

Safety Evaluation of Ethanol Extract from Torbangun Leaves (*Coleus amboinicus* Lour.) on Mouse Fetal Development

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ABSTRACT

The purpose of this study was to evaluate the safety of ethanol extract from torbangun leaves on mouse fetal development. This research used 24 female pregnant mouse. The leaves extract was administered orally at dose level of 0; 0.56; 1.68 and 3.36 g/kg body weight. The extract was given from the first day until the eighteenth day of pregnancy period. The ethanol extract of Torbangun leaves contains flavonoids, saponins, tannins, and steroids. The result showed that consumption of 3.36 g/kg torbangun leaves extract had led to significant differences in the decrease of maternal weight change, number of live fetuses, fetal weight, and fetal length. Consumption of Torbangun leaves extract during pregnancy should be avoided due to its potency to interfere the fetal development.

Keywords: ethanol extract, pregnancy, teratogenicity, torbangun

INTRODUCTION

The use of plants as functional food/ *nutraceutical* is increasing worldwide in the past three decades. About 4 billion people (80% of world population) use plant products to improve their health quality health (Ekor 2013). Due to the high usage of plant products for health improvement, health workers and consumers need to know the advantages, efficacy, and possible side effects of those products.

Torbangun is one kind of plant that is used in various regions, such as Africa, South America, Asia, The Caribbean and Pacific Islands (Lukhoba *et al.* 2006). In Indonesia, torbangun leaves are commonly consumed by Bataknesse women in North Sumatra after delivery to increase milk production (ASI) and this local wisdom has been proven scientifically (Damanik *et al.* 2001; Damanik *et al.* 2004; Damanik *et al.* 2006; Damanik 2009). Since breastmilk is the most suitable source of nutrition for the infant growth and development as well as its immune system, the question can torbagun be consumed earlier to increase breastmilk production before the baby is born is imperative. However, Bataknesse women in North Sumatra also believe torbangun leaves can act as a uterine cleansing agent that can ac-

celerate the release of blood and placental remnants after delivery (Damanik 2009). Cleansing of blood and placental remnants related to stimulation of uterine contraction (Ho *et al.* 2011). Johns and Sibeko (2003) stated that there is a potential of pregnancy complications if pregnant women consume plants which can stimulate uterine contraction.

Aside from its role as lactagogue and uterine stimulant, torbangun was also proven to have cytotoxic and antioxidants effects, as well as helps in reducing the symptoms of premenstrual syndrome (Rosidah & Hasibuan 2014; Suryowati *et al.* 2015; Devi *et al.* 2010). Cytotoxicity of Torbangun leaves that makes it a potential anticancer (Rosidah & Hasibuan 2014) could also cause potential side effects for fetal development by triggering cell death and necrosis (Timbrell 2009). Thus consumption of Torbangun leaves during pregnancy needs to be evaluated thoroughly because of the potential side effects that it may cause on fetal development.

Damanik *et al.* (2009) conducted a study to see the effect of torbangun leaves administration (0%, 2.5%, 5%) on pregnant mouse, in one group the torbangun was administered orally from the 14th day of pregnancy and in another one was from the delivery day. The results showed

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that the group given 5% of torbangun leaves on 14th day of pregnancy had higher milk production and higher newborn weight gain compared to the other groups. The result also indicated that there was no side effect in the form of fetal death on delivery day. Nevertheless, the safety of torbangun leaves consumption during pregnancy remained uncertain. The exposure period over 14th day of rodentia pregnancy tends to not provide a comprehensive safety information, due to the fact that usual side effect generated during this period often is in the form of functional abnormalities which is not easily recognizable. Therefore, it is suggested to conduct earlier safety evaluation for consumption of torbangun leaves during pregnancy where exposure is given within the sensitive period of before the 12th day of pregnancy (Lu 2006).

Review on many scientific publications documenting benefits of torbangun leaves consumption for health shows the possibility of this plant to be consumed by all groups in the community especially for post partum mothers in relation to lactation and prevention of post partum hemorrhage. However there is a gap of scientific information on the safety of its use during early pregnancy, whether it can be administered sooner before the post partum period. Therefore, the purpose of this research is to study the safety of ethanol extract from Torbangun leaves on mouse fetal development.

METHODS

Design, location, and time

The design of this research was experimental study using pregnant mouse as the experimental animal. The experimental design carried out in this study was Completely Randomized Design (CRD) with multi-stage dosing of torbangun leaves extract as the treatment factor. The level of torbangun leaves extract given were 0 (Treatment 0); 0.56 (Treatment 1); 1.68 (Treatment 2); and 3.36 (Treatment 3) g/kg mouse body weight.

This research was carried out in November 2016-April 2017. The torbangun leaves extract was made at the Research and Development Laboratory of Fish Disease Control, Depok; and Biochemistry Laboratory of Community Nutrition Department, IPB (Bogor Agricultural University). Phytochemical analysis was performed at the IPB Biopharmaceutical Study Laboratory. The mouse were kept in the Animal Management Unit Laboratory and the analysis was done in the Embryology Laboratory, FKH-IPB (the Faculty of Veterinary Medicine).

Materials and tools

The material used in this research was 96% ethanol extract from Torbangun leaves. The leaves sample had been identified by Center for Plant Conservation Botanic Gardens-LIPI (The Indonesian Institute of Science) to ensure that the leaves used were from the right species.

The number of experimental animals was determined using the Federer formula: $(t-1)(r-1) \geq 15$. Based on the formula, the sample size used in this study were 24 healthy female mouse (*Mus musculus*, DDY strain), 8-11 weeks old and had never been mated. Each treatment group consisted of 6 female mouse. The total of 8 male mouse aged ≥ 12 weeks were used for the mating process. All mouse were obtained from Indonesia National Agency of Drug and Food Control. The ethical consent in this study was obtained from the Animal Ethics Commission of the FKH-IPB (Faculty of Veterinary Medicine, Bogor Agricultural University), with certificate number: 053/KEH/SKE/I/2017.

Vaginal swab method's tools and materials were object glasses, cotton buds, methanol, and Giemsa stain 10%. Vaginal swab was done to ensure that female mouse had entered maturity for the mating process by having 4 estrus cycles, and was needed in order to determine the day for mating process. Other tools and materials used in this study were freeze dryer (ScanVac, Labogene, H 1115 0023) and vacuum evaporator that were used for leaves drying process, as well as stainless-steel feeding needles & Polisorbat 80 [1%v / v] for oral administration of Torbangun extract. Light microscopes, digital stereo microscope and 0.9% NaCl, were used for the observation.

Procedures

The research stages began with the preparation of torbangun leaves' ethanol extract and phytochemical analysis, after that, the mating process, administration of torbangun leaves extract and observation of the intervention effects on experimental animals were conducted.

The preparation of experiment materials and phytochemical analysis. The leaves were cleaned 3 times using flowing water and followed by one time with aqueous solution, then drained. The leaves were then dried by using freeze dryer for 52 hours and processed into powder. A total of 100 grams of torbangun leaves powder was extracted using 1000 ml of 96% ethanol solvent. The leaves residues then being extracted again using 96% ethanol solvent. The same procedures were repeated for 5 times in total. The ethanol solvent

was then evaporated using a vacuum evaporator with a temperature of 50 °C. Phytochemical contents of torbangun leaves extract were analyzed qualitatively using color visualization method. The analysis was performed to see the presence of alkaloids, saponins, tannins, triterpenoids/steroids, and flavonoids.

The mouse mating process and intervention of ethanol extract from Torbangun leaves.

At the age of 8-11 weeks, female mouse entering the proestrus and estrus phases were mixed with male mouse for the mating process. Mixing can be done as 1:1 to 3:1 for female and male comparison. The day of vaginal plug or sperm were found in the vagina was set as the 1st day of pregnancy.

The ethanol extract of torbangun leaves was given daily to the pregnant mouse orally. The administration was done from the 1st to the 18th day of pregnancy, after that, the mouse were sacrificed on the 18th day of pregnancy. The extract was dissolved in Polysorbate 80 [1%] solution as an emulsifier for oral administration. Treatment 0 group was given only the polysorbate solution of 80 [1%].

The observation of the intervention effects. Maternal weight observation was performed daily during pregnancy. The observations done on the 18th day of pregnancy were conducted for parameters as follow: the uterine weight with fetuses inside it, number of live and dead fetuses, weight and length of the fetuses, and the weight of the placenta.

Data analysis

The results were presented in the average value and standard deviation. All data was processed using Microsoft Excel 2013. The data was analyzed using ANOVA to compare the significance ($P < 0.05$) between treatment groups and further analysis with Duncan Multiple Range Test (DMRT) using SPSS version 21.0 for Windows.

RESULTS AND DISCUSSION

The qualitative phytochemical contents of ethanol extracts of torbangun leaves.

Phytochemicals are metabolites in plants that have the ability to improve human health (Liu 2003). The results of qualitative phytochemical analysis in this study showed that ethanol extract of torbangun leaves contains flavonoid, tannin, saponin, and steroid compounds.

The previous research has shown that torbangun leaves has cytotoxic effects and could act as a potential anti-cancer in HeLa cells (cervical cancer cells) and lung cancer cells (A549) (Rosidah & Hasibuan 2014; Ramalakshmi *et al.* 2014). Those can be caused by the presence of flavonoid, saponins, and steroids compounds in the torbangun leaves.

The phytochemical responses in the body depend on the type and the amount consumed. The cytotoxic agents contained in the torbangun leaves have a potency to cause side effects for fetal development. The consumption of large amounts of tannins during pregnancy also has a potency to inhibit the intake of maternal and fetal nutrients. Exposure to cytotoxic agents and lack of nutrient intake during pregnancy potentially inhibit the intensive cell proliferation and differentiation process on fetal development (Timbrell 2009).

Maternal weight changes during pregnancy

Weight gain during pregnancy is one of the parameters that needs to be observed to evaluate the safety of an exposure for maternal health. The result of this study indicated that there was a significant difference ($p < 0.05$) between the Treatment 3 group with the other groups on the maternal weight gain during pregnancy (Table 1). The decrease of maternal weight gain average occurred along with the dose addition.

Table 1. The average of maternal weight gain according to treatment groups, from the 1st to 18th day of pregnancy

Groups	Maternal weight gain during pregnancy (g)
Treatment 0	19.2±0.8 ^a
Treatment 1	18.8±3.8 ^a
Treatment 2	17.1±4.8 ^a
Treatment 3	7.5±8.4 ^b

Description: The average values with different superscript letters in one column indicate the significant difference based on Anova test results ($p < 0.05$).

Variations in maternal weight gain may be affected by food consumption, systemic stress toxicity, anorexia due to treatment, and estrogen and progesterone hormone instability. Maternal weight gain is also a manifestation of the increased in uterine weight with the fetuses in it (Hood 2006). The corrected maternal weight gain was also calculated from all treatment groups. The corrected weight gain of the Treatment 3 group was the lowest among the others, but there was no significant difference ($p>0.05$) between the treatment groups that were given torbangun leaves extract (Treatments 1, 2, and 3) with the control group (Treatment 0).

The data of uterine weight with the fetuses in it and the corrected maternal weight gain data (1st-18th day of pregnancy) are presented in Table 2. The corrected maternal weight gain may provide the information of maternal toxicity because it does not include the weight gain due to the increase of litters' weight and/or number (Hood 2006; Jahnke *et al.* 2006). There was no significant difference in corrected maternal weight gain indicated that the low weight gain of Treatment 3 group was not caused by maternal toxicity. The condition described only the low uterine weight with the fetuses in it. Ehrenbreg *et al.* (2003) said that low maternal weight gain during pregnancy is associated with lower fetal weight and an increase of preterm delivery incidence.

The effect of treatment on number of living and dead fetuses

The results of this study indicated that the average number of live and dead fetuses in the Treatment 3 group were significantly different ($p<0.05$) with the other treatment groups. The Treatment 3 group had the lowest number of live fetuses, followed by the highest number of death fetuses when compared with the others (Table 3).

Torbangun leaves can act as a cytotoxic agent that has a potency to cause fetal death. Previous research has shown that cytotoxic agents could cause fetal death by affecting the mitotic division process (Yakubu & Bukoye 2009).

The effect of treatment on fetal weight, fetal length, and placental weight

The results of this study indicated that the average value of fetal weight decreased along with the dose addition. There was a significant difference ($p<0.05$) between the fetal weight of Treatment 3 group with the other treatment groups, in which the Treatment 3 group had the lowest average of fetal weight (Table 4).

Low fetal weight is associated with the incidence of Intra-Uterine Growth Retardation (IUGR). IUGR is a condition in which the fetal development in uterine is delayed, characterized by the incompatible of fetal weight according to its age (Sutomo *et al.* 2015). The low fetal weight in the treatment group with the highest dosage indicated that there was fetal developmental disorder due to the ethanol extract of torbangun leaves administration along with the dose addition.

There was a decrease in the average value of fetal length along with the dose addition. The statistical test showed that there was a significant difference ($p<0.05$) between fetal length of Treatment 3 and Treatment 0 groups (Table 4). The fetal length and weight are sensitive parameters in the safety evaluation test. The decrease in fetal length and weight are indicative of the disturbances in proliferation process and the rate of biosynthesis on fetal development phase (Setyawati 2009). The cytotoxic ability of torbangun leaves extract may act as an antiproliferation agent that has a potency to cause fetal developmental disorder. The appearance of fetuses in each treatment group are presented in Figure 1.

The results of this study showed that there was no significant difference ($p>0.05$) of placental weight in all treatment groups (Table 4). The placental weight has a significant relationship with fetal birth weight (Haeussner *et al.* 2013). The results of this study indicated that the administration of ethanol extract of torbangun leaves based on the observation of placental weight has shown no observable specific disorder in placental organ.

Table 2. Uterine weight with the fetuses in it (day 18th), and corrected maternal weight gain (day 1st-18th) according to treatment groups

Groups	Uterine weight with the fetuses in it, on 18 th day of pregnancy (g)	Corrected maternal weight gain from 1 st -18 th days of pregnancy (g)
Treatment 0	12.8±0.9 ^a	6.4±0.9 ^{ab}
Treatment 1	10.6±2.8 ^a	8.2±2.3 ^a
Treatment 2	10.6±2.9 ^a	6.4±3.1 ^{ab}
Treatment 3	4.4±3.1 ^b	3.1±6.2 ^b

Description: The average values with different superscript letters in one column indicate the significant difference based on Anova test results ($p<0.05$).

Table 3. The average number of live and dead fetuses according to treatment groups, on the 18th day of pregnancy

Groups	Number of live fetuses	Number of dead fetuses
Treatment 0	11.7±0.5 ^a	0.0±0.0 ^a
Treatment 1	11.0±2.1 ^a	0.3±0.5 ^a
Treatment 2	10.5±3.2 ^a	1.3±0.5 ^a
Treatment 3	2.2±3.7 ^b	8.8±3.5 ^b

Description: The average values with different superscript letters in one column indicate the significant difference based on Anova test results ($p < 0.05$).

Table 4. The average of fetal weight, fetal length (*crown-rump*), and placental weight according to treatment groups, on the 18th day of pregnancy

Groups	Fetal weight (g)	Fetal length/ <i>crown-rump</i> (mm)	Placental weight (g)
Treatment 0	1.15±0.04 ^a	23.4±0.6 ^a	0.13±0.01 ^a
Treatment 1	1.02±0.09 ^a	21.1±1.0 ^{ab}	0.12±0.01 ^a
Treatment 2	0.98±0.24 ^a	21.0±2.4 ^{ab}	0.12±0.02 ^a
Treatment 3	0.65±0.45 ^b	17.4±5.2 ^b	0.12±0.02 ^a

Description: The average values with different superscript letters in one column indicate the significant difference based on Anova test results ($p < 0.05$).

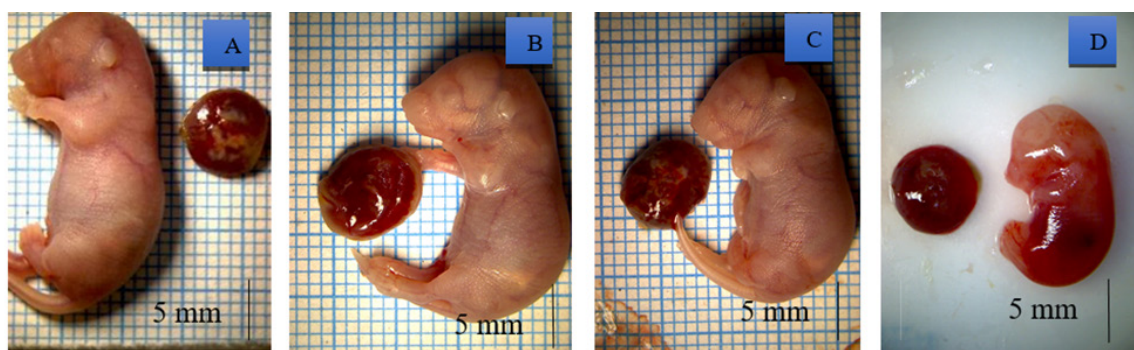


Figure 1. Fetuses from groups: (A) Treatment 0; (B) Treatment 1; (C) Treatment 2; (D) Treatment 3

CONCLUSION

The ethanol extract from torbangun leaves (*Coleus amboinicus* Lour.) based on the qualitative phytochemical analysis showed to contain flavonoids, saponins, tannins, and steroids. Administration of the extracts with highest dosage showed the greatest effects on fetal development parameters and were significantly different with the lower dosage groups. The parameters observed were decrease in maternal weight gain during pregnancy, number of live fetuses, fetal weights, and fetal length. The consumption of torbangun leaves during pregnancy, especially in the form of extract, should be avoided because it potentially can inhibit fetal development.

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