Evaluation Lactogenic Activity of Ethyl Acetate Fraction of Torbangun (*Coleus amboinicus* L.) Leaves

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**Abstract.** This study aimed to assess the lactogenic property of ethyl acetate fraction of torbangun (*Coleus amboinicus* L.) leaves and to identify the compounds that responsibility as ‘milk booster’ using LC-MS approach. Lactagogue activity was evaluated in terms of quantity of milk produced from the rats treated with commercial milk booster (AF), ethyl acetate fraction of torbangun leaves (EA), water extraction of torbangun (AQ) and kaempferol (KP). The feed was given orally every two days and starting from Day 2 after giving birth until Day 28. The performance of milk production was measured along the experimental period by weight-suckle-weight method. The level of prolactin serum was determined by ELISA methods. Histopathological analysis of mammary gland, liver, intestines and kidney tissues was carried out. Moreover, in order to profiling and identification of compounds of ethyl acetate fraction, ultra-performance liquid chromatography quadrupole time of flight to electrospray ionization mass spectrometry (UPLC-QTOF-ESI-MS) in the positive-ion mode was performed. The ethyl acetate fraction of torbangun leaves (EA) was induced milk production about 17%, and AF 22% and KP 51% compared to the control group. Meanwhile, the EA was not significantly stimulate the synthesis of serum prolactin at Day 14 and Day 28 (p>0.05). Administration of EA did not cause any signs or symptoms of toxicity. In addition, a total of ten compounds was identified by UPLC-QTOF-ESI/MS in the ethyl acetate fraction of the leaves of *C. amboinicus*, mostly phenolic compounds, flavonols and some of their glycoside derivatives, such as: digiprolatone, and kaempferol-3-7-O-di-rhamnopyranoside. The present study reveals the ethyl acetate fraction of torbangun leaves and its bioactive compounds has the potency as a remedy for stimulating and improving milk production.

1. **Introduction**

In recent decades, bioactive compounds derived from nature were beginning to gain attention to be developed as alternative therapies. A secondary metabolite of the plant is widely investigated for its bioactivity. According to [1] reported that about 80% of the world's population (4 billion people) used plant products to improve quality of health (nutraceuticals).

A systematic analysis of non-communicable diseases (NCD) risk factors, globally in 21 countries in 1999-2010, showed that not optimal of breastfeeding was one of the risk factors contributing to the...
development of NCD [2]. World Health Organization [3] recommended breastfeeding infants for 6 months. According to [4] reported that limited of milk production is the main reason for mother’s not giving exclusive breastfeeding to the infant. Limitations of milk production are influenced by factors, i.e., nutritional and endocrine factors (hormones) [5]. As an alternative solution to treat these problems, used galactopoetics herbs are highly recommended [6].

Prolactin plays an important role in the development of mammary glands during early gestation and the postpartum period [7]. Interaction of prolactin and its receptor (PRLR) on the glandular epithelial of membrane cell will determine the amount of milk production. The prolactin receptor is needed for the expression of milk proteins and lactation [8]. The medicinal-herb interactions associated with galactagogues have concrete evidence, but the evidence explaining the mechanism of action of herbs as galactagogues is still very limited and scares. One of the pharmacological-used plants has been used as galactagogues, is Coleus amboinicus L [9].

Torbangun (Coleus amboinicus L.), family Lamiaceae, believed can be used as food, food additives and medicines in treating various diseases, such as diarrhoea, flatulence, constipation, cough, chronic asthma, bronchitis, kidney, liver and malaria [10]. In vitro studies reported that the extract of C. amboinicus has antioxidant capacity, antibacterial and cytotoxic, anti-inflammatory and hepatoprotective [11-14]. Meanwhile, in vivo studies using animals and human experiments showed that this plant had anti-diabetic activity, immunomodulator, analgesic and lactagogue [15-21]. Many studies have examined extensively the bioactivity of these plants, but studies of exploration and identification of responsible components are scarce and rare.

In our previous studies, ethanolic extract and its fractions of C. amboinicus showed potent antioxidant activities [22]. Ethyl acetate fraction of this plant also has potential to increase milk production by 17% comprised with control and increasing rat pup’s performance [21]. Hence, the present work to evaluate the lactogenic property of ethyl acetate fraction of torbangun (C. amboinicus L.) leaves and to identify the compounds that responsibility as ‘milk booster’ using liquid chromatography – mass spectrophotometry (LC-MS) approach.

2. Material and methods

In this section, material and methods of this study are discussed.

2.1. Plant material

Coleus amboinicus (Lour.) leaves were collected from Sukabirus Garden-Bogor, Indonesia, ("S 06°39'58.3”; “E 106°52’28.7”; 558 mdpl), during the month of October 2015 – March 2016. The cutting interval of torbangun leaves were 60 days, and each cutting was cultivated with 25 cm radius from the other [23-24]. Botanical identification was performed by Dr. J. S Rahajoe, Botanist from “Herbarium Bogoriense”, Research Center for Biology, Indonesian Institute of Sciences, where the voucher specimen has been deposited (No.145/IPH.1.01/II.8/II/2015).

2.2 Procedure analysis

2.2.1 Quality control of medicinal plant materials

Standardization (quality control) of medicinal plant material, i.e., simplisia, comprises various analytical and phytochemical procedures. The analytical such as moisture content, ash values were described by [25], microbial content, heavy metal analysis measured by atomic absorption spectrophotometer (AAS) and phytochemical analysis.

2.2.2 Preparation samples of C. amboinicus

Samples of C. amboinicus (200 g) were washed and then extracted with ethanol 96% for 24 h for three times. The supernatant was filtered through filter paper (Whatman No. 1), then dried by vacuum evaporator. The ethanolic extract (20 g) was dissolved in 10 ml of methanol: water (with ratio 1:1), and filtered again, thus obtaining the crude extract. This crude extract was then partitioned with hexane (500 ml fractions repeatedly up to decoloration of the organic solvent), thus obtaining both the hexane fraction
and the “defatted” crude extract. The defatted crude extract was then successively partitioned with ethyl acetate (as in the hexane partition), thus obtaining the ethyl acetate (7.5 g), and aqueous fractions (2.7 g). The fractions, then dried by vacuum evaporator.

2.2.3 Preliminary phytochemical screening
The powdered of ethanolic extract and its fraction of C. amboinicus (Lour.) spreng were tested for the presence of sterols or triterpenes, flavonoids, tannins, saponin and alkaloids [26-28]. The results are given in Table 2.

2.2.4 Effect of oral treatment on milk production
Twenty five lactating dams weighing 250-300 g at the beginning of lactation and suckling six pups were used for this experiment [29]. The females were divided into five groups of five animals each. Group I (KO) was given aqueous as a control with vehicle orally, Group II (AF) was orally administered commercial milk booster (composition: 114 mg katuk leaf extract, 20 μg vitamin B12, 25 mg vitamin B2, 10 mg vitamin B1) at a dose of 50 mg/kg BW. Group III (EA) and Groups IV rats were orally administered 30 mg/kg of ethyl acetate fraction of torbangun and 80 mg/kg BW of aqueous extract of torbangun, respectively [18-19, 30]. Group V (KP) animals were treated orally kaempferol compound at a dose of 60 mg/kg BW. Milk production was measured from day 3 to day 15 of lactation by using method described by [30]. The ethical clearance approved by The Commission on Supervision of Animal Welfare and Veterinary Research, Research Institute and Community Service (LPPM), IPB No. 37-2016 IPB.

2.2.5 Serum prolactin and histopathological examination
On 14th day and 28th day of 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 was estimated by enzyme-linked immunosorbent assay (ELISA) method. Histopathological analysis, after termination of experiment, whole mammary gland and other vital organs viz., intestines, liver and kidney were excised from each experimental group of animal. Fibrosis as the effect of treatments was estimated by blind scoring method [31]. Similar fibrosis levels were subjectively grouped into 4 groups of scoring criteria, i.e., 0 (none, very little), +1 (mild), +2 (moderate), +3 (severe).

2.2.6 LC-MS analyses
An aliquot of ethyl acetate fractions (10 mg) of C. amboinicus was dissolved in methanol (8 ml) and 2 ml of ultra pure water for LC, respectively. The extract and fractions were filtered through 0.2 μm filters, and 1 μl of each extract and fraction were automatically injected into the LC system (UPLC-QTOF-ESI-MS: Acquity; Electrospray Ionization system; column UPLC@HSS T 3 1.8μm C18 (2.1 mm x 100 mm)), and the absorbance changes were monitored at 280 nm. Mobile phase: 0.1% formic acid in water solution (A) and 0.1% formic acid in methanol solution (B). The gradient elution: 5% B (1 minute), 5-40% B (7 minutes), 40-100% B (3 minutes), 100% B (2 minutes), 100-5% B (2 minutes). Total running time was 15 minutes with the flow rate used was 0.3 ml/min. The MS analyses were performed with the following conditions: positive mode scanning, the electrospray voltage was 4.7 Kv, and the capillary temperature and voltage were maintained at 250°C and 15v, respectively. The molecular ions were scanned from 100 to 1500 m/z. Peak identification: the bioactive compounds were identified by comparing their retention time and their mass spectra with those reported in literature.

2.3 Statistical Analysis
Data were presented in mean ± standard deviation, and then tested for normality. All statistical analysis was performed using Microsoft Excel 2010. The differences between treatments were analysed using ANOVA. Significant differences between mean values were determined using Duncan’s Multiple Range Test (α=5%).

(1.4 g)
3. Results

3.1 Quality control of Coleus amboinicus L.
In recent years, standardization and quality control of medicinal plants of therapeutic potential have been emphasized. Controlling of starting material is most important. The evaluation of chemical composition, microbial and heavy metal analysis should be carried out before any tests are undertaken. The qualities of simplisia of *C. amboinicus* were shown in Table 1.

| Parameters        | Values | Indonesia Food and Drug Regulation* |
|-------------------|--------|-------------------------------------|
| Water content     | 10.3%  | ≤ 10 %                              |
| Total ash         | 14.2%  | Max. 33.3%                          |
| Heavy metal:      |        |                                     |
| Arsenic           | < 0.003 mg kg\(^{-1}\) | ≤ 5 mg kg\(^{-1}\) |
| Lead              | < 0.040 mg kg\(^{-1}\) | ≤ 10 mg kg\(^{-1}\) |
| Mercury           | < 0.005 mg kg\(^{-1}\) | ≤ 0.5 mg kg\(^{-1}\) |
| Cadmium           | < 0.005 mg kg\(^{-1}\) | ≤ 0.3 mg kg\(^{-1}\) |
| Microbial content:|        |                                     |
| Total Plate Count | 1.58 \(\times\) 10\(^2\) CFU ml\(^{-1}\) | Max. 10\(^6\) CFU ml\(^{-1}\) |
| *E.Coli/ S. aeurus*| Negative | Negative |
| Mold or yeast     | Negative | Max. 10\(^4\) CFU ml\(^{-1}\) |

*Source: Indonesia National Agency of Drug and Food Control (INADFC) [32]*

3.2 Phytochemical screening
Phytochemical screening of ethanolic extract and its fraction of *C. amboinicus* (L.) showed that ethanolic extract is rich in phenolic compounds, this indicated by the presence of flavonoids, alkaloids, tannin, triterpenoids and saponin (Table 2).

| Phytoconstituents | EtOH | FHex | FEA | Fair |
|-------------------|------|------|-----|------|
| **Alkaloid**       |      |      |     |      |
| *Bouchardat*       | ++   | -    | ++  | -    |
| *Mayer*            | +    | +    | +   | +    |
| *Dragendorf*       | +    | ++   | ++  | ++   |
| **Triterpenoids**  | ++   | +++  | +   | -    |
| **Saponin**        | ++   | +++  | -   | +    |
| **Tannins**        | +    | -    | -   | ++   |
| **Flavonoids**     | ++   | -    | +++ | +    |

(+) = present; (-) = absent. EtOH: ethanolic extract; FHex: hexane fraction; FEA: ethyl acetate fraction; Fair: aqueous fraction
3.3 Milk production
The influences of lactagogue compounds with the ability to increase milk production were stimulated by different treatments are shown in Fig. 1.

![Figure 1](image1.png)

**Figure 1.** Average of milk yield (A) and change fold of milk production (B) during lactation period (days 2 – 14) (n=5). KO: control groups, AF: commercial milk booster groups, EA: ethyl acetate fraction of CA groups, AQ: aqueous fraction of CA groups, KP: kaempferol groups. a>b>c>d>e; the difference alphabetic showed significantly (p<0.05).

3.4 The levels of serum prolactin
The effect of lactagogue compounds on the level of serum prolactin is shown in Fig. 2.

![Figure 2](image2.png)

**Figure 2.** The levels of serum prolactin of lactating rats during days 14 and days 28. KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group. a>b>c>d>e; difference alphabetic showed significantly (p<0.05).
3.5 Toxicity profile and histological

Table 3. Scoring level of organ damage (fibrosis) at day 28 of lactation period

| Samples | Mammary gland | Liver | Intestine | Kidney |
|---------|---------------|-------|-----------|--------|
| KO      | 0             | 0     | 0         | 0      |
| AF      | 0             | 0     | 0         | +2     |
| EA      | 0             | 0     | 0         | 0      |
| AQ      | 0             | 0     | 0         | 0      |
| KP      | 0             | 0     | 0         | 0      |

(+2): more than one (mineralized) multifocal focus (2-5 focus of mineralization) in the cortex or renal medulla. KO: control group, AF: commercial milk booster group, EA: ethyl acetate fraction of CA group, AQ: aqueous fraction of CA group, KP: kaempferol group.

3.6 LC-MS analyses of ethyl acetate fraction of C. amboinicus

The ethyl acetate fraction of C.amboinicus leaves was injected into LC-MS to identify bioactive compounds. Ten major peaks were detected in the chromatogram from the ethyl acetate fraction of C.amboinicus leaves (Fig.3).

![Figure 3. LC-MS chromatogram of blank (A) and ethyl acetate fraction of C. amboinicus L. leaves (B) with retention time (Rt) at λ = 280 nm.](image)
Table 4. Predicted compounds from ethyl acetate fraction of C. amboinicus L. leaves

| No. | RT   | Mass Spectra | Compounds                                      | References |
|-----|------|--------------|------------------------------------------------|------------|
| 1   | 7.266| [M+H]+ 225.109 | 9-((2-Aminoethoxy)methyl)guanine | [41]       |
| 2   | 8.042| [M+H]+ 197.118 | Digiprolactone                               | [41]       |
| 3   | 8.641| [M+H]+ 225.109 | 9-((2-Aminoethoxy)methyl)guanine               |            |
| 4   | 8.705| [M+H]+ 197.118 | Digiprolactone                               | [41]       |
|     |      | [M+Na]+ 219.098 |                                               |            |
| 5   | 9.876| [M+H]+ 609.278 | Reserpine                                     | [42]       |
| 6   | 10.126| [M+H]+ 623.296 | Pectolinarin                                  | [43]       |
| 7   | 10.644| [M+H]+ 608.304 | Methyl phaeophorbide a                        | [44]       |
| 8   | 10.746| [M+H]+ 607.299 | unknown                                       |            |
| 9   | 11.142| [M+H]+ 775.535 | N9-Allylazithromycin                        | [41]       |
| 10  | 11.307| [M+H]+ 579.259 | Kaempferol-3, 7-O-di-rhamnopyranoside (kaempferitin) | [45]       |

4. Discussion

Phytochemical screening of ethanolic extract and its fraction of C. amboinicus (L.) showed that ethanolic extract is rich in phenolic compounds, this indicated by the presence of flavonoids, alkaloids, tannin, triterpenoids and saponin (Table 2). Phenolic compounds are accumulated at a fraction with high polarity, i.e. ethyl acetate and aqueous fractions. These results were consistent with the finding of [33] which reported C. amboinicus contained flavonoids, tannin and saponin. Table 2 also shows that the ethyl acetate fraction of C.amboinicus were negatively on saponin and tannin compounds. Moreover, the preliminary phytochemical screening results agreement with previous works that we have done, which showed the highest total phenolics content and antioxidant activity were present in the ethanolic extract followed by aqueous fraction and ethyl acetate fractions [22].

Fig. 1a showed that the milk yield of the ethyl acetate fraction of C.amboinicus (EA), kaempferol (KP) and commercial ‘milk booster’ (AF) groups was significant difference compared to the control group (P<0.05). The kaempferol group contained the highest on average of yields produced (9.17±2.00 gram/pup/day) by the rats during lactation period followed by EA and AF>KO and AQ. The figure also showed that the total milk production for 14 days of breastfeeding observation, kaempferol group (KP) had the highest total milk production (55 gram/14 days), followed by AF (45 gram/14 days)> EA (43 gram/14 days)> KO (36 grams/14 days) and AQ (35 grams/14 days) (P<0.05). Ethyl acetate fraction of torbangun leaves (EA) was induced milk production about 17%, AF 22% and KP 51% compared to the control group (Fig. 1b). These results agreement with [30] and [34], which reported administration extract and soup of C.amboinicus was given positive respond to increase milk production in lactating rats. It can be assumed that in the ethyl acetate fraction of C.amboinicus contained bioactive compounds as “galactagogue” which can modulate hormone in lactogenesis and lactation period.

According to [35] reported in galactopoetics herb plants, phytochemical compounds viz., polyphenols, tannins, and alkaloids have an effect to increase milk production, protein concentration and ovulation rate, bioavailability of protein digestion and secretion milk. Moreover, [36] suggested phytoestrogenic action and some molecules of phytoestrogen in herbal galactagogues may have effects similar to 17β-estradiol (E2) that promotes the proliferation of mammary epithelial cells (MEC) [37]. Some phytoestrogen molecules are mentioned, viz., diosgenin, estragole, silybin, shatavarine, kaempferol, and quercetin, could induce the expression of prolactin receptor (PRLR), upregulate casein production, and lactose synthetase activity in MEC [38]. In these results, kaempferol positively induced milk production about 51% compared to the control group (see Fig. 1b). In addition, present work shows
that the ethyl acetate fraction of *C. amboinicus* contained phenolic compounds i.e., digiprolactone and kaempferol derivatives.

Fig. 2 showed that concentration of serum prolactin not affected by all treatment in day 14 and day 28 (P>0.05). In contrast with these results, [39] reported that fenugreek increased milk production and concentration of serum prolactin was found higher in the fenugreek fed goats compared to control group. This difference results are probably due to the ethyl acetate fraction of *C. amboinicus*, kaempferol, or phytoestrogen molecules could not affect the prolactin (PRL) expression in the anterior pituitary, but they induce the expression of PRL receptor (PRLR), upregulate casein production, and lactose synthetase activity in MEC to increase milk production. According to [8] prolactin receptor (PRLR) is needed for the expression of milk proteins and lactation. Interaction of prolactin and its receptor (PRLR) on the glandular epithelial of membrane cell will determine the amount of milk production.

In this study, all treatment did not cause any behavioural alterations or deaths in rat when oral administration. In histological profile, generally no significant changes were observed in ethyl acetate fraction of *C. amboinicus* and all treated animals and controls (Table 3). It concludes that ethyl acetate fraction *C. amboinicus* and all treatment is safe and non toxic.

The ethyl acetate fraction of *C. amboinicus* leaves was injected into LC-MS to identify bioactive compounds. Ten major peaks were detected in the chromatogram from the ethyl acetate fraction of *C. amboinicus* leaves (Fig.3). The eluted peak at retention time of 10.64 minutes is the most numerous peak based on peak size. In the structure of components, a limitation of the database on LC-MS has forced the mass spectra analysed by manually [40]. Predicted compounds of the ethyl acetate fraction of *C. amboinicus* presented in Table 4.

According to [46] that digiprolactone had oestrogen activity, which can selectively activate the oestrogen receptor (ER). This compound identified may be used to increase proliferation of any cell that is capable of proliferation. In addition, kaempferol-3,7-O-di-rhamnopyanoside (kaempferitin) had phytoestrogenic action and have effects similar to 17β-estradiol (E2) that promotes the proliferation in MEC [38]. Meanwhile, as an antioxidant, pectolinarin also possess anti-inflammatory activity and might inhibit eicosanoid formation in inflammatory lesions [43]. The presence of digiprolactrone and kaempferol derivative following the synergic action between the two components in the ethyl acetate fraction is a factor that can be predicted to determine the ability to stimulate and increase milk production as shown by the ethyl acetate fraction of *C. amboinicus*.

5. Conclusion

The ethanolic extract is rich in phenolic compounds, this indicated by the presence of flavonoids, tannin and saponin. Kaempferol group (KP) with dose 60 mg/kg body weight had the highest total milk production followed by AF>EA> KO and AQ. Ethyl acetate fraction of torbangun leaves (EA) with dose 30 mg/kg body weight was induced milk production about 17% compared to the control group. Meanwhile, there was not significantly affected to stimulate the synthesis of serum prolactin at Day 14 and Day 28. Generally, administration of EA and other treatment did not cause any signs or symptoms of toxicity. The presence of digiprolactrone and kaempferol derivative following the synergic action between the two components in the ethyl acetate fraction is a factor that can be predicted to determine the ability to stimulate and increase milk production as shown by the ethyl acetate fraction of *C. amboinicus*.

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