

## Evaluation of Teratogenic Effect of Tempuyung (*Sonchus arvensis*) Extract on Wistar Rats

Elin Yulinah Sukandar\*, Dewi Safitri

Pharmacology and Clinical Pharmacy Research Group School of Pharmacy, Institut Teknologi Bandung, Indonesia

Available Online: 1<sup>st</sup> May, 2016

### ABSTRACT

**Objectives:** this study was conducted to obtain safety data of ethanolic extract of tempuyung leaves (EETL) on pregnant Wistar rats. **Methods:** Four groups of rats were used for this study, including control group, ethanolic extract of tempuyung leaves (EETL) at doses of 100 mg.kg<sup>-1</sup>, 400 mg.kg<sup>-1</sup> and 1000 mg.kg<sup>-1</sup>, in which each group consisted of ten pregnant rats. Extracts were administered once daily starting from day 6 to day 15 of pregnancy. At day 20 of gestation period, laparotomy was performed to isolate the fetuses. For evaluation, all parameters related to fetal state such as fetal appearances, skeletal condition, organ weight, as well as fetal weight were determined. **Results:** There was no difference between EETL treated group with the control group in parameters: fetal weight, organ weight, mortality rates, macroscopically fetal appearance, skeletal condition. Moreover, abnormalities of organs were not presented in all extract treated group. **Conclusion:** administration of EETL at doses of 100, 400 and 1000 mg.kg<sup>-1</sup>bw in rats did not show teratogenic effects.

**Keyword:** tempuyung leaves, *Sonchus arvensis*, teratogenic effects

### INTRODUCTION

For the past decades, natural product based medicine has been growing and people's believe to go back to nature has slowly increased. Thus, herbs that are used in treating certain ailments should be standardized as herbal medicines or clinical based traditional medicines. It allows people to utilize them in health care facilities. All types of drugs in health care should meet requirements of safety, efficacy, and quality. In terms of safety, affirmation efforts are crucially needed to suffice all requirements. It can be obtained through toxicity and pharmacological study, which may be followed by clinical trials. The toxicity test is widely known divided into two classes: the general toxicity test and specific toxicity test. In terms of general toxicity test, there are three types of test including acute toxicity test where subjected animals receive single or multiple doses of test substances for 24-hour period, sub-chronic toxicity test in which animals are exposed repeatedly by particular test substance for period of a month or 3 month, and chronic toxicity test which is conducted by giving test substance for approximately one year. Another class, specific toxicity is performed to investigate carcinogenic, mutagenic, and teratogenic effects of particular substance. Teratogenic toxicity test is classified as specific toxicity which is conducted to observe toxic effect by giving animal laboratories. Tempuyung (*Sonchus arvensis*) is considered as traditional plant that is commonly utilized especially in Indonesia. According to our previous researches, tempuyung extract showed potential effect as xanthine oxidase inhibitor and antihyperuricemic

activities<sup>2-4</sup>. With a broad range of activity, there is always a possibility that this herbs is taken by pregnant women. The safety aspect on prenatal exposure of tempuyung extract should be confirmed. Developmental toxicity of compounds may result in spontaneous abortion, growth retardation, structural birth defects, or functional deficit<sup>5</sup> which are considerably unintended effects. However, no human data are available regarding the potential effect of tempuyung extract to cross the placenta and to be exposed to the fetus. The study on human developmental toxicity is considered as unethical experiment, thus utilizing animal laboratory is a possible way to uncover teratogenic effect of any substance.

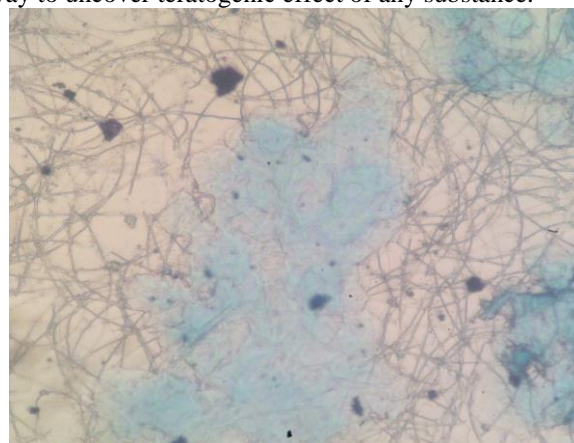


Figure 1: Vaginal smear. An arrow pointed at the presence of sperms

Table 1: The Number of Alive Fetuses for each Dam and Its Body Weight

Groups	Alive fetus for each dam/parent/mother		Undeveloped Fetuses		Body Weight (g)
	$\Sigma$	%	$\Sigma$	%	
Control	10.9 ± 1.4	100.0 ± 0.0	0	0	1.7 ± 0.1
Group 100 mg/kg bw	9.6 ± 2.6	98.3 ± 5.3	0.1 ± 0.3	1.7 ± 5.3	1.5 ± 0.3
Group 400 mg/kg bw	8.9 ± 2.0	91.7 ± 8.6	0.9 ± 1.0	8.4 ± 8.6	1.6 ± 0.1
Group 1000 mg/kg bw	10.4 ± 1.6	98.2 ± 3.8	0.2 ± 0.4	1.8 ± 3.8	1.8 ± 0.1

The values are expressed as mean ± standard deviation, with n=10. \* means there is a significant difference on particular paramaters compared to control group (P<0.05)

Table 2: Percentage of vertebral malformation in alive fetuses

Groups	$\Sigma$ pregnant rats	$\Sigma$ total fetus	$\Sigma$ fetuses observed	Malformation Occurrence (%)			
				Cervical	Thorax	Lumbar	Sacrocaudal
Control	10	109	55	0	0	0	0
Group 1	10	97	50	0	0	0	0
Group 2	10	98	53	0	0	0	0
Group 3	10	106	55	0	0	0	0



Figure 2: Physical appearance of foetus from each group. (1) control, (2) group 1, (3) group 2, (4) group 3

Therefore, this research was carried out to observe the safety of ethanolic extract of tempuyung leaves extract in pregnant rats through the teratogenic test. The results of this study are expected to become consideration in extract consumption during pregnancy.

## MATERIALS AND METHODS

The teratogenic study was performed according to the OECD toxicity study guidelines for testing chemicals and prenatal development<sup>6</sup>. OECD guidelines are a regular protocol conducted in School of Pharmacy, Bandung Institute of Technology. The similar procedure has been conducted before as in the institution, OECD protocol are generally accepted<sup>7</sup>.

### Chemicals

Chloroform, solution for skeletal and connective tissue staining, alizarin red S 0.0025%, Bouin's solution, potassium hydroxide (0.5 and 1%), ethanol 95%, glycerin (25, 50, 80, 100%), and hydrogen peroxide (1%). All utilized solutions were meet pharmaceutical grades.

### Plant Materials

Tempuyung leaves were purchased from Banjaran, Bandung, Indonesia. This plant was then determined in Herbarium Bandungense, School of Natural Science and Technology ITB, Indonesia.

### Preparation of Extract

Crude leaves of tempuyung which have been collected were then dried and powdered by using grinding machine. Powdered tempuyung leaves was extracted by using ethanol 96% in the reflux apparatus. Liquid extract was filtered through Whatman filter paper. It was followed by evaporation by using rotary evaporator until the viscous extract was obtained which was referred as tempuyung extract. It was kept in the refrigerator 4°C until it was utilized for the study.

### Animals

Male and female Wistar rats with approximately 2 to 3 month in age and 150 to 300 gram in weight were used in this study. Rats were provided by Animal Laboratory of The Wistar, Bandung and kept under usual management condition in this institution. All conducted methods have followed the standard of management care for laboratory animals.

### Experimental Design

Investigation was carried out according to the principles of Good Laboratory Practice with regards to OECD<sup>6</sup>, which are essential in conformity with FDA. Before mating, rats were fed to the standard chow and had free access for drinking water. Then, four females were placed into a cage of one male overnight for mating. Successful mating was determined through microscopic observation in which sperms were presented in the vaginal smear.

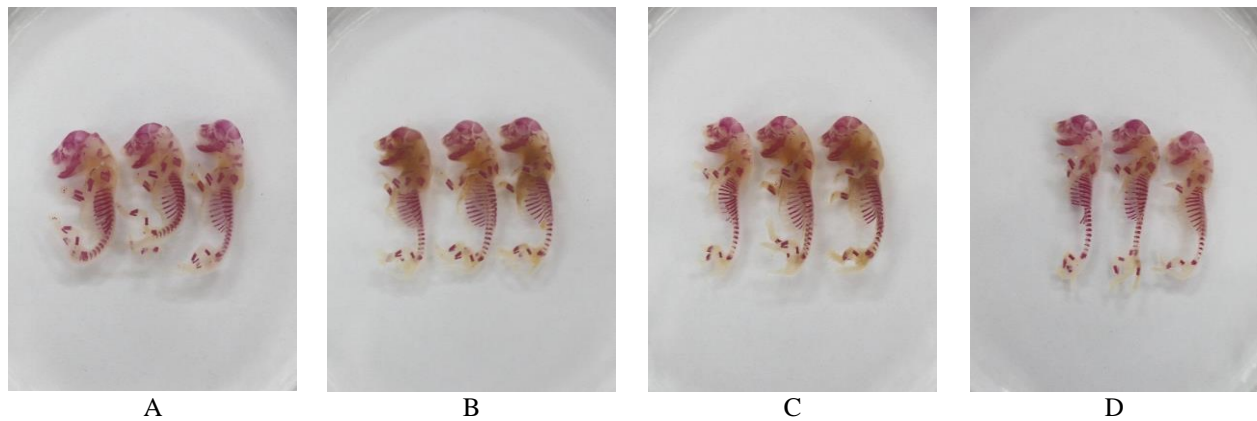


Figure 3: Skeleton observation on alive fetuses. (A) control, (B) group 1, (C) group 2, (D) group 3

Table 3: Percentage of Skeletal Limb Completeness

Groups	ΣPregnant rats	ΣTotal fetuses	ΣObserved fetuses	Percentage of fetuses with malformed limb (%)					
				Forelegs			Hindlegs		
				DP	PP	Mc	DP	PP	Mt
Control	10	109	55	0	0	0	0	0	0
Group 1	10	97	50	0	0	0	0	0	0
Group 2	10	98	53	0	0	0	0	0	0
Group 3	10	106	55	0	0	0	0	0	0

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

Table 4: Organ malformations on fetal soft tissue

Groups	ΣPregnant rats	ΣTotal fetuses	ΣObserved fetuses	Percentage of fetuses with malformed organ (%)										
				Hs	Es	Cp	Ht	Lv	Kd	Tt	Ov	Fl	HI	TI
Control	10	109	54	0	0	0	0	0	0	0	0	0	0	0
Group 1	10	97	47	0	0	0	0	0	0	0	0	0	0	0
Group 2	10	98	45	0	0	0	0	0	0	0	0	0	0	0
Group 3	10	106	51	0	0	0	0	0	0	0	0	0	0	0

Hs = Hydrocephalus, Es = eyes, Cp = Cleftpalate, Ht = heart, Lv = liver, Kd = kidneys, Tt = Testes, Ov = Ovarium, Fl = forelegs, HI = hind legs, TI=tail

Vaginal smears were stained by using methylene blue 0.1% and then observed under optical microscope with the magnification of 100 folds. The following first 24 h was determined as day 0 of gestation. All rats which had mated successfully were randomly divided into 4 groups including control, EETL treated group with a dose of 100 mg/kg bw which referred as group 1, EETL treated group with a dose of 400 mg/kg bw which referred as group 2, and EETL treated group with a dose of 1000 mg/kg bw which referred as group 3. All treated groups received extract according to each doses, meanwhile the control group only was given by vehicle only (CMC Na 1%). All dosage forms were administered once daily beginning from day 6 of gestation to day 15. The pregnancies were interrupted just prior the calculated date of delivery at day 20 of gestation and all groups were subjected to cesarean section for teratogenic examination. The uterine horns were exposed and fetuses were examined for any external malformation, including event of resorptions and the number of live fetuses. Cleft palate and full details were recorded. Alternate fetuses then were placed in alcohol 90% for skeletal examination and Bouin's solution for visceral examination.

#### Skeletal Examination

**Preparation:** After fixation in alcohol 90% in the period of one to two weeks, the procedure was followed by skinning methods. Freehand surgical blade sections were made using the technique described by Wilson<sup>8</sup>, immediately after the fetus was examined for external malformations. Eyes and blobs of fat on the nape were removed and the trachea was trimmed. Groin legs and underarms should be taken apart from the main body. Afterwards, the abdominal was dissected to take out all internal organs. After it was skinned and organs were removed, fetuses were transferred into fixation glass which contained alcohol 90%.

**Cleaning:** after skinning procedure and withdrawing organs, ethanol solution was replaced by KOH 0.5%. The cleaning process was conducted for a day. Often at times, it should be shaken to ensure that there were no air bubbles trapped in the thorax cavity.

**Bleaching:** upon accomplishing the cleaning process, KOH solution was discarded. Fetuses were then rinsed by a plenty of water and remaining fat should be removed. On the same container, water was replaced by 1% hydrogen peroxide solution, then fetuses were soaked into that for 2 to 3 hours. It should be sometimes shaken

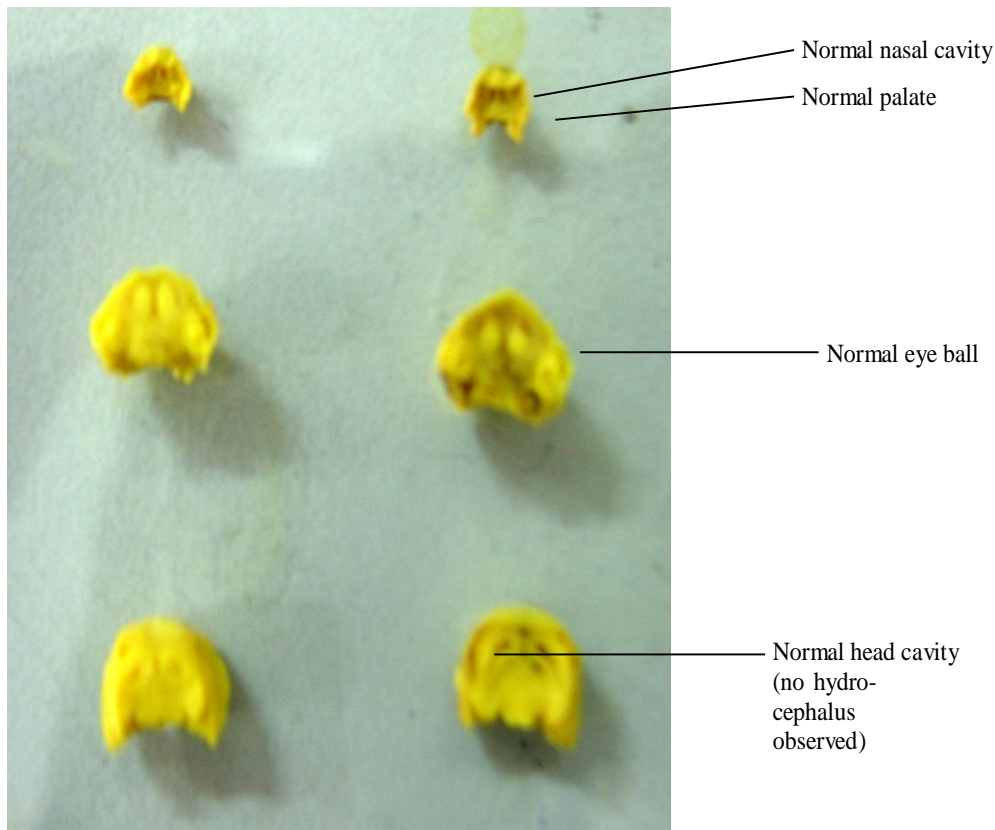


Figure 4: Visceral organs in the head cavity

occasionally. Bleaching was complete if the inner part of the bone has become white.

**Staining:** bleaching was followed by staining procedure. Fetuses were rinsed and soaked into a glass of water for 10 minutes. Subsequently, water was then replaced by alizarin solution in which fetuses should be soaked into this solution for maximum 24 h. The process was accomplished if the skeleton was stained perfectly.

**Finishing:** alizarin solution was withdrawn and fetuses were rinsed by a plenty of water for several times. Thereupon, they were immersed gradually in solutions of glycerol with concentration-dependent manner, starting from 5%, followed by 20%, 40%, 80%, and 100%, respectively. Soaking in each type of solution took approximately a week.

**Evaluation:** evaluation was carried out after fetuses soaked in 80% of glycerol. The first observation was made for fetal posterior including skull, vertebral column, and ribs. Then, fetuses were observed according to inferior parts, such as mouth cavity skeleton, shoulder and hips wrapped bone, forelimbs, and hindlimbs. A systematic record was made on a specially designed form of all malformations and anomalies.

#### **Soft Tissues Assessment**

Being soaked in Bouin solution for a week or two weeks, fetuses from the same dam were transferred into a water filled glass container. By using the tissue or filter paper, fetuses were dissected into particular pattern. For the upper part of the head, a cut was being made through the jaws and just above the ears. After examination of the palate, the cut surface of the head is

placed on a cork board and 1 mm slices are then made through the nose to the posterior of the head. Sectioning was continued from the region of the shoulder joint through the thoracic and abdominal regions to the kidney region. The sections were then examined under a dissecting microscope for any malformations. Any malformations and abnormalities were recorded.

## **RESULTS**

Day 0 was determined by the presence of sperms in vaginal smears, which was shown in the fig.1. According to the results of breeding, there were 10 pregnant rats in the group of: control, group 1, group 2, and group 3. In other words, all rats in each group were a hundred percent pregnant.

#### **Body Weight, Mortality Rates, and The Number of Undeveloped Fetuses**

There were no significant differences in all treated group compared to control group. The results were presented in table 1. Results showed that in the control group, there were no undeveloped fetuses observed. However, in group of 100 mg/kg bw, one of six fetuses from one dam undeveloped. At a dose of 400 mg/kg bw, there were found 1-2 undeveloped fetus per parent of 6 parents and at a dose of 1000 mg/kg bw were found two undeveloped fetus from 2 parents. The increase of undeveloped fetus was not correlated with the dose increase.

#### **External Malformations**

There were no malformations observed in all groups. Extract treated groups in all doses have no differences compared to the control group (fig.2).



### Vertebral Malformation

According to the table 2, vertebral of each fetus in all groups was formed completely with regards to normal skeletal number (fig.2). Arrangement and total number of vertebral column in normal development include: 7 cervical, 13 thoracic, and 4 sacral vertebrae. Meanwhile, that of normal forelimb consists of 5 distal, 4 proximal, and 4 metacarpal bones; and the hind column involves 5 distal, 4 proximal, and 5 metatarsal bones. A representative from each group was depicted in figure 2. With regards to evaluation on limb organization, there were no abnormalities in all groups (table 3). The similar tendency has been shown in table 4, in which represented the condition of fetal soft tissues. All visceral organs was normal, with no abnormalities observed in the formation of eyes, head cavity, nasal cavity, palate, and mouth cavity. In addition, there were no hydrocephalus discovered in this study (fig 4.)

### DISCUSSION

In our previous studies, tempuyung (*Sonchus arvensis*) has been proven that this extract showed diuretic effect and could be employed for treating gout arthritis<sup>9</sup>. The lowest dose of extract was chosen according to the former experiment<sup>9</sup>. Meanwhile the intermediate dose was calculated 4 times higher to the lowest dose, regarding to toxicity test guideline from OECD<sup>6</sup>, it was followed by a dose of 1000 mg/kg bw as a dose limit, which was mentioned in OECD as the highest limit in which no need to increase the dose if there were no toxic effects observed or teratogenicity happen on the embryos. Determining the estrous cycle should be carried out before conducting this study. Nucleated epithelial cells are commonly found in the proestrous phase. Female rats that were in this phase were subjected to mating with male rats. After mating, early pregnancy was confirmed by the presence of vagina plugs or sperms in vaginal smears, which is referred as day 0 gestation. Either extract or vehicle suspension was administered starting from day 6 to day 15 of gestation period. The administration was performed during the organogenesis phase which was the most critical period during gestation and teratogenic effect due to external influences may occurred in this timeframes. There is an implantation of blastocyst to the uterine wall and it allows further development and differentiation into organs and important cavities. Increased sensitivity of almost all tissues during organogenesis may cause lethal effect and/or disabilities<sup>7,8,10</sup>. Absorbed tempuyung extract will be transferred to all tissues over the body through the cardiovascular system, including to reproductive organs and to fetus through placental cord. In this pre-clinical study, placental transfer of tempuyung extract during pregnancy showed there is no incidence of resorption. Cederberg<sup>11</sup> explained that by giving antioxidant compounds during pregnancy, such as vitamin E and vitamin C, it can decrease fetal malformation rate, diminish oxygen radical-related damage, and reduce oxidative stress in the liver, which contribute to fetus protection. *Sonchus arvensis* has been reported to be able

to suppress free radical scavenging activity through several experimental methods<sup>12</sup>, which may attribute on preventing teratogenicity. The results of our teratogenic investigation indicated that ethanolic extract of tempuyung does not possess developmental toxicity which is referred by no indication of fetal abnormalities, vertebral, skeletal, and visceral malformations. Underdeveloped fetuses have been observed in this study, thus consuming tempuyung extract should be warned because it may attributed to underdeveloped or risk of abortion.

### CONCLUSION

In general, administration of tempuyung leaves extract did not cause any abnormalities on developed fetuses. However, through the observation in which it may contribute to undeveloped fetus, women should be aware to take it during pregnancy or in women who desire a pregnancy.

### Abbreviation

EETL: ethanolic extract of tempuyung leaves, bw: body weight

### REFERENCES

1. Anindya AL. 2011. Xanthine oxidase inhibitory activity and antioxidant of traditional medicinal plants extract (Unpublished Undergraduate Thesis). Bandung: Bandung Institute of Technology; 2011. p. 23-30. 5.
2. Lidinilla NG. 2104. Activities test of binahong leaves ethanol 70% extract to decrease blood uric acid levels in white male rat induced by caffeine. Jakarta: UIN Syarif Hidayatullah.
3. Susanti A. 2011. Effects of *Sonchus arvensis* extract on in vitro activity of xanthine oxidase as basic of kinetics test (Unpublished Undergraduate Thesis). Bogor: Bogor Agricultural University.
4. Hendriani R, Sukandar EY, Anggadiredja K, Sukrasno. 2104. In vitro evaluation of xanthine oxidase inhibitory activity of *Sonchus arvensis* leaves. Int J Pharm PharmSci;6(2):501-3
5. National Research Council. 2001. Evaluating Chemicals and Other Agent Exposures for Reproductive and Developmental Toxicity. Washington: National Academic Press, p.206-214
6. OECD. 2001. OECD Guideline for The Testing of Chemicals Document No.414: Prenatal Developmental Toxicity Study, 1-11
7. Sukandar EY, NF Kurniati, V Fitria. 2014. Evaluation of Teratogenicity Effects of Ethanolic Extracts of Binahong Leaves (*Anredera Cordifolia* (Ten) Steenis) In Wistar Rat. Int J Pharm Pharm Sci, Vol 6, Issue 11, 422-426
8. Wilson JG, Fraser FC. 1978. Handbook of teratology vol 4: Research Procedures And Data Analysis. New York: Plenum Press,p. 223.
9. Widyaningrum KD, Sukandar EY, Fidrianny I. 2015. Xanthine oxidase inhibitory and antihyperuricemic activities of *Anredera cordifolia* (Ten) Steenis,

- Sonchus arvensis*, and its combination. Int J Pharm Sci, 7(3),86-90
10. Taylor P. 1986. Practical Teratology. London: Academic Press, 77-108
11. Cederberg, J., Simán, C.M., Eriksson, U.J., 2001. Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. Ped. Res. 49 (6), 755–762.
12. Khan RA. 2012. Evaluation of flavonoids and diverse antioxidant activities of *Sonchus arvensis*. Chemistry Central Journal, 6:126