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Original Article

EVALUATION OF TERATOGENICITY EFFECTS OF ETHANOLIC EXTRACTS OF BINAHONG LEAVES (*ANREDERA CORDIFOLIA* (TEN) STEENIS) IN WISTAR RAT

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ABSTRACT

Objective: The aim of this study was to evaluate teratogenicity effect of ethanolic extract of binahong (*Anredera cordifolia* (Ten.) Steenis) leaves in Wistar rats.

Methods: The teratogenicity test were done in 5 groups of rat *i. e.* control group, trimethoprim at a dose of 360 mg/kg bw, ethanolic extract of binahong at doses of 100, 400, and 1000 mg/kg bw. Test substance was given orally on day 6 to day 15 of rats pregnancy. At day 20 of gestation, laparoctomy was performed to retrieve the fetuses. Evaluation was done towards the fetus skeletal, organ and fetal weight.

Results: Teratogenicity test results showed that there was the ungrowth fetus observed in all groups with the highest incident in trimethoprim group (39.5%). Average of fetus body weight of trimethoprim, control, binahong extract at doses of 100, 400 and 1000 mg/kg bw group was 2.32 ± 0.66 g, 3.58 ± 0.24 g, 3.64 ± 0.26 g, 3.67 ± 0.25 g and 3.93 ± 0.61 g, respectively. Skeletal evaluation showed that there was malformation of sacral vertebrae (65.2%), convulated costal stucture (82.6%), palate (33.3%), head cavity (88.9%), nasal cavity (5.6%), eye cavity (5.6%), ear (5.6%) and liver (43.1%) which were observed only in trimethoprim group.

Conclusion: Ethanolic extract of binahong at doses of 100, 400 and 1000 mg/kg bw in rat did not have the teratogenic effect.

Keywords: Binahong, *Anredera cordifolia* (Ten.) Steenis, Teratogenic effect, Trimethoprim.

INTRODUCTION

Nowadays, traditional medicines are expected to evolve into a standardized herbal medicines or phytopharmaca (clinical based herbal medicines) class so they can be used in health care facilities. Drugs used in health care facilities must meet the requirements of a safe, beneficial and have been standardized. Security affirmation efforts are necessary to meet the requirements through some toxicity and efficacy tests, which will be followed by clinical trials.

Toxicity testing is divided into two categories. First, the general toxicity tests include acute toxicity test (conducted by giving animal a single or multiple doses of a test substance for 24-hours period), sub chronic toxicity test (conducted by giving animal repeated exposure of a test substance, usually for 3-months period), and chronic toxicity test (conducted to observe the toxic effects by giving animal long-term repeated administration of a test substance usually for 1-year period). Second category is special toxicity tests which investigate the carcinogenic, mutagenic, and teratogenic effects of a substance [1].

Binahong (*Anredera cordifolia* (Ten.) Steenis) plant is one of the plants used in traditional medicines. The leaves of binahong plant have been used by most people to treat bacterial infection [2], peptic ulcers [3], diabetes [4], and kidney failure [5]. With such properties of binahong than it is possible for pregnant women to consume it during their pregnancies. However, there has been no publication about the teratogenic effects of the use of binahong extracts yet.

This research was conducted to observe the teratogenic effect of ethanolic leaf extracts of binahong in pregnant rats. The results of the teratogenic test were expected to be a material consideration in its use during pregnancy.

MATERIALS AND METHODS

Methods used in this study were based on a OECD toxicity study guidelines for testing chemicals and prenatal development [6,7]. OECD guidelines are a protocol accepted by animal ethics committee in School of Pharmacy, Bandung Institute of Technology.

Wistar rats were randomly divided into five groups: a control group that received 2% tragacanth, comparative controlled group that administrated with a dose of 360 mg/kg bw of trimethoprim and three experimental groups, each was administrated with 100, 400 and 1000 mg/kg bw of ethanolic leaf extracts of binahong (in a suspension form in 2% tragacanth). The lowest dose used was derived from the conversion of therapeutic dose in humans, while the highest dose was a limit dose on teratogenicity test. If the embryo toxicity or teratogenicity was not found at the highest dose, then it was not necessary to test at the other doses. The drug was administered orally once daily on day 6 to day 15 of gestation. The drug administration period was in the organogenesis phase of rat gestation.

Determination of early pregnancy

Female rats were caged with sexually matured male in the ratio of two males to three females. The following day, vaginal smear examination was performed in female rats. A small amount of saline was flushed into the vagina hole of rats using a pipette tip. One or two drops of the resulting vaginal fluid contained cell suspension then dripped into a slide. As soon as the smears were dried out, they were stained with 0.1% methylene blue then examined under a microscope using magnification of 400x. Gestational day 0 was determined by the presence of vaginal plugs or sperm in vaginal smears, as can be seen in fig. 1.

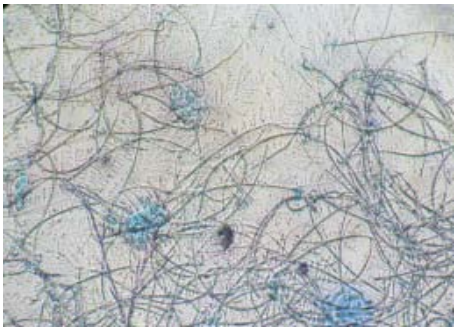

Fig. 1: Photomicrograph of rat vaginal smear after copulation. There were a lot of sperm in the smear, as shown by the arrow

DISSECTION AND OBSERVATION

On day 19 of gestation, pregnant rats were dissected to retrieve fetuses. Each fetus was then dried and weighed. The fresh normal fetuses taken from the uterus was shown as in fig. 2.

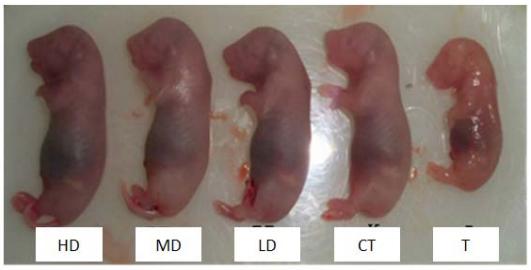

Fig. 2: Fresh fetuses of each group taken out from the uterus CT: control group, LD: lower-dose group (100 mg/kg bw), MD: middle-dose group (400 mg/kg bw), HD: higher-dose group (1000 mg/kg bw), T: trimethoprim group (360 mg/kg bw).

Preparation of skeletal preservation

Preparation: Fetuses was fixed in 90% ethanol solution for at least 1-2 weeks. After drying out, fetuses were skinned completely. Eyes and blobs of fat on the nape were removed, and the trachea was trimmed. Groin legs and underarms were cut out of the body. The abdominal wall was opened. After skinning and removing the internal organs, the fetuses were transferred back to the fixation bottle.

Cleaning: After skinning and removing the internal organs, ethanol solution was replaced with 0.5% KOH. Cleaning lasted for one day. The containers were shaken to remove the air from the chest cavity occasionally.

Bleaching: After purification was considered perfect, KOH solution was removed, the fetuses were washed with water and the remains fat were removed. The water was then replaced with 1% hydrogen peroxide solution. Fetus remained in it for 2-3 hours, shaken occasionally. Bleaching was considered perfect if the inner bones were in white color.

Staining: After bleaching, fetuses then washed and soaked in the water for approximately 10 minutes. The water was then replaced with Alizarin dye solution. Fetus remained in the dye for not more than 24 hours. Staining was considered perfect if the skeleton had been seen clearly.

Final cleaning: After staining was considered perfect, alizarin dye solution was discarded, the fetuses then were washed out with water several times and then gradually immersed in a solution of 5%, 20%, 40%, 80% glycerol and lastly pure glycerol respectively for 1 week each.

Skeletal evaluation: The evaluation was conducted after the slides were immersed in 80% glycerol for at least 1 week. Firstly the posterior of fetal skeleton were observed: the skull, spine, ribs were examined. The skeletons were then turned over to observe the anterior parts: the skeleton of oral cavity, bone wrapped around the shoulders and hips, forelimbs and hind limbs. The results of the examinations including structure, morphology, number and position of the bones were then recorded.

Soft tissue assessment

After being fixed in Bouin solution for 1-2 weeks, the fetuses from the same dam were collected in a beaker glass filled with water. Fetuses then dried and cut up with a particular pattern. The head part between the upper and lower jaw was cut using a razor blade, and the plate was examined. Cross section was made from the nose to the hind at the upper head-cut with a maximum distance of 1 mm from the nose, eyes, and lateral ventricles on the priority. Abdominal skin was incised with a scalpel, the fore part was cut and removed, and the organs inside the abdominal cavity were carefully taken out. Condition status of the ureter, genital glands, and bladder was then examined. All the abnormalities were recorded.

Statistical Method and Data Analysis

Statistical analysis of the results was analyzed with one-way ANOVA followed by Bonferroni post hoc analysis. p values <0.05 were considered to be significant.

RESULTS AND DISCUSSION

The methods used in this study were *in vivo* methods in rats. Rats were divided into 5 groups: a control group that received 2% tragacanth, comparative controlled group that administrated with a dose of 360 mg/kg bw of trimethoprim and three experimental groups, each was administrated with 100, 400 and 1000 mg/kg bw of ethanolic leaf extracts of binahong (*Anredera cordifolia* (Ten.) Steenis). The lower-dose (100 mg/kg bw) was based on the previous studies which had pharmacological activity [5]. The middle-dose (400 mg/kg bw) was derived from conversion of 4 times of the lower-dose. The higher-dose (1000 mg/kg bw) is a dose test limit, at which if there was not found toxic or teratogenic effects on the embryos, then the dose should not be increased or in other words the extract used was considered safe.

Table 1: The Number of Live Fetuses, Underdeveloped Fetuses, And Their Body Weight Observations

Groups	Live Fetus		Underdeveloped Fetus		Average fetal weight (grams)
	Total number	%	Total number	%	
Control	160	97.6	4	2.4	3.58 ± 0.24
Binahong extract 100 mg/kg bw	166	93.3	12	6.7	3.64 ± 0.26
Binahong extract 400 mg/kg bw	182	97.3	5	2.7	3.67 ± 0.25
Binahong extract 1000 mg/kg bw	191	91.8	17	8.2	3.93 ± 0.61*
Trimethoprim 360 mg/kg bw	118	61.1	77*	39.5	2.32 ± 0.66*

Note: Statistical calculations of live fetuses, underdeveloped fetuses and fetal weight using ANOVA followed by a Bonferroni's post hoc test at p<0.05, *significantly difference compared to control group.

This study was preceded by the determination of the estrous cycle. Proestrus phase was characterized by the presence of nucleated epithelial cells, which were more dominant in the vaginal fluid of rats examined under the microscope at 400x magnification. After that proestrus female rats were mated with male rats and mating was confirmed by examining rats' vaginal smears on the following day. After mating was confirmed, pregnancy was determined by the presence of vaginal plugs or sperm in vaginal smears. The presence of sperms in the vaginal smears was designated as gestation day 0 (fig. 1).

Drug and extract were administrated orally during the organogenesis period on day 6 to day 15 of gestation daily. Organogenesis phase was the most critical period during gestation and outside environmental influences could cause teratogenic effects. During this phase, the attachment of the blastocyst to the uterine wall called implantation occurred, the structure formation developed and the cells differentiated into organs very rapidly. Increased sensitivity of most tissues during organogenesis caused death and disabilities in the embryos easier [8,9]. Laparotomy was performed to retrieve the fetuses on day 20 of gestation.

Observations were done towards the number of live fetuses, underdeveloped fetuses, and morphological abnormalities.

Table 2: Examination of Vertebral Malformation Occurrence in 20-Day-Old Rat Fetuses

Groups	Total number of fetus	Number of fetus observed	Malformation occurrence (%)				
			Ce	T	L	Sa	R
Control	160	58	0	0	0	0	0
Binahong extract 100 mg/kg bw	166	63	0	0	0	0	0
Binahong extract 400 mg/kg bw	182	66	0	0	0	0	0
Binahong extract 1000 mg/kg bw	191	70	0	0	0	0	0
Trimethoprim 360 mg/kg bw	118	46	0	0	0	30 (65.2)	38 (82.6)

Note: Ce: Cervical, T: Thoracic, L: Lumbar, Sa: Sacral, R: Ribs.

Table 3: Examination of Skeletal Limb Completeness in 20-Day-Old Rat Fetuses

Groups	Total number of fetus	Number of fetus observed	Malformation occurrence (%)					
			Forelegs			Hind legs		
			DP	PP	Mc	FD	FP	Mt
Control	160	58	0	0	0	0	0	0
Binahong extract 100 mg/kg bw	166	63	0	0	0	0	0	0
Binahong extract 400 mg/kg bw	182	66	0	0	0	0	0	0
Binahong extract 1000 mg/kg bw	191	70	0	0	0	0	0	0
Trimethoprim 360 mg/kg bw	118	46	0	0	0	0	0	0

Note: DP: Distal Phalanges, PP: Proximal Phalanges, Mc: Metacarpal, Mt: Metatarsal.

In number of live fetuses (Table 1), there were 2.4 % underdeveloped fetuses observed in the control group. In trimethoprim group, there were only 61.1% live fetuses and 39.5% out of it were underdeveloped fetuses which statistically significant compared to the control group. Meanwhile, in ethanolic extract of binahong at doses of 100, 400 and 1000 mg/kg bw, there were 6.7%, 2.7% and 8.2% respectively underdeveloped fetuses, which was not statistically significant compared to control group. It suggests that ethanolic extract of binahong does not affect development of the fetus.

Table 4: Examination of Organs Malformation in 20-Day-Old Rat Fetuses

Groups	Total number of fetus	Number of fetus observed	Organs Malformation Occurrence (%)													
			Uj	No	Ey	Hc	Hr	Ln	Lv	Sm	Sp	Kd	Tt/ Ov	Fl	Hl	Er	Tl
Control	160	102	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Binahong extract 100 mg/kg bw	166	103	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Binahong extract 400 mg/kg bw	182	116	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Binahong extract 1000 mg/kg bw	191	121	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trimethoprim 360 mg/kg bw	118	72	24 (33.3)	4 (5.6)	4 (5.6)	64 (88.9)	0	0	31 (43.1)	0	0	0	0	0	0	4 (5.6)	0

Note: Uj: upper jaw, No: nose, Ey: eyes, Hc: head cavity, Hr: heart, Ln: lung, Lv: liver, Sm: stomach, Sp: spleen, Kd: kidney, Tt/Ov: testes/ovaries, Fl: foreleg, Hi: hind leg, Er: ear, Tl: tail.

Average fetal weight in control,trimethoprim, 100, 400 and 1000 mg/kg bw of ethanolic leaf extract of binahong groups were 3.58 ± 0.24 g, 2.32 ± 0.66 g, 3.64 ± 0.26 g, 3.67 ± 0.25 g, and 3.93 ± 0.61 g, respectively (Table 1 and fig. 2). Fetal weight of trimethoprim group was significantly lower compared to control group. The increase in dose of leaf extracts of binahong followed by the increase in fetal weight. Dose of 1000 mg/kg bw of ethanolic group showed significantly higher compared to control group.

Evaluation of the fetal skeletons aimed to see the abnormalities in the skeletons, which stained with alizarin sulfonate dye solution after undergoing the series of processes including cleaning, bleaching and final cleaning. Collecting of fetuses for skeletal examinations was based on teratogenicity test protocols. Skeletal preparation was done from one third of live fetuses. The examination included the number and abnormalities of fetal skeletons that might be occurred.

The composition and number of normal vertebral columns are 7 cervical, 13 thoracic, 6 lumbar and 4 sacral vertebrae. Observations of the anterior and posterior column were performed using a magnifying glass. The composition and number of normal forelimb columns are 5 distal, 4 proximal, and 4 metacarpal, whereas the hind limb columns are 5 distal, 4 proximal, and 5 metatarsal [10].

Skeletal evaluations in trimethoprim group showed that there were abnormalities in form of wavy ribs structure (82.6%), but found no abnormality in the number of ribs (Table 2 and fig. 3B). The

abnormalities were also found in the sacral (65.2%), in which only 2 bones were in normal growth while the other 2 bones were abnormal (Table 2 and fig. 3C). Malformations observed in skeletal evaluation confirmed that trimethoprim might also affect bone formation in the fetus during pregnancy, while no abnormality was observed in all fetuses in control and 100, 400 and 1000 mg/kg bw of ethanolic leaf extract of binahong groups (Table 2 and 3; fig. 3A, 3B, and 3C).

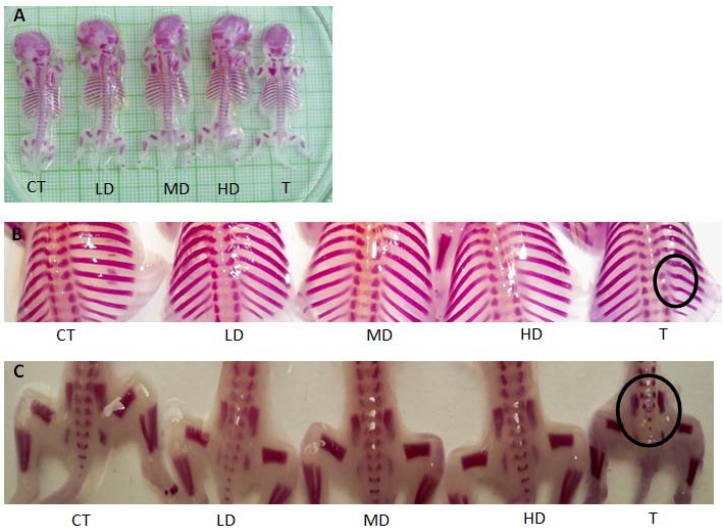

Fig. 3: A. Nomal skeleton. B and C. Skeletal malformations in trimethoprim group. Abnormalities in wavy ribs structure form (B) and incomplete sacro-caudal growth (C) were observed. Note: CT: control group, LD: lower-dose group (100 mg/kg bw), MD: middle-dose group (400 mg/kg bw), HD: higher-dose group (1000 mg/kg bw), T: trimethoprim group (360 mg/kg bw)

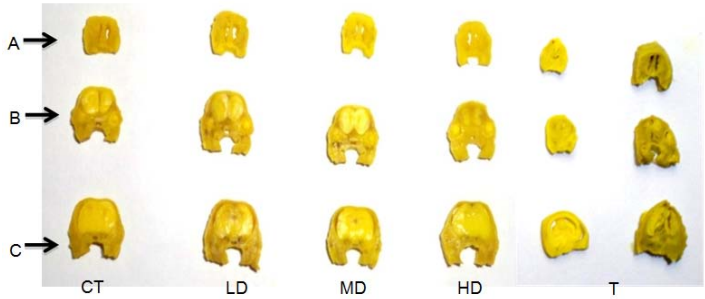

Fig. 4: Fetal head section of each group

A: nasal cavity section, B: eye socket section, C: cranial cavity section. Note: CT: control group, LD: lower-dose group (100 mg/kg bw), MD: middle-dose group (400 mg/kg bw), HD: higher-dose group (1000 mg/kg bw), T: trimethoprim group (360 mg/kg bw).

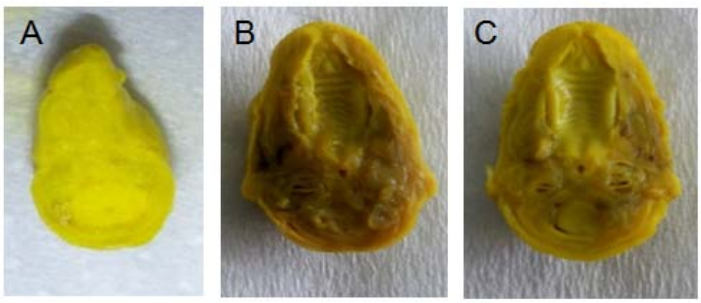

Fig. 5: Organ abnormalities in trimethoprim group

The incomplete jaw formation was observed in trimethoprim group (Fig. 5A and 5B). A: Palate formation was absent. B: Palate formation was incomplete. C: Normal palate formation

Soft tissue or organs examinations in trimethoprim group showed some abnormalities such as incomplete upper jaw formation, absence of the nasal cavity formation, brain shape malformation, absence of eye socket and ears formation and also color of liver abnormalities, which were darker than the color of control group's liver (Table 4, fig. 4,5, and 6).Neither control group nor experimental groups showed abnormality in organ formation.

Fig. 6: Liver colour abnormalities in trimethoprim group
Note: T: trimethoprim group, CT: control group.

CONCLUSION

Ethanolic leaf extracts of binahong (*Anredera cordifolia* (Ten.) Steenis) at doses of 100, 400, 1000 mg/kg bw in pregnant rats did not have teratogenic effects like trimethoprim (360 mg/kg bw) as a comparative drug. The increase in dose of leaf extracts of binahong followed by the increase in fetal weight.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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