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Teratogenic test of *Pandanus conoideus* var. yellow fruit extract to development of rat embryo (*Rattus norvegicus*)

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Abstract. *Muna L, Astirin OP, Sugiyarto. 2010. Teratogenic test of* Pandanus conoideus *var. yellow fruit extract to development of rat embryo (*Rattus norvegicus*). Nusantara Bioscience 2: 126-134.* This experiment was performed to examine the effect of *Pandanus conoideus* Lam. var. yellow fruit extract on the percentage of the living foetus, the death intrauterus, heavy and long of foetus, foetus morphology, and skeleton structur of foetus. The experiment was used by 25 pregnant mice that randomly were divided into 5 groups, they contained 5 mice. Each group was given with the different dose. The P1 group (control) was given 1 mL sesame oil, the group P2, P3, P4 and P5 were respectively given the yellow fruit extract 0,02 mL, 0,04 mL, 0,08 mL and 0,16 mL. The *P. conoideus* var. yellow fruit extract was given orally on day 5 to 17 of gestation (organogenesis periode). Observation was carried out on day 18 of gestation by caesarean section to take the foetus from the uterus. Foetus morphology was observed after taking foetus from uterus, whereas observation of skeleton structure was made *wholemount* preparat with dual colourization, they are *Alcian blue* and *Allizarin Red-S*. The result was analyzed with *one way anova.* Results showed that giving yellow fruit extract didn't influence to the percentage of the living foetus, the death intrauterus, heavy and long of foetus. The effect of giving yellow fruit extract to the maternal were abnormality skeleton (lordosis) of foetus in the dose 0.16 mL and obstacled to the ossification of foetus.

Key words: *Pandanus conoideus* var. yellow fruit, teratogenic, white rat.

Abstrak. *Muna L, Astirin OP, Sugiyarto. 2010. Uji teratogenik ekstrak* Pandanus conoideus *varietas buah kuning terhadap perkembangan embrio tikus putih (*Rattus norvegicus*). Nusantara Bioscience 2: 126-134.* Penelitian ini betujuan untuk mengkaji pengaruh pemberian ekstrak *Pandanus conoideus* Lam. var. buah kuning terhadap persentase fetus hidup, kematian intrauterus, berat dan panjang fetus, keadaan morfologi fetus, serta struktur skeleton fetus tikus putih. Dalam penelitian ini digunakan 25 tikus bunting yang dibagi menjadi lima kelompok secara acak, sehingga masing-masing kelompok terdiri dari lima ekor tikus. Setiap kelompok diberi dosis yang berbeda. P1 (kontrol) diberi 1 mL minyak wijen, P2, P3, P4 dan P5 diberi ekstrak masing-masing: 0,02 mL, 0,04 mL, 0,08 mL dan 0,16 mL. Ekstrak tersebut diberikan secara oral pada kebuntingan hari ke 5 sampai hari ke 17 (fase organogenesis). Pengamatan dilakukan pada hari ke 18 dengan cara bedah sesar untuk mengambil fetus dari uterus. Morfologi fetus diamati setelah fetus dikeluarkan dari uterus, sedangkan untuk pengamatan struktur skeleton dibuat preparat wholemount dengan pewarnaan ganda *Alcian blue* dan *Allizarin Red-S*. Hasil percobaan dianalisis dengan ANAVA satu jalur. Hasil penelitian menunjukkan bahwa pemberian ekstrak tidak berpengaruh terhadap persentase fetus hidup, kematian intrauterus, serta berat dan panjang fetus ($P \geq 0,05$). Pemberian ekstrak pada induk mengakibatkan kecacatan skeleton (lordosis) fetus pada dosis 0,16 mL dan menghambat osifikasi fetus.

Kata kunci: *Pandanus conoideus* var. buah kuning, teratogenik, tikus putih.

INTRODUCTION

Cancer is a disease characterized by uncontrolled cell growth. Cancer is the second cause of death in the world after cardiovascular disease (Ganiswara 2001; Foye 1996). Cancer cells in the organs of the body will grow and develop in an abnormal, rapid and uncontrolled with the cells shape, nature and movement different from its origin and cause damage to the form and function of organs (Dalimartha 2004). Uncontrolled growths of cancer cells push the normal cells around them, because cancer cells can metastasize to other body parts (Harkness 1989).

Treatments to suppress or cure these diseases are surgery, radiation, and treatment with chemical compounds (Harkness 1989; Alatas 2005). Anti-cancer drug or sitostatica are medications that can stop the growth of malignant cells or even kill normal cells (Tjay and Rahardja 2002). Anti-cancer drugs are teratogenic and can not only affect the cancer cells, but can affect normal cells (Foye 1996; Ganiswara 2001). The high cost and side effects by the presence of cancer therapy (Harkness 1989; Harmanto 2001), encourage communities to use anti-cancer drugs from plants.

In Indonesia, there are no less than 2039 species of medicinal plants from tropical forests. This situation makes Indonesia as one of the world's storehouse of biodiversity important for pharmaceutical or medicinal ingredients for human health (Zuhud 2009). One of the plants that acts as anti-cancer is a red fruit (*Pandanus conoideus* Lam) varieties of *P. conoideus* var. yellow fruit. These plants

contain various useful chemical compounds (Budiman 2000). According to Mun'im (2006) mampung red fruit extract inhibits tumor growth. Atsirin (2009) and Pratiwi (2009) independently proved that the extract of yellow varieties of *P. conoideus* fruit can inhibit the growth of T47D breast cancer cells. Hidayati (2010) proved that this plant extract could inhibit Hela cell growth. *P. conoideus* var. yellow fruit is a endemic plant of Papua, which has the tocopherol and beta-carotene content that act as antioxidants. As an antioxidant, both compounds were able to ward off free radicals and are thought to help the healing process of cancer (Budi and Paimin 2005).

Gysin et al. (2002) revealed that α-tocopherol could inhibit the growth of DU145 prostate cancer cells by 50%, LNCaP, prostate cancer cells as much as 48%, and 50% in adenocarcinoma colon cancer cell (Caco-2). Consuming 30-60 mg of beta-carotene daily for 2 months will make the body have natural killer cells and more T cells and lymphocyte-helpers which are more active. The increase in natural killer cells is essential to fight cancer cells and control free radicals that disturb health badly (Budi and Paimin 2005). The same is stated by Russell (2002) that the cancer risks are lower in people who consume vegetables and fruits that contain high carotenoids.

Tocopherol is a form of vitamin E, when consumed excessively; it can cause poisoning (Almatsier 2002). And, beta-carotene is an vitamin A. The results of experiments on animals showed the occurrence of birth defects as a result of hipovitaminosis and hipervitaminosis of vitamin A during pregnancy (Pergament 1996).

Anti-cancer drug is used by all cancer patients with including pregnant women, while pregnant women are particularly vulnerable to drugs, especially during organogenesis. Some of drugs that are not allowed to eat by pregnant women are anti-cancer drug, because the anti-cancer drugs are capable of stopping cell division (Nogrady 1992) and drugs that reach the fetus can cause miscarriage, malformation and death of the fetus (Suryawati 1990).

This study aims to examine the effect of the extract by oral administration of external abnormalities in the form of percentage of live fetuses, intrauterine death, fetal weight and length, and the state of fetal morphology, and internal abnormalities of fetal skeleton structure of the white rat (*R. norvegicus*).

MATERIALS AND METHODS

Material

Test animals used were white rat (*R. norvegicus*), with zero day to 2.5 months of pregnancy and an average weight of 200 g, pellets Br 2 (PT. Japfa Comfeed Indonesia, Sidoarjo) as the daily feed, extracts of *P. conoideus* var. yellow fruit, water to drink and sesame oil as a solvent. Sesame oil is packed by PT. Heinz ABC Indonesia, Jakarta).

Study design

This research was conducted with experimental methods used 25 white rats which were divided into 5 groups with completely randomized design (CRD). Each treatment consisted of 5 replicates. The treatment is as follows:

P1: Control = 1 mL of sesame oil

P2: The dose of 0.02 mL of extract of *P. conoideus* var. yellow fruit oil and 0.98 mL of sesame oil/200 g BW

P3: The dose of 0.04 mL of extract of *P. conoideus* var. yellow fruit oil and 0.96 mL of sesame oil/200 g BW

P4: The dose of 0.08 mL of extract of conoideus var. yellow fruit oil and 0.92 mL sesame oil/200 g BW

P5: The dose of 0.16 mL of extract of *P. conoideus* var. yellow fruit oil and 0.84 mL sesame oil/200 g BW

Extracts *P. conoideus* var. yellow fruit and sesame oil given orally in the morning and afternoon on gestation day of 5 to 17.

Procedures

Pre-treatment. Twenty-five white adult female rats (*R. norvegicus*) in estrus cycle were gathered in one cage with 10 male of white rats. The following day the female rats were examined in way of vaginal plug (vaginal plug), if there was vaginal plug or after viewed microscopically with vaginal smear method and the spermatozoa was found, then that day was considered as the first day of gestation. Furthermore, female rats were separated from white male rats and they were divided into 5 groups, each group consisted of 5 female rats.

Preparation of the animals test. The 2.5 months pregnant rats with an average weight of 200 g were kept in a cage, each cage contained five mice with the same treatment group. The total number of white rats used was 25. They were divided into five treatment groups. Before being used in the study, rats were acclimatized for 4 days, and they were given by food and drink.

Extraction. Preparation of extracts of *P. conoideus* var. yellow fruit refers to Budi and Paimin (2005) as follows: the fruit that is mature was selected, the maturity was marked with a yellow fruit color and the distance between bulges which were rare. The fruit was cleft and the pith was discarded, then it was cut and washed. Fruit flesh was boiled for 1-2 hours, when it was soft, it was removed and cooled. Fruit meat was kneaded until the seeds were separated. Water was added up to 5 cm above the surface of the material (3:1). This material was squeezed again until the seeds seemed white and clean of meat. The result was the digest of *P. conoideus* var. yellow fruit which look like coconut milk. The digest was refined to separate juice from seeds. The refined digest was cooked in ± 40ºC for 5-6 hours as it was stirred. If yellow oil was appeared on the surface, the flame is turned off and continued the stirring for 10 minutes to cool it quickly. Juice was removed and was left alone for 1 day until there was the formation of 3 layers, namely the pulp (bottom layer), water (middle layer), and oil (top layer). Oil was taken slowly with a spoon and moved into a transparent container, and then it was left alone for 3 hours until the oil, water and waste were completely separated. With a spoon, the oil was moved again carefully into the container.

Determination of dose usage. The recommended dosage of *P. conoideus* var. yellow fruit is 2-3 times of one tablespoon daily (Budi and Paimin 2005), where one

tablespoon is equivalent to about 15 mL (Wiryanta 2007). That dose is for people with 70 kg of bodyweight. If the dose is applied on white rats weighing 200 g, it is obtained: X/200g = 15 mL/70 kg → X = 0.043 mL = 0.04 mL. The determination of dose is also based on the research by Pratama (2009) who uses the extract with a concentration of 0.03125 mL to T47D breast cancer cells. The results of these studies indicate that with this concentration, not all T47D breast cancer cells die. While the use of the same oil solvent is based on the research by Mun'im (2006) using 1 mL of sesame oil on the control. The results show that there is no abnormality in the fetus due to the use of 1 mL of sesame oil.

Treatment of the animals test. Previously, all rats were weighed to determine their initial weight. The extract was given orally in each treatment group from day 5th to the 17th day of gestation, respectively. On the 18th day, the surgery was done. Before the surgery process, all rats were weighed to determine the final weight. Observations were carried out by taking a fetus from the uterus and then it is cleaned from the placenta and the mucous membrane enveloped it. Fetus external observations began with the count and record the number of implantation which consists of the number of living fetus, the number of dead fetuses, and the number of fetuses that were resorption. Furthermore, the body weight, the body length, and the fetal morphology were observed including: body shape, number of extremities, skull, tail, etc that were considered abnormal. The observation was also done on the internal skeleton system (bone shape, the amount of bone and the process of reinforcement). To study the fetal skeleton structure, a wholemount preparations with dual staining method of Allizarin red-S and Alcian blue is made (Inouye 1976).

Wholemount preparations making process was as follows: Fetuses were fixed into 95% alcohol for 3 days. Viscerasi is the disposal process of skin, fat tissue and internal organs of the fetus. This process was done very carefully as not to damage the fetus nor change the fetal organ position. White rat fetuses were put into acetone for 1 day to dissolve fat. Fetuses were stained on day 4th using a double staining namely Allizarin red-S and Alcian blue for 1-3 days at 37 º C temperature. Fetuses were washed with running water for several times until they were clean. Fetuses were clarified with 1% of KOH solution in water for 2 days until the tissues that wrap the body become transparent and the ones that have red or blue color were only bone tissues. Fetus transferred into 20% of glycerin solution in 1% of KOH for 1-4 days. In a row, fetuses were inserted in a solution of glycerol 50% and 80% in KOH 1% respectively for 1 hour, and then were stored in 100% of glycerin for later observation. Observations on the results of ossification were based on of the dye absorption of skeleton. True normal bone turns red and inhibited growth bone will be blue or no color at all as a dyeing reaction of Allizarin Red-S. The photographing of fetal was performed at the time of abnormalities observation, both external (abnormalities of morphology, hemorrhage, and resorption) and internal (ossification abnormalities) using a digital camera.

Collection of data. The quantitative data was obtained by observing the number of implantation consists of the number of live fetuses, number of dead fetuses, fetal weight, and fetal body length. The qualitative data was obtained by observing the morphology of the fetus (eyes, ears, knuckles, skulls, tails and others that are considered abnormal) and its skeleton system (bone shape, number of bones, and the process of reinforcement).

Data analysis

Quantitative data were analyzed using single lane of analysis of variance (ANOVA) with significance level of 5% to know the real differences among the treatments. If the analysis of variance obtained significant results, it continued with Duncan multiple range test (DMRT) to know the difference. For the observation of external and internal abnormalities (the abnormalities of ossification results) a descriptive analysis was conducted.

RESULTS AND DISCUSSION

Pandanus conoideus var. yellow fruit is one of the alternative medicines to cope with cancer (Budi and Paimin 2005). Anti-cancer drug is used by all cancer patients, including pregnant women, while pregnant women are particularly vulnerable to drugs, especially during organogenesis (Briggs et al. 2008). Anti-cancer drugs are teratogenic; not only affects the cancer cells, but can affect the normal cells around it (Ganiswara 2001; Foye 1996).

Fetal tissue grows rapidly, the cells divide rapidly so it is very vulnerable to anti-cancer drug. In addition, the drugs consumed by the parent will move to the fetus through the placenta, namely the same path in which the nutrients needed for growth and development of the fetus go through. Drugs that reach the fetus can cause miscarriage, malformation or even death of the fetus (Suryawati 1990).

White rat fetal external abnormalities

In this study, external abnormalities were observed in a morphometry way by seeing the occurrence of reproduction of white rats parent by counting the number of live fetuses, the number of intrauterine death (dead fetuses and resorption), measuring weight and length of fetal body and observing the abnormalities in the form of hemorrhage and deformity in some parts of fetal body (Table 4).

Fetal morphometry

Fetal weight and length are important parameter to be observed in teratogenic research. Wilson (1973) states that the decrease in fetal body weight and length is the lightest form of an effect of teratogenic compound. Fetal body weight and length is a parameter that is sensitive enough to determine the effect of foreign compounds on the growth of a fetus. There were changes in fetal body weight and length ranging from the control group until the group treated with the highest dose (Table 4).

Based on statistical analysis, weight and average length of fetuses between treatment groups were not significantly

different (P> 0.05). The result of variance analysis is supported by the analysis of correlation between the dose with fetal weight and between fetal length with doses. R value between the dose with body weight was 0.265 (weak correlation). Probability value was > 0.05 (0.200> 0.05), then H0 was accepted. It means that the relationship between doses with fetal body weight was not significant at the level of 95%. While the value of r between dose with fetal length is 0.123 (the correlation was very weak). Probability value was > 0.05 (0.559> 0.05), it could be concluded that the relationship between dose with fetal length is not significant at the level of 95%.

Beta-carotene and tocopherol in a certain amount are needed by the body because it is a vitamin that is important for the body, but if there's too much, it would be toxic (Almatsier 2002). The results of Azman's study (2001) said that, vitamin A and E effect weight gain of normal rats and rats with treatment of diovariectomy (removal of two ovaries). In this study, weight gain primarily is due to the increase of fat mass. Tocopherol and beta-carotene are fat soluble vitamins that are lipophilic, allowing it to easily cross the placenta.

Tocopherol and beta-carotene are absorbed in the intestines along with the fat or oil consumed. One of the reasons why the extract is taken orally is because tocopherol and beta-carotene are not water soluble, which means it also not soluble in blood plasma. In order for these vitamins can be transported into the blood circulation, it must bind to proteins (lipoproteins), which is then absorbed by the lymphatic system. From the lymphatic system, these vitamins along with VLDL (Very Low Density Lipoprotein) go into the blood circulation. Some go to the needed part and the others go to the liver through the ductus toracicus which later merged with triglyceride-rich VLDL and HDL (High Density Lipoprotein) which is rich in phospholipids, cholesterol and esters. VLDL and HDL are synthesized by the liver. Then E vitamin will be back into the blood vessels and then converted to LDL (Low Density Lipoprotein) by the help of the enzyme of lipoprotein lipase in the blood. Furthermore, the vitamins in LDL transported to adipose tissue.

There are three types of entry of drugs through the placenta, namely: Type 1, drugs with a balanced concentration between mother and fetus; Type II, drugs that have a concentration in fetal plasma is higher than the concentration in maternal plasma or an excessive transfer is happened. This may occur because of the flowing out transfer of fetal drug is slower; Type 3, drugs that have a concentration in fetal plasma is lower than the concentration in maternal plasma or an incomplete transfer is happened (Nindya 2001).

Fetal body weight tends to increase due to the tocopherol contained in *P. conoideus* var. yellow fruit. Tocopherol is stored in the liver and fat tissue, so that if fetuses are deficient in E vitamin, the tocopherol may be used again soon. In addition, if there is damage to fetal cells, then the fetus can immediately make the recovery, because the fetal cells are still actively dividing, so damage to the cell can be easily replaced with another normal cell.

Normal levels of A vitamin in plasma is 100-120 units/dL. The requirements of A vitamin in pregnant women is > 200 RE (Dewoto 2007). The content of A vitamin in *P. conoideus* var. yellow fruit is 240 ppm (240 mg/L), so that meet nutrient adequacy rate for pregnant women. While the requirement of E vitamin for pregnant woman is 10 mg. The content of E vitamin on *P. conoideus* var. yellow fruit is 10,400 ppm (10,400 mg/L). E vitamin acts as protective of fatty acids from free radical oxidation (Almatsier 2002), so the use of high concentration of E vitamin has no effect.

Average normal fetal body length on day 17th of gestation is 19.31 mm, whereas on day 18th is 20-23 mm (Kauffman 1992). Average length of fetuses in this study is 25 cm, so it can be said that the length of fetuses in this study was normal. Besides influenced by the external factors namely the inclusion of A and E vitamin in the body of the fetus, growth is also influenced by genetic factors (Wilson 1973). The decrease or increase in fetal body weight and length are associated with the genes contained in each individual and the room for its growth. PKH (2009), states that the consumption of fat will affect the production of progesterone which is essential for implantation and as nutrients that are essential for the formation of early embryos. Number of embryos in the uterus also affects the availability of space for embryonic development and blood supply.

Fetuses which are derived from one uterine sac with a little amount of implantation have relative heavier and longer size than the fetus from the uterus with a lot of implantations. This relates to nutrition received by the fetus. The fewer the number of implantation in the uterus, the availability of nutrients for the fetus are met, so that the fetal weight and length will increase (Zahrah 2008). This can be seen in the control group with the largest number of implantation compared with treatment group. The control group tends to have average lowest body weight and length. The treatment group of 0.08 mL and 0.16 mL of extract that has the same number of implantation had nearly the same weight.

Percentage of intrauterine death and fetal life

Fetal death or resorption is a form of intrauterine death. Intrauterine death occurred due to the inability of cells to repair (recovery) to replace damaged cells with normal cells. This is probably because of the large number of damaged cells, so there is no balance between damaged cells with normal cells. Fetal cells are able to do recovery causing fetal survival. Based on the analysis of variance with a level of 95%, there are no significant differences for the percentage of live fetuses, fetal death and resorption between the control group and treatment group. This is evidenced by the value (P> 0.05). Fetal death in this study was at a dose of 0.02 mL of extract. Fetus is categorized into fetal death, when the fetus is fully developed and there are no signs of Autolisis, and it gives no respond to a touch (Hutahean 2002). The provision of *P. conoideus* var. yellow fruit on the parent does not affect fetal death, because the dosages are very small. In addition, A and E vitamin contained in *P. conoideus* var. yellow fruit are necessary for fetal growth. Fetal death in this study is likely

caused by the process of cell division and disrupted cell differentiation, so the fetus can no longer continue its development or can be caused by severe functional abnormalities so that the fetus can not survive. In addition, the dead fetus in the womb has not finished its development, so it has smaller size compared to fetuses born alive (Setyawati 2009).

Fetuses which experience resorption are marked by a clump of red or yellow-brown color that does not respond when touched (Hutahean 2002). Resorption is a manifestation of the death of the conceptus (Lu 1995) that may occur due to improper body morphology with various disabilities and ended with the death (Rugh 1968).

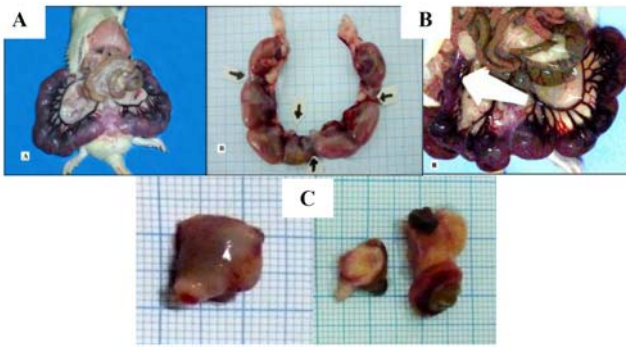


Figure 9. The morphology of the fetus inside the mother of the control and that fed by stem extracts. The arrows show where resorption. (A) The uterus containing a normal fetus, (B) The uterus containing the fetus resorption, (C) The form resorption.

In this study, resorption presents in all treatment groups. Treatment group with the highest number resorption is found in 0.02 mL doses of extract (Table 4). In the early stages of proliferation, the embryo will respond to die or grow normally, because at this stage, cell differentiation does not happen yet, so there is no selective effect of a teratogenic agent. This is because the cell is still totipotent, whereas if it occurs during cell intensive differentiation, mobilization and organogenesis, it will cause malformations or birth defects, but if it happens after the phase of organogenesis, it will cause abnormal function. If the effects of teratogens can not be handled by the embryo, it can cause embryonic death followed by abortion or resorption on rodensia if it occurs in early pregnancy, and fetal death in late pregnancy.

In addition, genetic plays an important role in the death in the phase of pre-implantation (resorption). Embryo death in pre-implantation stage often occurs due to inbreeding marriage. Before implantation, the embryo is more susceptible to the influence of genetic mutations and chromosomal abnormalities, followed by early embryonic death (PKH 2009). Abnormalities of chromosomes can be distinguished on the number of chromosome abnormalities and chromosome structure. This can occur because of failure of the spread of chromosomes or arrangement of chromatin in cells that occur during meiosis and mitosis of the egg or sperm cells that produce 2 forms poliploid cells. Aneuploid is chromosome abnormalities in animals that may occur due to the reduction of the normal chromosome number (2n-1), while poliploid is the addition of a normal chromosome number (2n+1). The abnormalities caused resorption.

Morphology of the fetus-hemorrhage

There are 4 forms of embryo developmental disorders, namely death, deformity, growth inhibition and impaired function (Hutahean 2002). Overall, the fetal organs are fully developed in this study (body components are complete), although there are fetuses with less size than the others. In this research, there are three types of external abnormalities, such as: transparent skin, growth inhibition and humpbacked body (Table 5).

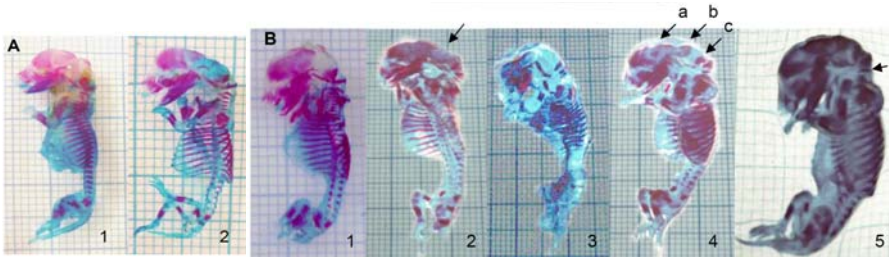
Transparent skin or which is often referred to hemorrhage is an event of blood discharge from the cardiovascular system which is accompanied by accumulation in tissues (Price and Wilson 1984 in Widiyani and Sagi 2001). Hemorrhage is a form of external abnormalities that often occur as an effect of a teratogen. In this study, Hemorrhage is found on the head, the neck, the back and the stomach.

Fetuses with transparent skin can be found in all treatment groups, including the control group (Figure 11). This possibility occurs because the extract is given repeatedly in high enough doses, so there is high concentration in the blood, resulting in osmotic imbalance. In normal circumstances, the embryo develops in the amniotic fluid that is isotonic with body fluids. The entry of foreign substances in tissue can alter the osmotic pressure. Osmotic imbalance can cause pressure and viscosity of liquid in different parts of the embryo, between blood plasma and extra capillary space or between extra-embryonic and intra-embryonic fluid. This difference causes the blood vessels rupture and hemorrhage occurred (Wilson 1973).

In cases of hemorrhage, there is no difference between control (sesame oil provision) with treatment group (the provision of a mixture of sesame oil and extract). If the red blood cells are at hipotonic solution, ie solution in which concentration of dissolved substance outside the cell is lower than inside the cell, then the red blood cells are lysis (rupture). This is due to the absence of cell wall which may hinder the process of red blood cell lysis (Zulti 2008).

Table 5. Percentage of external fetal abnormality of *R. norvegicus* after the provision of extracts to the parent.

Types of external abnormalities	Dose extract				
	0 mL	0.02 mL	0.04 mL	0.08 mL	0.16 mL
Number of fetuses	58	52	44	46	44
Hemorrhage/transparent skin	14 (24.14%)	15 (28.85%)	4 (9.09%)	12 (26.09%)	6 (13.64%)
Barriers to growth	0 (0%)	1 (1.92%)	0 (0%)	0 (0%)	0 (0%)
Body of humpback	0 (0%)	0 (0%)	3 (6.82%)	0 (0%)	0 (0%)
Total	14 (24.14%)	16 (30.77%)	7 (15.91%)	12 (26.09%)	6 (13.64%)



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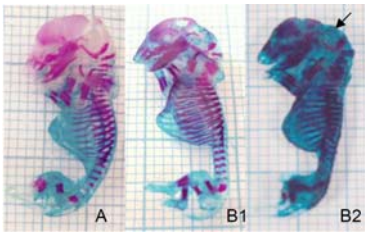

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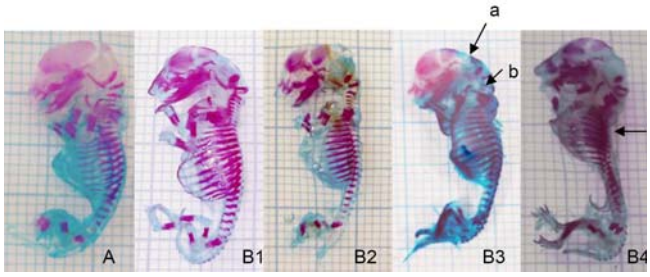
Figure 14. The development of fetal skeleton *R. norvegicus* as a result of the provision of 0.02 mL of *P. conoideus* var. yellow fruit extract on the stem. The arrows indicate the area which experiences delayed ossification. A) Skeleton control group: A1. Perfect ossification, A2. Transparent skin, perfect ossification. B. Skeleton of 0.02 mL extract treatment groups: 1. Ossification perfect, B2. Delayed ossification of os interparietal, B3. Skeleton of stunted fetuses, delayed ossification in the frontal os, os parietal, os interparietal, cervical vertebrae, lumbar vertebrae, sacral vertebrae, tibia and fibula, B4. Skeleton of normal fetuses, delayed ossification of os parietal (a), os interparietal (b), and cervical vertebrae (c), B5. Skeleton of delayed ossification in the cervical vertebrae.

Figure 15. The development of fetal skeleton *R. norvegicus* as a result of the provision of 0.04 mL of the extract on the parent. A. Skeleton of the normal control group. B. Skeleton of groups of 0.04 mL extract: B1. Normal Skeleton, B2. Skeleton of delayed ossification in os interparietal



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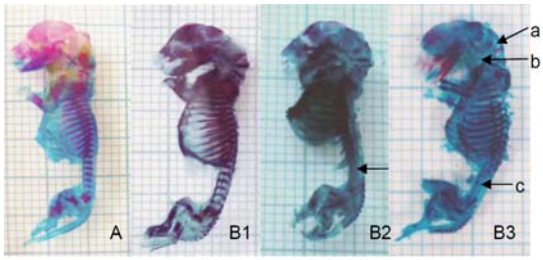

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Figure 16. The development of fetal skeleton *R. norvegicus* from the treatment of 0.08 mL of the extract on the parent. A. Control. B. Skeleton 0.08 mL extract treatment groups: B1. Normal fetus, ossification perfect, B2. Fetus transparent skin, perfect ossification, B3. Normal fetus, but delayed ossification of interparietal os (a) and cervical vertebrae (b), B4. Skeleton lordosis.

Figure 17. The development of fetal skeleton *R. norvegicus* from the provision of 0.16 mL of the extract on the parent. A. Skeleton control group. B. 0.16 mL extract skeleton groups: B1. Ossification perfect, B2. Skeleton delayed ossification in the lumbar vertebrae, B3. The delay in ossification of the cervical vertebrae (a), clavicula (b) and lumbar vertebrae (c).

Fetal morphology-growth retardation

In this study, fetal which experienced growth inhibition is only in the treatment group of 0.02 mL of extract (Table 6). According to Ritter (1977), compounds with low doses of teratogens are capable of causing the death of some cells and can also cause cell turnover, because the fetal cells have high regeneration ability. If one or a group of cells are damaged by the interference of toxic agents, the normal cells around it will divide and replace the damaged cells. The replacement of fetal damaged cells will be maintained during the period of organogenesis in order to form a normal morphology. If that fails or does not reach the target in the phase of organogenesis, it will cause fetal malformations, forming morphologically normal, but small.

Growth inhibition in the fetus may be caused by disruption of cell division, namely the nucleic acid and protein synthesis are disrupted and then the damaged cells can not be repaired. Fetal growth occurs because of cell proliferation by mitosis and proliferation rapidity is a function of growth velocity (Herbold 1985). In this study, growth inhibition on fetuses is caused by cell death due to the provision of extract as an anti-cancer which is given at the time these cells begin to actively divide, resulting in inhibition of cell division. If a foreign substance is given continuously, in the process of time, these cells will die. If more and more cells die, it will be difficult for fetus to develop.

Fetal morphology-humpback body

In this study, Humpback body was only found in group of 0.04 mL of extract treatment (Figure 13). Normal embryogenesis ended with the formation of new individuals that shape and structure is the same as its parent, but abnormal embryogenesis will end with the formation of individuals who vary (Wilson 1973). Abnormal shape in the form of humpback body in this study is likely caused by a deformity of the vertebrae (spine) which is caused by the death of bone cells making up some vertebrae, resulting in bone growth rate of one to another is not the same, so the bone bends. Fitrianna (2009) states that the vertebrae are formed on day 12^{th} . In this study, the extract is given to the parent from day 5^{th} to day 17^{th}. Presumably this cell death is caused by the extract given to the parent. Given that the immune response against the teratogens substance of each individual is different (Wilson 1973), the fetus with a low immune system is unable to repair cells, especially spine constituent cells which are damaged or die by the presence of substances such teratogens.

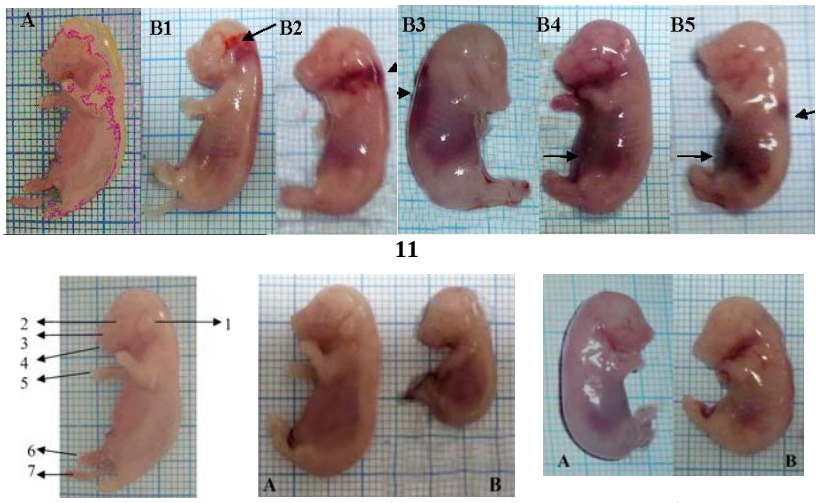

Figure 10. Morphologically normal fetuses of *R. norvegicus*. Note: 1. Pinnae, 2. Eye, 3. Vibrisae, 4. Mouth, 5. Anterior limb, 6. Posterior extremity, 7. Tail

Figure 11. Comparison of fetal normal skin and fetal transparent skin after administration of extract on the parent. Arrows indicate hemorrhage. (A) Fetal normal skin, (B1) Fetus transparent skin doses of 0 mL; (B2) Fetus transparent skin dose of 0.02 mL; (B3) Fetus transparent skin dose of 0.04 mL; (B4) Fetus transparent skin dose of 0, 08 mL, (B5) Fetus transparent skin dose of 0.16 mL of extract.

Figure 12. Comparison of normal fetuses (A) with an experienced fetal growth inhibition (B) effect of extract to the parent.

Figure 13. Comparison of normal fetuses (A) and fetal body humpbacked after administration extract to the parent.

Internal fetal abnormality of white rats

The observation of internal abnormalities, namely the observation on the development of fetal skeleton of *R. norvegicus* wistar strain is by making the wholemount preparation. The preparations were made using double staining, ie Allizarin Alcian Blue and Red-S. Alcian Blue and Red Allizarin-S is a special chemical substance for staining bone tissue (Inouye 1976). Alcian Blue will perform affinity with the matrix in cartilage tissue, so the cartilage will be stained in blue. While Allizarin Red-S will perform affinity with the matrix in bone tissue, so the bones will be stained in red. The internal abnormalities observed included bone structure and results of its ossifications.

Skeleton comes from the mesoderm. In the mesoderm, differentiation occurs including mesoderm of head, body and tail which in this level of development is called embryonic Mesenchyme. Mesenchyme develops into mesoderm structures of the body, including connective tissue namely cartilage (cartilage) and bone (Sagi 1997). Calcification process occurs in two ways, namely intra-membrane and endochondric ossification. Intra-membrane ossification is the process of bone formation of mesenchymal tissue into bone tissue, such as the formation of lamellar bone. While ossification endokondral the bone formation process that occurs in which mesenchymal cells first differentiate into cartilage (cartilage tissue), then transformed into bone tissue, such as the formation of long bones, vertebrae and pelvis.

According Loegito et al. (1995) in Ekawati (2002), there are three benchmarks to determine the growth and development of the skeleton, namely: number of components of skeleton and their ossification level, the perfect or not the ossification process, and the presence or absence of abnormalities in the formation of skeleton. Based on the observation, the results show the presence of delays in the process of ossification in all treatment doses ranging from lowest to highest dose (Table 6).

The development consists of increasing bone size, maturity and age. Changes from the development of membranous and cartilaginous hard bone are called bone maturation. There are five periods of bone formation, namely: (i) the embryonic period: mandible, maxilla, humerus, radius, ulna, femur, and fibia, (ii) fetal period: scapula, illium, fibula, (iii) cartilage: epiphisis on the limb, carpal, tarsal, and sesamoids; (iv) adolescent bones: scapula, ribs, hip/waist, (v) adult bone (Jessop 1988). Abnormalities of skeletal malformations found in this research is a form of lordosis skeleton which is found in 0.16 mL of extract treatment group and delayed ossification process found in all treatment groups. However, the treatment group with deformity skeleton is mostly found in 0.02 mL of extract treatment group (Table 6).

Table 6. Percentage of deformity of fetal *R. norvegicus* skeleton as the result of the provision of the extract to the parent.

Dose of extract of *P. conoideus* var. yellow fruit	Amount of parent	Amount of fetus	Amount of observed fetus	fetal defects percentage
P1 0	5	58	20	0%
P2 0.02 mL	5	52	12	33.33%
P3 0.04 mL	5	44	18	5.56%
P4 0.08 mL	5	46	21	9.52%
P5 0.16 mL	5	44	9	22.22%

In this study, the bones experiencing delays in ossification were found in the Cranium, cervical vertebrae, clavicula, lumbar vertebrae, sacral vertebrae, and tibia and fibula. The delay in ossification is blue in the fetal skeleton and indicates that these bones are still cartilage (cartilage).

The results shows that 0.02 mL of the extract treated group experienced delayed ossification compared to other groups. This is most likely influenced by a lack of nutrients to the mothers of white rat, so that the metabolic processes are inhibited. It is indicated by the presence of very light fetal weight compared to fetal weight of other groups, namely 0.7 g. The most important nutrient for growth and development of bone is calcium (Dewoto 2007). According Setiyohadi (2009), calcium holds two important physiological roles in the body. In bone, calcium salts play a role in the calcification process, so the bones become harder. Hardening of the bone serves to sustain weight loss. While in the extracellular fluid and cytosol, calcium plays a

role in various biochemical processes of the body in the form of calcium ions.

Norazlina et al. (1999) states that the consumption of 1% of calcium in rats with low E vitamin can increase bone mineral density. The body normally contains 1100 g of calcium for body weight of 70 kg. If the dose is converted in rats weighing 200 g, then there are 3.14 g of calcium in the body of rats. Yuliati et al. (2007) revealed that the addition of 27 mg/200 g BW/day can increase the thickness of the trabecular bone. Histologically, the bone is divided into 2, namely cortical bone and trabecular bone. Cortical bone has a mechanical and protective role, whereas trabecular bone is metabolical. Bone thickness indicates the strength of the bone, because in thick bone, there is an abundant amount of minerals.

Calcium in *P. conoideus* var. yellow fruit was 15.3 mg/100 g of material. The dose is very small compared to the intake of calcium that is used in research by Yuliati et al. (2007). Calcification is the deposition of calcium salts and phosphorus in bone matrix. Vitamin D is also needed in this process. Vitamin D is a prohormone, so that if the body does not get enough sunlight, D vitamin needs to be met through food. The conversion of D vitamin into active vitamin D3 (calcitriol) occurred in the kidney. In the intestine, calcitriol can increase the absorption of calcium and phosphorus. The calcitriol synthesis is regulated by calcium and phosphorus levels in the blood. When blood calcium levels are low, parathyroid hormone will stimulate the kidney to produce calcitriol. Role of vitamin D3 in the process of bone calcification is by making the calcium and the phosphorus available in the blood to be precipitate in the bone matrix. Because the amount of calcium used is not sufficient with the levels of calcium in the blood, the calcification process is inhibited. As a result, the bones still have the quality of cartilage.

There are two primary metabolisms in bone formation that are vulnerable to nutritional deficiencies, namely: the synthesis protein process to form the organic matrix of bone tissue consisting of collagen and non collagen protein. The next process is the bone calcification, in this stage; minerals such as calcium and phosphorus are precipitated in the bone matrix. If there are obstacles in the formation of organic matrix, then there will be obstacles in the process of calcification of bone resulting in decreased levels of bone minerals, including calcium and phosphorus. The occurrence of bone calcification resistance will lead to obstacles in the formation of osteoclast cells (Setiyohadi 2009).

Bone regeneration was influenced by two cells, namely osteoclasts and osteoblasts. Osteoclasts play role in bone reconstruction using acids and enzymes (bone resorption); while Osteoclasts play a role in the formation of new bone to replace the old bone which is dismantled by Osteoclasts (Rebecca and Brown 2007). Osteoclasts are motile cells. These cells will do resorption on bone to form lacuna, then they will move to another part of the bone. At the time of osteoclast cells move, resorption of bone does not occur. But when the osteoclast cell stops moving, then the process of resorption happens. The increase of bone resorption causes a reduction in the amount of bone.

Santoso (2004), in his study, states that a teratogen agents can affect the thickness of cells from the femur of mice. The layers on the femur of mice become thinner and thinner, and some even die. Teratogen agents are accumulated in several organs, especially the organ that is undergoing calcification which would result in abnormalities in fetal development. This is caused partly because the fetus does not have enzymes that can metabolize these toxic agents perfectly.

The presence of fetal skeleton with perfect ossification is due to internal factors, namely the hormone that maintains bone mass. Rebecca and Brown (2007) states that the hormone is one of the factors that influence the bone to be strong or not. Hormones are natural substances produced by specialized cells in the body. Hormones circulate in the bloodstream and can affect cell activity at various places in the body. In addition, the hormone can also help limiting the amount of bone resorption, because it regulates calcium levels in the blood. This is clarified by the study of Masyita (2006), that lack of estrogen can cause osteoporosis in mice. Osteoporosis occurs due to loss of bone mass and increased bone absorption. Estrogen is assessed to affect the process of bone destruction by inhibiting cytokine production. Estrogen plays an important role in the process of homeostasis, namely supporting the secretion of calcitonin, as an inhibitor resorption bone and can increase levels of D3 vitamin which serves to increase the degree of calcium absorption in the intestine. Low cytokines may decrease osteoclast activity that plays a role in the overhaul of the bone. Cytokines are proteins that play a role in the process of bone resorption. There are two kinds of cytokines, namely IL-1 and IL-6. IL-1 plays a role in stimulating bone resorption, replication of bone cells and increase synthesis of IL-6. IL-6 also plays a role in undergoing resorption bone by cells to activate osteoclasts. Synthesis of IL-6 would be inhibited by estrogen, so the intake of estrogen can reduce bone resorption (Norazlina et al. 2007). There is a relationship between estrogen with E vitamin, namely E vitamin which is a fat soluble vitamin may be converted into cholesterol, while cholesterol is the precursor of estrogen that its construction is through a series of enzymatic reactions.

Shuid et al. (2010) stated that E vitamin has potential as an anabolic agent in bone and can improve bone strength. In addition, E vitamin can improve bone structure. Anabolic agents are agents that can improve bone strength by increasing bone mass. In addition, E vitamin also acts as anti-osteoporotic agents (Nazrun et al., 2010). Tocopherol contained in *P. conoideus* var. yellow fruit is E vitamin, which plays a role in bone formation. Tocopherol along with calcium and vitamin D plays a role in bone metabolism process. Calcium plays a role in increasing bone mineral density, thus reducing resorption activity on bone. E vitamin can increase the density of calcium and D3 vitamin is required for calcium absorption in intestine and for calcium storage in bones (Norazlina et al. 1999).

Xu et al. (1995) stated that E vitamin can stimulate the growth of trabecular bone. Lack of E vitamin can reduce the transport of calcium in intestine. E vitamin intake of 10-30 mg/day is sufficient to maintain normal levels in the blood (Dewoto 2007). Norazlina et al. (1999), states that

the use of E vitamin by 60 mg/kg BW/day can increase the calcium content in rat femur. The content of tocopherol in *P. conoideus* var. yellow fruit is 10,400 ppm (10,400 mg). Excess tocopherol will not be discarded, because tocopherol is stored in the liver and adipose tissue in the form of glycerol and it can be reused by the body any time.

CONCLUSION

Morphometry of white rats fetus changes from control to highest dose treatment group. There are fetuses that have: mortality and growth inhibition at doses of 0.02 mL extract; resorption in all treatment groups, ie at a dose of 0.02 mL, 0.04 mL, 0.08 mL, and 0.16 mL extract; or transparent skin presents in the control and in all treatment groups. The extract can cause fetal abnormalities in the skeleton structure of the white rat. Abnormalities in the form of lordosis skeleton is found at a dose of 0.16 mL of the extract and the occurrence of ossification barriers can be found in all treatment groups.

REFERENCES

Alatas Z. 2005. Teratogenic effects of ionizing radiation. Bul Alara 6 (3): 133-142. [Indonesia]

Almatsier S. 2002. The basic principle of nutrition science. Gramedia. Jakarta. [Indonesia]

Astirin OP, Harini M, Handajani NS. 2009. The effect of crude extract of *Pandanus conoideus* Lamb. var. yellow fruit on apoptotic expression of the breast cancer cell line (T47D). Biodiversitas 10 (1): 44-48.

Azman A, Kholid BAK, Ima-Nirwana S. 2001. The effects of vitamin E on bodyweight and fat mass in intactand ovariectomized female rats. Med J Islamic Acad Sci 14 (4): 125-138.

Briggs GG, Freeman RK, Yaffe SJ. 2008. Drugs in pregnancy and lactation; a reference guide to fetal and neonatal risk. 8th ed. Lippincott Williams & Wilkins. Philadelphia.

Budi IM. 2000. Study nutrients and physical-chemistry properties of various types of red fruit (*Pandanus conoideus* Lam.) oil traditionally extracted in Jayawijaya, Irian Jaya Province. [M.Sc.-Thesis]. Graduate Program, Bogor Agricultural University. Bogor. [Indonesia]

Budi IM, Paimin FR. 2005. Red fruit. Penebar Swadaya. Jakarta. [Indonesia]

PKH. 2009. Causes of early embryonic death in cattle. Center for Animal Health. http://www.vet-klinik.com. [Indonesia]

Dalimartha S. 2004. Early detection of cancer and anticancer simplisia. Penebar Swadaya. Jakarta. [Indonesia]

Dewoto HR. 2007. Pharmacology and therapy: vitamins and minerals. 5th ed5. Faculty of Medicine, Indonesian University. Jakarta. [Indonesia]

Foye WO. 1996. The principles of medicinal chemistry. Gadjah Mada University Press. Yogyakarta. [Indonesia]

Ganiswara SG. 2001. Pharmacology and therapy. Gaya Baru. Jakarta. [Indonesia]

Gysin R, Azzi A, Visarius T. 2002. α-Tocopherol inhibits human cancer cell cycle progression and cell proliferation by down regulation of cyclin. FASEB J 16: 1952-1954. [Indonesia]

Harmanto N. 2001. Healthy with a traditional herb medicine heirloom crown god of the gods. Agro Media Pustaka. Jakarta. [Indonesia]

Herbold B. 1985. Micronucleus test on the mouse to evaluate for mutagenic effects. Report No. 14102E (Study Nos. T6019042 & T8019675). Institute of Toxicology, Pharmaceutical Division, Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer AG, Leverkusen, Germany (report No. 74164).

Hidayati MN. 2010. Cytotoxicity test of *P. conoideus* Lam. var. yellow fruit in vitro against Hela cell growth and chemical profile of the most active. [Thesis]. Department of Biology, FMNS, Sebelas Maret University. Surakarta. [Indonesia]

Hutahean S. 2002. The principles of toxicology development test. Laboratory of Animal Structure, Department of Biology, FMNS, North Sumatra University. Medan. [Indonesia]

Inouye M. 1976. Differential staining of cartilage and bone in fetal mouse skeleton by Alcian Blue and Allizarin Red S. Congenital Anomalies J 16 (3): 171-173.

Jessop NM. 1988. Theory and problem of zoology. B&JO Entreprise. Singapore.

Kauffman MH. 1992. The atlas of mouse development. Academic Press. New York.

Lu FC. 1995. Basic toxicology: principles, target organs and risk assessment. UI Press. Jakarta. [Indonesia]

Masyita D. 2006. Microscopic structure of mandibular bone in ovarectomic rats and feeding ratio phosphate/calcium high. Media Kedokteran Hewan. 22 (2): 112-117. [Indonesia]

Mun'im A, Retnosari A, Heni S. 2006. Constraints test tumorigenesis Red Fruit (Pandanus conoideus Lam.) juice against female White Rats induced by 7,12 Dimetilbenz (a) Antrasen (DMBA). Majalah Ilmu Kefarmasian 3 (3):153-161. [Indonesia]

Nazrun AS, Norazlina M, Norliza M and N S Ima. 2010. Comparison of the effects of tocopherol and tocotrienol on osteoporosis in animal models. Intl J Pharmacol 6 (5): 561-568.

Nindya S. 2001. Changes of pharmacokinetics drugs in Pregnant Women and its clinical implication. Cermin Dunia Kedokteran 133: 40-43. [Indonesia]

Nogrady T. 1992. Medicinal chemistry: The biochemical approach. Bandung Institute of Technology. Bandung. [Indonesia]

Norazlina M, Ima-Nirwana S, Khalid BAK. 1999. Effect of palm vitamin E, vitamin D and calcium supplementation on bone metabolism in vitamin E deficient rates. Med J Islamic Acad Sci 12 (4): 89-96.

Norazlina M, Lee PL, Lukman HI, Nazrun AS, Ima-Nirwana S. 2007. Effect of vitamin E supplementation on bone metabolism in nicotine-treated rats. Singapore Med J 48 (3): 195-199.

Pergament E, Schechtman A, Curell C. 1996. Vitamin A and pregnancy. ITIS Newslett 4 (6). http://fetal-exposure.org.

Pratiwi AP. 2008. Cytotoxicity test of mixture extract of *Pandanus conoideus* Lam. var. yellow fruit and lauric acid of VCO to T47D breast cancer cells in vitro. [Thesis]. Department of Biology, FMNS, Sebelas Maret University. Surakarta. [Indonesia]

Rebecca F, Brown P. 2007. Simple guides to osteoporosis. Penerbit Erlangga. Jakarta.

Ritter EJ. 1977. Altered biosynthesis In: Wilson JG, Fraster FC (eds). Hand book of teratology. Plenum Press. New York.

Rugh R. 1968. The mouse, its reproduction and development. Burgess Publising. Minneapolis.

Russel RM. 2002. Betacarotene and lung cancer. Pure Appl Chem 74 (8): 1461-1467.

Sagi M. 1997. Comparative embryology of vertebrates. Faculty of Biology, Gadjah Mada University. Yogyakarta. [Indonesia]

Santoso HB. 2004. Skeleton anatomical abnormalities of mice due to defects of caffeine. Bioscientiae 1: 23-30. [Indonesia]

Setiyohadi B. 2009. The role of calcium and vitamin D on bone metabolism. Subsections of Rheumatology, Department of Medicine Faculty of Medicine/RSCM. Jakarta. [Indonesia]

Setyawati I. 2009. Morphology of fetal mice (*Mus musculus* L.) after administration of bitter leaf extract (*Andrographis paniculata* Nees. J Biologi 13 (2): 41-44. [Indonesia]

Shuid AN, Zulfadli M, Norazlina M, Norliza M, Ima-Nirwana S. 2010. Vitamin E exhibits bone anabolic actions in normal male rats. Bone Miner Metab J 28: 149-156.

Suryawati S. 1990. Use of drugs in pregnancy. Laboratory of Clinical Pharmacology, Faculty of Medicine, Gadjah Mada University. Yogyakarta. [Indonesia]

Takekoshi S. 1964. The mechanism of vitamin A induced teratogenesis. J Embryol Exp Morph 12 (2): 263-271.

Tjay TH, Rahardja K. 2002. Properties of essential drugs, the use and side effects. PT. Elex Media Komputindo. Jakarta. [Indonesia]

Wilson JG. 1973. Environment and birth defects. Academic Press. New York.

Wiryanta BTW. 2007. Wonders of red fruit; testimony of those who healed. http://www.deherba.com/khasiat-buah-merah.html. [Indonesia]

Xu H, Watkins BA, Seifert MF. 1995. Vitamin E stimulates trabecular bone formation and alters epiphyseal cartilage morphometry. Calcif Tissue Int 57: 293-300.

Zahrah S. 2008. Teratogenic effects of water extract of sarang semut (*Myrmecodia pendens* Merr. & Perry) in rats (*Rattus norvegicus* L.) organogenesis phase of wistar strain. [Thesis]. Department of Biology, FMNS, Sebelas Maret University. Surakarta. [Indonesia]

Zuhud EAM. 2009. Development of pharmaceutical ethnoforestry in Indonesia. Plant Biodiversity Conservation Section, Faculty of Forestry, Bogor Agricultural University. Bogor. [Indonesia]

Zulti F. 2008. Mechanism of transport through the membrane. http://www.crayonpedia.org. [Indonesia]

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