 2 times higher compared to water fraction. This suggested that flavonoid contents such as luteolin, myricetin, kaempferol, quercetin, and apigenin in torbangun leaf extract were highly soluble in ethyl acetate solvent. The characteristics of each solvent (polar, semi polar and nonpolar) will affect its ability to extract the chemical components [22] .
The highest flavonoid contents were found in ethyl acetate fraction (89.85 ± 3.55 mg QE/g extract), while the lowest was in water (48.82 ± 1.66 mg QE/g extract). Flavonoid contents in each fraction from the highest to the lowest were as follows ethyl acetate> n-hexane> methanol> chloroform> water. Total flavonoid in ethyl acetate fraction were 2 times higher compared to water. This showed that flavonoid contents in torbangun leaf extract were highly soluble in ethyl acetate solvent. Ethyl[23]. acetate can extract flavonoids from various kinds of spice plants Consumption of foods containing flavonoid causes flavonoid content in breast milk to increase [24]

B. *Toxicity*
MTT assay showed that extract concentration significantly affected viability of MCF-12A cells. At a low concentration of 7.81 µg/ml, viability with all extracts was high. At a high concentration of 62.5 µg/ml, viable cells in n-hexane and chloroform fractions decreased to 36 and 38%, respectively. Meanwhile, in ethyl acetate and water fractions, the numbers were still above 80%. This result suggested that potentially toxic compounds in torbangun leaf were more extractable in n-hexane solvent and chloroform, compared to using ethyl acetate and water. IC_{50} values of torbangun leaf extract fractions are presented in **Fig 1**. Based on IC_{50} values, the order of toxicity levels was n-hexane> chloroform> ethyl acetate. In water fraction, the use of high concentrations up to 1000 µg/ml produced viability of above 50%, which limited the calculation of IC_{50}. According to *U.S Cancer Institute*, plant crude extract could be considered toxic when *in vitro* tests possessed IC_{50} values with a concentration below 20 µg/mL. Based on the results, all fractions of torbangun leaf extract were safe and potentially non-toxic in mammary gland epithelial cells, at least in MCF-12A model. In vivo experiments in rat model showed that the aqua extract of torbangun leaf was non-toxic by acute and sub-acute toxicity tests [25].

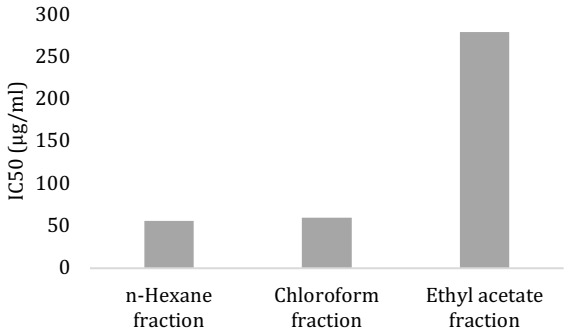

Fig 1. IC_{50} torbangun leaf extract on cell MCF-12A

C. *Expression of Lactation-related Genes*
This study was conducted to observe the effects of bioactive compounds in torbangun leaf extract on genes related to lactation regulation, at a cellular level using a human mammary gland epithelial cell line. Prolactin and GR mRNA expression were examined, as both are major hormones included in alveologenesis process and milk protein formation during

lactation. Ethyl acetate and water fractions were used due to their high IC_{50} values. The result showed that water fraction of torbangun leaf extract produced greater expression of PRLR by 1.5 times higher than the control, while this was not found with the treatment of ethyl acetate fraction (**Fig 2**). Prolactin and PRLR play an important role in the process of milk synthesis, with increased levels of mRNA and protein PRLR leading to higher milk yield [26]. However, rats lacking PRLR showed a formation of alveoli structures with small lumen. PRLR mRNA expression is known to increase in the mammary gland of pregnant experimental animals [27] and during lactation[9]. The interaction between prolactin and PRLR in lactation process is only determined by the number of PRLR [28]. In this study, increased expression of PRLR was relatively similar to MCF-12A cells treated with 0.1 µg/ml prolactin hormones [29]. Based on the results, bioactive compounds in torbangun leaf extract showed potential to have a similar effect to prolactin hormone in mammary gland epithelial cells. Moreover, extraction of bioactive compounds could use water and ethyl acetate which produced better results.

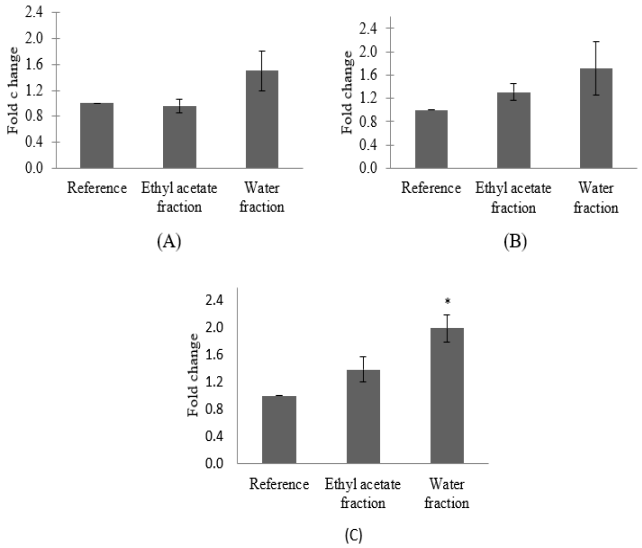

Fig 2. Effect of torbangun leaf extract (25 µg/ml; incubation 4 hours) on the expression of mRNA PRLR (A), GR (B), and STAT5A (C) in human MCF-12A cell line, compared to control (untreated cells, as reference). The value is the means ±SEM (n=3). * indicate significantly different results by t-test, $p<0.05$.

Water and ethyl acetate fractions of torbangun leaf increased GR mRNA expression by 1.7 and 1.3-fold respectively, relative to control (**Fig 3**). GR is required for cell proliferation during lobuloalveolar development without influencing differentiation and milk production. The amount of GR increases during pregnancy peaks at parturition and will remain high in lactation, until mammary gland involution at weaning [30].

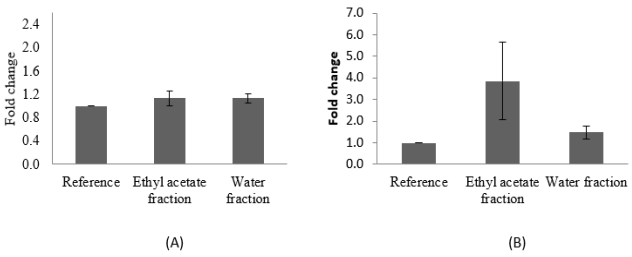

Fig 3. Effect of torbangun leaf extracts (25 µg/ml; incubation 4 hours) A) and (50 µg/ml; incubation72 hours) B) on the expression of mRNA CSN2 in human MCF-12a cell line, compared to control (untreated cells, as reference). The value is the means ±SEM (n=3).

In addition to prolactin hormone receptors and glucocorticoid, this study also evaluated STAT5A and CSN2 expression. Ethyl acetate and water fractions increased STAT5A expression (**Figs 2 and 3**) by 1.4-fold and 2-fold, respectively. STAT5A is a gene that plays an important role in cell proliferation, increasing differentiation, and forming milk protein. The pathway Jak2 and STATs genes play an essential role in milk production [31], [32]. STAT5A expression increased by 2.5-fold during the first 72 hours after birth [10] and affects alveologenesis processes in the mammary gland epithelial cells. Without STAT5A, the number of luminal progenitors is significantly reduced, along with ductal growth that lacks alveoli formation during pregnancy. In previous reports, rats without STAT5A had lower alveolar numbers by 50%, and gene expression of whey acidic protein (WAP) and α-lactalbumin (LALBA) reduced by 32 and 23%, respectively [33]. The increase of STAT5A in this study was a similar response to the effect of prolactin hormone on STAT5A mRNA expression found in previous reports. The results showed that administration of 1 µg/ml of prolactin hormone and 1 µg/ml of hydrocortisone in HC-11 cells increased STAT5A mRNA expression by approximately 3.5-fold in 4 hours [34]. The administration of 1 µg/ml of prolactin hormone in MCF-12A cells also increased phosphorylated STAT5A protein [29]. STAT5A could be activated through PRLR, as well as growth hormone receptor (GHR) and epidermal growth factor receptor (EGFR). However, GHR and EGFR in epithelial cells were not needed for alveolar development and formation in the mammary gland. Signaling through PRLR and STAT5A are essential factors in milk production [35].

Administration of torbangun leaf extract with a concentration of 25 µg/ml and 4-hour-long incubation time showed relatively no effect on CSN2 mRNA expression (**Fig 3A**). However, with a concentration of 50 µg/ml and 72-hour-long incubation time, ethyl acetate and water fractions increased CSN2 expression by 3.9-fold and 1.5-fold, respectively (**Fig 3B**). The concentration of the extract influenced the increase in CSN2 mRNA expression. The effect of torbangun leaf extract on CSN2 mRNA expression in this study was in line with the results in MCF-12A cells treated with 0.1 µg/ml prolactin hormone. Meanwhile, expressions of CSN2 mRNA and CSN2 protein

increased by 2-fold on day 7 [29]. CSN2 expression is regulated by lactogenic hormones and transcription factors of STAT5A. The effects of prolactin are mediated by binding to extracellular PRLR, which binds and activates intracytoplasmic targets of Jak2/STAT signaling pathway, such as CSN2 [26]. Prolactin and glucocorticoid hormones synergized to induce CSN2 expression in mammary epithelial cell [36].

CSN2 in mammary glands was produced through PRLR, as well as GHR. The data showed that torbangun leaf extract increased expression of genes associated with proliferation, lobuloalveolar differentiation, and milk protein formation, which were PRLR, GR, STAT5A, and CSN2. These results were believed to be the underlying mechanism of torbangun leaf extract potential to affect breast milk production. The proposed pathway of how torbangun leaf extract increases CSN2 is presented in **Fig 4**.

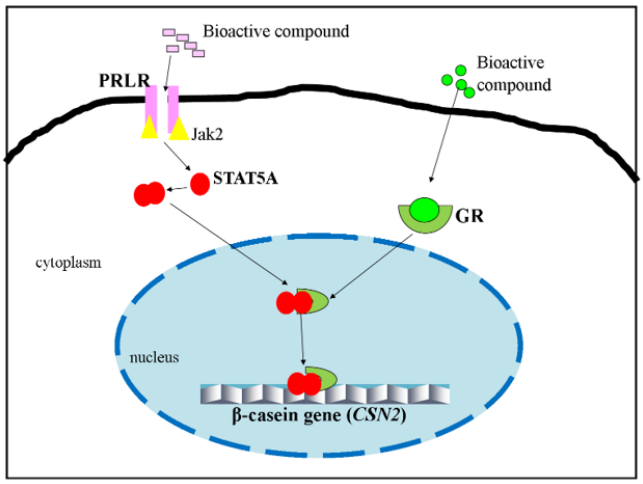

Fig 4. The proposed pathway on how torbangun leaf extract increases breast milk at the molecular level in human mammary gland epithelial cell.

This study proposes the mechanism of how torbangun leaf extract increases breast milk at the molecular level in human mammary gland epithelial cell. Initially, the bioactive compounds of torbangun leaf bind to PRLR in epithelial cells' membrane to further activate STAT5A. An activated STAT5A will translocate to the cell nucleus and transcribe CSN2 gene. Compounds in torbangun leaf that may presumably bind to PRLR are flavonoid such as quercetin and kaempferol. In silico method has previously shown that quercetin and kaempferol bind to active PRLR, with the amino acid sequences of Phe, Ile, Tyr, His, Arg, Val, and Asp [37]. Meanwhile, the results showed that bioactive compounds in torbangun leaf extract could also affect GR. As a coactivator of STAT5A in transcribing CSN2 gene, GR activated by torbangun leaf extract would translocate into the nucleus and further interact with STAT5A to transcribe CSN2. The bioactive compounds that can increase GR expression are steroid, triterpenoid, saponin, and flavonoid. Some triterpenes and steroids have a similar structure to hormones such as steroid, sex hormones, and cortisone which modulate hormone response. In some cases, steroid, triterpene, and saponin have a similar structure to endogenous anti-inflammatory hormones such as glucocorticoid[38]. The bioactive compounds of torbangun leaf extract such as phenol and flavonoid are polar, which will bind to PRLR in the epithelial cell membrane, before transcribing CSN2 gene in the nucleus. Meanwhile, triterpenoid and steroid can directly enter the epithelial cells because their non-polar nature.

The phenolic compounds were largely found in torbangun leaf extract. Pearson's correlation coefficient value of 0.5 showed that the effect of phenolic compounds on CSN2 mRNA expression was relatively strong. **Fig 3B** shows that CSN2 mRNA expression was higher in ethyl acetate fraction compared to water fraction. These results were in line with a previous study, where phenolic compounds from grape seeds elevated prolactin serum levels in blood, the number of alveoli, and alveolar diameter, with reduced distance between alveoli [39] .

This study showed that torbangun leaf extract contained bioactive compounds capable of modulating the response of endogenous prolactin and glucocorticoid in improving CSN2 production in mammary gland epithelial cells. As prolactin and glucocorticoid were not used in this study, further investigation should be carried out to compare hormones with torbangun leaf extract, particularly in examining their effect on various lactation-related genes downstream of prolactin and GR. From this study, the synergy of various bioactive compounds in torbangun leaf was found to serve in regulating genes associated with lactation function. The main compounds in torbangun leaf contributing to breast milk production include phenolic, flavonoid, tannin, steroid, and saponin. Therefore, further studies should be carried out to investigate the role of each content in affecting breast milk production.

IV. CONCLUSION

In conclusion, this study showed that torbangun leaf extract contained tannin, flavonoid, steroid, and saponin, but there were no alkaloid or quinone. At the cellular level, water and ethyl acetate fractions increased the expression of genes important to lactation function regulation in mammary epithelial cells. Torbangun leaf extract increased CSN2 mRNA due to the effect on the pathway, including PRLR, STAT5A, and GR.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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