

Anti-Implantation Activity of Kepel (*Stelechocarpus burahol*) Pulp Ethanol Extract in Female Mice

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Received 6 October 2015; Revised 11 November 2015; Accepted 21 December 2015

ABSTRACT

Kepel (*Stelechocarpus burahol*) has been used as a contraceptive remedy in Indonesian traditional Javanese royal family. Its fruit contains toxic substances to the fetus. However, few studies have been conducted on the toxic effect of kepel fruit. This paper discloses the effects of kepel's fruit extract on the percentage of fetus death, ovaries weight, and endometrial thickness in female mice. This study applied a post-test control group design. A 28 female mice were divided into 4 groups. Group I (control), II, III, and IV were groups treated with kepel pulp extract using dosage 0.65, 1.30, and 3.60 mg/kg body weight (BB). The extract was dissolved in DMSO and given in 1.0 mL/mouse. The result gives implantation numbers of each group I-IV were 5.60±1.14; 6.20±1.64; 7.60±1.51; and 8.00±1.58, respectively. The percentages of fetus death 0.00%±0.00; 48.89%±22.78; 35.83%±25.27; and 30.87%±23.01 and fetus re-sorption in all groups were recorded as 0.00±0.00. Moreover, the ovaries weight of each groups I, II, III, and IV were recorded in 0.11±0.01; 0.08±0.01; 0.11±0.32; and 0.07±0.02 g, respectively. In addition, endometrial thickness was calculated as 584.86; 841.68; 659.72; and 624.10 µm. The anova test showed that insignificant difference for ovaries weight and endometrial thickness both for control and treated groups. Conversely, the LSD test showed the percentage of fetus death has a significant difference between control and treatment groups. These results suggest that kepel pulp ethanol extract has potency for anti-implantation.

Key word: endometrium, fetus, implantation, ovarium, *Stelechocarpus burahol*

INTRODUCTION

Teratogenic substances could trigger varied growth abnormalities such as growth detention, physiological detention, structural disability, and even fetus death [1]. In Indonesia, the miscarriage rate is estimated around 2-2.5% every year, and this can significantly decrease birth rate to approximately 1.7% a year. An external cause of the high rate of misbirth is in consequence of public ignorance to harmful substances consumed during pregnancy, e.g traditional herbs and preservatives [2]. Increase in fetus death level occurs in toxic compound consumption simultaneously.

Traditionally, Kepel (*Stelechocarpus burahol*) fruit has been utilized as perfumery ingredient, to reduce uric acid levels in human body, prevent kidneys inflammation and as a regulator of pregnancy [3,4,5]. Kepel fruit contains alkaloids, saponin, tannin, and flavonoid [6]. Kepel fruit is also used as oral deodorant, that oral dose of kepel fruit for human is suggested as much as 100 gr of the raw fruit [5]. Phytochemical test and anti-implantation

The journal homepage www.jpacr.ub.ac.id

p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

effect of ethanol extract from several fruits including kepel fruit have been conducted and found that kepel fruit extract also contains polyphenols, besides alkaloids [7] Application of alkaloid, triterpenoid, saponin and flavonoid in excessive doses can lead toxicity [8], however the study of fetal death due to consumption of kepel fruit has not been much investigated. This research aims to investigate anti-implantation activity of kepel fruit pulp extract by determining its effect on percentage of fetal death in female mice, ovaries weight, and endometrial thickness.

EXPERIMENT

Plant material and extraction procedure

The ripe fruits of kepel (*Stelechocarpus burahol*) were obtained from the inhabitant park in Ambarawa, Semarang, Indonesia. The seeds were removed from the ripe fruits and the fruit pulp was sun-dried to a constant weight over a 7-day period. The dried fruit pulp was then powdered using a mechanical grinder. The powdered fruit pulp (250 g) was extracted by soxhletation method in 500 ml of 96% ethanol for 24 hours. The material was then filtered, concentrated to dryness under reduced temperature and pressure in a vacuum evaporator (yield = 2%). The dried extract was stored in air-tight clean glass container at 4°C until ready to be used in next treatment. The stock solution of ethanol extract in various concentration was prepared in DMSO (Dimethyl sulfoxide) with determined concentration for oral administration.

Animal and anti-implantation procedure

Healthy virgin normal cyclic female Swiss albino mice, aged 90–120 days old and weighing between 20–30 g were used in the experiments of following studies. The mice were exposed to 12:12 light:dark regime at 23±1°C. Daily vaginal smears were recorded throughout the experiments. Animal procedures are performed according to the guidelines for animal experiments at Lembaga Pusat Penelitian Terpadu Unit 4 Universitas Gadjah Mada, Yogyakarta. Animal procedures for the use of laboratory animals were approved by Ethics Committee of the Faculty of Medicine Universitas Islam Sultan Agung.

Mice showing proestrus phase were caged with mature healthy adult male to proven fertility with ratio 1:4 (female:male). The mating was confirmed by the vaginal contents examined and the presence of vaginal plug was considered evidence of impregnation and onset of pregnancy. The day was designated as day 1 of pregnancy and mice were randomly divided into control and experimental groups. The doses were given based on the empirical doses in human to mice as 100 g a day. The mice were divided into 4 groups, 3 groups were used for graded doses of kepel pulp extract and other 1 group was used for control. Each group consists of 5 mice. Graded doses of kepel pulp extract with 0.65, 1.3 and 2.6 mg/kg body weight/day were administered orally. The treatment was continued until 18 days of pregnancy. All the mice were autopsied by cervical dislocation on day 19, 24 hours after the last oral dose. The ovaries were dissected out freed from connective tissue and weighed to the nearest gram in single pan "OHAUS" electrical balance. The number of implantation sites and placental scars in both the uteri were counted along with corpora lutea in both ovaries.

Uterus of each mouse was fixed in Bouin's fluid passed through ascending series of ethanol and then through xylene and embedded in paraffin wax. Tissues were sectioned at 5 µm and stained with hematoxylin and eosin. The endometrial thickness were measured from the surface of endometrium functional layer to the basal layer of the endometrium using a microscope equipped with a 100x magnification with a micrometer connected to the OptiLab camera.

Statistical analysis

Data were expressed as the mean±SEM. Data were subjected to analysis of variance followed by LSD test by comparing the control group versus treated groups ($p < 0.005$).

RESULT AND DISCUSSION

Percentage of fetus death

Treatment with kepel pulp extract for 18 days of pregnancy causes no inhibition of implantation. The results indicate that 71.4% of the mated mice became pregnant and show normal number of implantation sites and show normal implantation sites. Treatment with 0.65 and 1.3 mg kepel pulp extract for 18 days of pregnancy causes death fetus higher than treatment 2.6 mg (Table 1). The percentages of fetus death were significantly different ($p < 0.05$) between the control and the treatment groups. However, increase in doses did not cause a significant difference in the percentage of fetus death ($p > 0.05$) (Figure 1). This indicates that increase in dose of kepel pulp extract did not increase the number of implantations in all pregnant mice. The resorbsion number in all treatment groups were 00.00 ± 0.00 .

Table 1. Anti-implantation activity of kepel pulp extract in mice model

Group	Treatment (mg)	Number of Mice		Number of Implantation		Number fetus life		Number of Fetus death		Number of Embryo Resorption		Percentages of fetus death	
		Mated	Pregnant at autopsy	Total	Mean**	Total	Mean**	Total	Mean**	Total	Mean**	Total	Mean**
I	Control	7	5	28	5,60±0.51	28	5,60±0.51	0	0.00±0.00	0	0.00±0.00	0	0.00±0.00
II	0.65	7	5	31	6.20±0.73	15	3.00±0.55	16	3.20±1.02	0	0.00±0.00	244.44	48.89±10.19*
III	1.3	7	5	38	7.60±0.68	22	4.40±0.40	16	3.20±0.97	0	0.00±0.00	191.66	38.33±10.97*
IV	2.6	7	5	40	8.00±0.71	29	5.80±1.32	11	2.20±0.66	0	0.00±0.00	154.36	30.87±10.29*

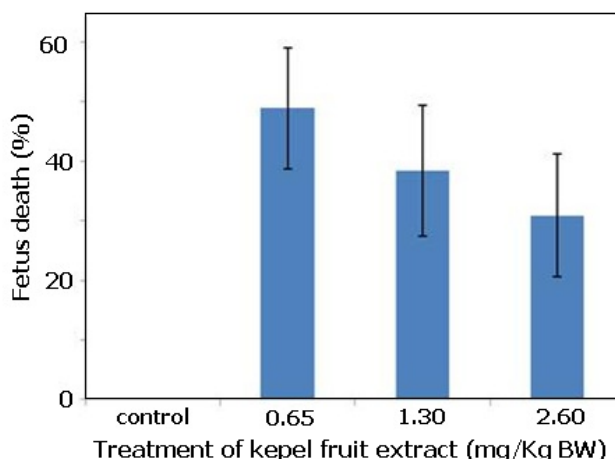
Note: * $p < 0.05$ as compared to the control; **value in (Mean±SEM).

The ability of benzoic acid to eliminate Cr(VI) using 3 methods of pH adjustment

Fetus deaths were allegedly caused by the content of Kepel fruit among other alkaloids, saponins, and tannins that cause toxicity when it is used in certain amount. Death can be caused by the direct effect of the tannins and alkaloids. Alkaloids from Kepel fruit allegedly have toxic properties which can inhibit the growth of the fetus, especially in the embryo development [9]. Saponins can cause fetal death indirectly. Saponin is the raw material of estrogen which can cause negative effect on the pituitary and suppress follicle stimulating hormone (FSH) levels resulting in lower levels of endogenous estrogen. Indirect lethal effect of saponins was not being found in this study. It can be seen from the absence of embryos resorbtion which shows no interference with ovulation, so that the embryo is still going on but experiencing a failure in growth and development.

Toxic substances can pass through the placental barrier and reach the embryo. It also depends on the molecular weight of compound substances and permeability of the placenta [10]. Fetus, which is not fully develop unable metabolize foreign substances properly. So that it have a negative effect and affect fetus development [11]. The nature of the toxic substance affects the cell depends on the sensitivity of the species, doses of toxic substances, and the critical period, i.e. during a period of differentiation and organogenesis which when it is disturbed, causing fetus death. Critical period in mice is on day 5 to day 15 of gestation [12]. The results are consistent with research by Siburian *et al.*, which indicates that there is significant effect of papaya seeds extract suspected to contain alkaloids and saponins on reproductive function of Swiss Webster female mice were visible from the decline in the number of live fetuses and an increased number of fetal death [13].

Figure 1 (right).
Effect of graded doses of kepel pulp extract on fetus death percentage in female albino mice



Uterus weight and endometrial thickness

The mice's uterus showed normal implantation sites. These mice also exhibited continuous diestrus. Treatment with 0.65 and 1.3 mg of kepel pulp ethanol extract for 8 days of pregnancy caused an increase of uterus weight compared to control group (Table 2). Anova test showed no significant difference in the ovaries weight ($p > 0.05$).

Treatment of 0.65 mg kepel pulp extract for 10 days before pregnancy and 8 days after pregnancy increased the thickness of endometrium, but no remarkable difference was shown compared to the control group. The mice treated with kepel pulp extract doses 1.3 and 2.6 mg showed no significantly decrease in endometrial thickness when compared to the control group ($p > 0.05$) (Table 2, Figure 2).

Table 2. Effect of extract doses to ovary weight and endometrium thickness in pregnant mice

Group	Treatment (mg)	Ovaries weight (g)	Endometrium thickness (μm)
I	Control	0.31 \pm 0.19	5.67 \pm 46.58
II	0.65	0.09 \pm 0.00	7.71 \pm 1.010
III	1.30	0.10 \pm 0.14	6.19 \pm 48.37
IV	2.60	0.07 \pm 0.01	6.33 \pm 60.28

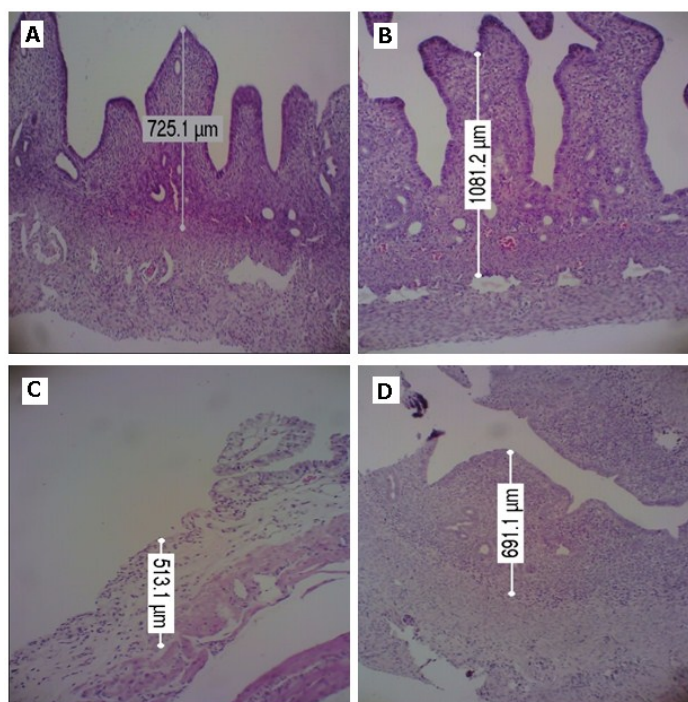
Note: Data are the mean \pm SEM values for five mice in each group.

* $p < 0.05$ as compared to the control

Ovaries weight linked to the development of ovarian follicles. The ovaries weight will be decreased if there is a decrease follicle [14]. The mean of ovaries weight treated with kepel pulp extracts were lower than those which did not given by the extracts, but there were no significant differences. Unfortunately, the indirect effects of saponins by decreasing endogenous estrogen levels that cause inhibition of ovulation and endometrial layer depletion does not occur in this study, so that the endometrial thickness were not significantly different among groups. Doses given supposedly have not given estrogenic and cytotoxic effect on the condition which disturbing growth and development of ovarium follicles, in consequences the ovarium weight on the treatment groups were almost similar to the control group.

Figure 2 (right).

Micrographs of mice's uterus tissue (100x, H&E / hematoxylin and eosin staining) in different groups: (a) representing the normal endometrial thickness and structure in the control group and (b) shows an increase in the endometrial thickness in the mice treated to kepel pulp extract dose 0.65 mg/kg BW, (c) and (d) shows a decrease in the endometrial thickness in the mice treated to kepel pulp extract dose 1.3 mg/kg BW and 2.6 mg/kg BW respectively (photo was taken with 100x magnification)



The phytoestrogens is needed in a very large amount to obtain adequate effects as estrogen. The substrate binds to the estrogen receptors will cause estrogenic effects [15,16,17]. The estrogen produced by the follicle de Graaf then stimulate the FSH [18]. Low levels of estrogen lead to reduction on follicle de Graaf, so that the ovaries weight also decreased. Moreover, uterus is an organ which has estrogen hormon reseptor, so as estrogen level can affect uterus' endometrium. Endometrial thickness was affected by the level of estrogen [19]. The higher the level of estrogen the thicker the endometrium tissue. Phytoesterogen is natural estrogen from plants containing compound which has an ability to affect estrogenic activity in the body by filling vacant endogen estrogen receptor site or competing with endogen estrogen which are available in the body with a lower intensity [20].

The result of this study is slightly different to that reported by Hikmah *et al.* that alkaloid, saponin, tannin and triterpenoid working based on their cytotoxic effect by disturbing ovum of healthy cell. Moreover is disruption on the progesterone and estrogen synthesis and also formation of endometrium cell. As an effect is endometrium depletion [21].

CONCLUSION

In conclusion, it can be said that the ethanol fruit pulp extract of kepel (*Stelecocarpus burahol*) is effective as potential source for natural anti-implantation compounds. However, it have no significant effect on ovaries weight and endometrial thickness. A further investigation is needed to advance and undestand the action mechanism of kepel extract on implantation in mice.

ACKNOWLEDGMENTS

This study was supported by the grant from Faculty of Medicine Universitas Islam Sultan Agung, Indonesia for group research with contract number 01/P-KEL/UPR-FK/VII/2012, fiscal year 2012/2013.

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