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Teratogenic Investigation of Bay Leaf (*Syzygium polyanthum* Wight.) Ethanol Extract on Morphology of Fetal Mice (*Mus musculus* L.) Strain DDY

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Abstract

This research was conducted to observe the teratogenic potential of *S. polyanthum* ethanol extract on morphology of fetal mice strain DDY. Thirty pregnant female mice were divided into 5 groups, normal group and treatment groups with dose 0.5; 5; 50; 500 mg/bw. The extract was given orally from 6th to 15th day of gestation. The results showed that the effect of *S. polyanthum* ethanol extract at a dose of 0.5 mg/bw did not cause resorption and external malformation. At a dose of 5 mg/bw, there were resorption (6.15%) and fetal open eyelids (1.63%). Resorption (7.69%; 9.34%) and fetal hemorrhage (1.63%; 1.47%) were found in mice given doses of 50 and 500 mg/bw. The treatment of *S. polyanthum* ethanol extract at doses 0.5; 5; 50; 500 mg/bw during the period of organogenesis did not have a significant influence on morphology of fetal mice strain DDY ($p > 0.05$).

Subject Areas

Toxicology

Keywords

Fetus, Mice, Organogenesis, Resorption, *S. polyanthum*

1. Introduction

Dengue hemorrhagic fever (DHF) is an infectious disease caused by the dengue virus and is transmitted by the mosquito *Aedes aegypti*. The disease is prevalent

in tropical and subtropical regions. One of the countries in Southeast Asia that have the highest incidence rate of dengue is Indonesia [1]. Another issue that occurs is dengue disease in Indonesia which affects all ages, including pregnant women [2]. Until now, there are no vaccines and standard drugs to prevent and treat the disease. Therefore, DHF in Indonesia still needs attention [1].

Several extracts of medicinal plants have been investigated to be developed as antiviral drugs for dengue. The phytochemical compounds in plant extracts such as alkaloids, saponins, eugenol, flavonoids, and tannins are reported able to kill *Aedes aegypti* larvae [3]. Indonesian plant such as *Myristica fatua*, *Acorus calamus*, and *Cymbopogon citratus* had been investigated and shown that the methanolic extract of these plants has an antiviral effect to DENV without any cytotoxic effect [4]. In addition, it has been reported that the water extract of the *Syzygium polyanthum* had a killing power to kill *Aedes aegypti* larvae, LC_{50} of methanolic and ethanol extract of these plant was 6576.68 ppm and 213 ppm [3] [4] [5].

Syzygium polyanthum (*Myrtaceae* family), is a tree species commonly known as salam in Indonesia and used as a seasoning. These plants spread in Sumatra, Borneo, and Java, that area is a DHF endemic area. Another advantage of *S. polyanthum*, the plant has a fast-growing stem. Thus, the utilization and preservation of the plant can be maintained. *S. polyanthum* leaves were reported to have antioxidant, antidiabetic, antimicrobial, antihypertensive, antitumor and cure fever. The leave of these plants contains tannins, alkaloids, steroids, triterpenoids, and flavonoids [6].

Preclinical trials that have been done are *in vitro* activity test and acute toxicity test on mice. *In vitro* activity test results, proved that the ethanol extract of the plant is potentially as antidengue (IC_{50} 1.25 ppm). Acute toxicity test results, showed that the LD_{50} value of *S. polyanthum* is 14.790 mg/kg bw [3]. The LD_{50} value is between the dosage range 5000 - 15,000 mg/kg bw, thus categorized as practically non-toxic [5]. Subsequent research is to test the *in vivo* activity. However, before performing *in vivo* activity test, teratogenic test needs to be done first.

The teratogenic test aims to determine the safety of preclinical a compound or a certain drug to the development of the fetus. Teratogenic test conducted on mice (*Mus musculus* L.) strain DDY. The metabolism of mice is similar to humans, so it can be used as animal studies for drug testing [6]. Mice also have regular estrous cycles and can be detected, thus facilitating the process of mating. In addition, the mice have a relatively short gestation period and the number of fetuses is relatively large. Mice gestation period ranges from 19 days, and the number of fetal mice ranges from 6 - 15 fetus [7] [8].

The extract of *S. polyanthum* administered orally from 6th to 15th day of gestation (the period of organogenesis). During that period germ layers differentiate to form specific organs. Therefore, this period is a critical period which is susceptible to exposure to teratogens, because the impact will be expressed on morphology of fetus [9]. The extract of *S. polyanthum* administered at doses 0.5; 5; 50 and 500 mg/kg bw. The dose is determined based on the result of the con-

version IC_{50} 1.25 ppm on dengue antiviral activity test [10]. Thus, this research aims to determine the effect of exposure *S. polyanthum* ethanol extract at dose 0.5, 5, 50, and 500 mg/kg bw during the period of organogenesis on morphology of fetal mice (*Mus musculus* L.) strain DDY.

2. Materials and Methods

2.1. Materials

Ethanol extract of *Syzygium polyanthum* was a stock of extract of Natural product laboratory Research Centre for Chemistry BRIN. Experimental animals were mice (*Mus musculus* L.) strain DDY. The total number of mice used as many as 40 mice consisting of 30 virgin females and 10 males with age ranges from 2 - 3 months. Mice were obtained from Institut Pertanian Bogor (IPB). Before the research began, animal experiments protocols have been reviewed by the Committee of Health Research Ethics Faculty of Medicine, Universitas Indonesia.

Chemicals used for fixation of fetus is composed of 70% ethanol [Merck] and Bouin solution, that comprising a solution of picric acid: formaldehyde: glacial acetic acid (15:5:1).

2.2. Methods

Research conducted an experimental study using a completely randomized design. Thirty pregnant female mice were randomly assigned to five different groups. There were five treatment groups tested, one of which was a control group (distilled water) (KK), while the other four were given therapy with *S. polyanthum* with dose 0.5 (KP1), 5 (KP2), 50 (KP3), and 500 (KP4) mg/kg bw. Research initiated by mating three female mice that were in the estrus phase with male mice. The next day was observed the presence of vaginal plugs that indicates there has been a sign of copulation and used as day 0 of gestation [11]. Pregnant mice were then transferred into a separate enclosure with the others and to label the date of the pregnancy and the description of a treatment.

The extract of *S. polyanthum* administered orally from 6th to 15th day of gestation (the period of organogenesis). Pregnant mice who entered the 18th day of gestation were sacrificed through inhalation anesthetic. Furthermore, the observation of intrauterine: fetal position, the number of corpus luteum, implantation, fetal life, fetal death, resorption, weight and length of fetus, and sex ratio of fetus. Then fetuses were fixed in Bouin solution for observation morphology of fetus.

Quantitative data included in mean ± SD and are presented in tables and charts using Microsoft Excel 2010. Data was tested with Levene homogeneity test and Shapiro-Wilk normality test. Data were normally distributed and homogeneous tested by one-way ANOVA followed by LSD test. Meanwhile, if data is not normal and is not homogeneous, then data tested by Kruskal-Wallis test followed by Dunnett test. All data were processed statistically using SPSS version 23.0, and using a significance level of 95% ($\alpha = 0.05$) [12].

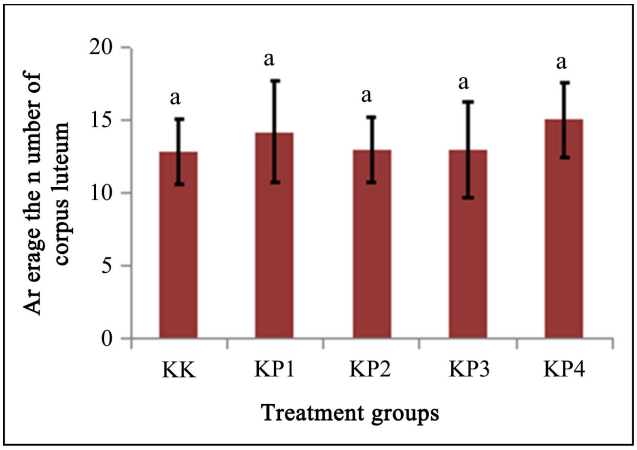
3. Results

3.1. The Number of Corpus Luteum

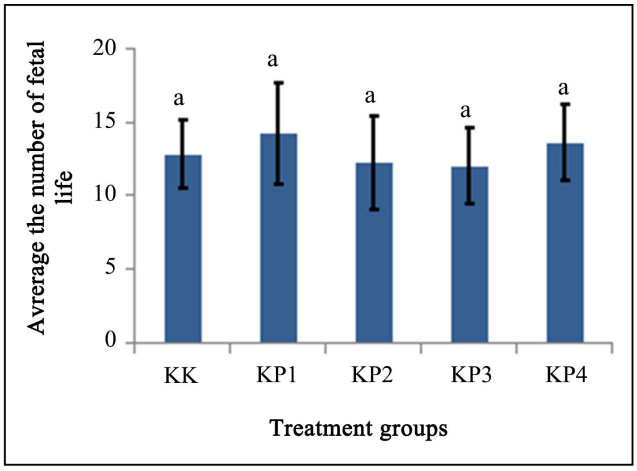
Calculation the number of corpus luteum aims to determine the number of implantation and intrauterine death. Statistical analysis showed no significant differences on the number of corpus luteum between treatment groups. Thus, exposure ethanol extract of *S. polyanthum* during the period of organogenesis did not affect the average number of corpus luteum in mice. The mean number of corpus luteum at doses 0.5, 5, 50, and 500 mg/kg bw in a row is 12.80; 14.20; 13.00; 13.00; and 15.00 (Figure 1).

3.2. The Number of Fetal Life and Resorption

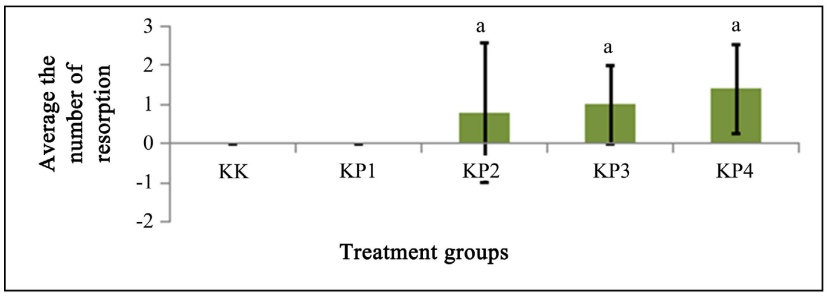
The results showed the entire embryo successfully implanted. Statistical test results showed no significant differences on the number of fetal life between treatment groups. Thus, exposure ethanol extract of *S. polyanthum* during the period of organogenesis did not affect the number of fetal life. The mean number of fetal life at doses 0.5, 5, 50, and 500 mg/kg bw in a row is 12.80; 14.20; 12.20; 12.00; and 13.60 (Figure 1).



(A)



(B)



(C)

Figure 1. (A-C) Diagram of the average number of corpus luteum (A), fetal life (B) and resorption (C). Line (bar) shows the standard deviation. Letter a indicates the level of significance (P > 0.05). *KK, KP1, KP2, KP3, KP4 : 0, 0.5, 5, 50, and 500 mg/kg bw.

Resorption found in the research consisted of late resorption and early resorption (Figure 2). Resorption found in group doses 5, 50, and 500 mg/kg bw, with the number of consecutive 4, 5, and 7 resorptions. Statistical analysis showed no significant difference between the number of resorption in the treatment group and control group. Percentage of total resorption was found in the control group and at doses 0.5, 5, 50, and 500 mg/kg bw in a row is 0%; 0%; 0.8%; 1.0%; and 1.4% (Figure 1).

Length and Weight of Fetus

Statistical test results showed no significant difference to the average fetal weight and length of the treatment groups. Thus, exposure ethanol extract of *S. polyanthum* during period of organogenesis did not affect weight and length of fetal mice. These results are supported by observations that showed the average weight and length of fetuses in the treatment group were still in the normal range. The mean weight of fetuses at doses 0, 0.5, 5, 50, and 500 mg/kg bw in a row is 1.38; 1.23; 1.25; 1.20; and 1.26. Meanwhile, the mean fetal length in a row is 24.76; 24.28; 24.14; 23.01; and 23.93 (Figure 3).

3.3. External Malformation

The results showed there were two forms of external malformation, fetal open eyelids and hemorrhage. One fetus with open eyelids found in the group at dose 5 mg/kg bw. Fetal hemorrhage found in the group at doses 5, 50, and 500 mg/kg bw, with the number 1 fetus at each dose (Table 1). Images of external malformation in fetal mice can be seen in Figure 4. Statistical analysis showed no significant difference to the number of fetal open eyelids in treatment group. Thus, exposure ethanol extract of *S. polyanthum* during period of organogenesis did not have a significant influence on fetal open eyelids. Fetal open eyelids were found allegedly occurred spontaneously. That is because only 1 fetus (1.63%) and only in the group at dose 5 mg/kg bw. Fetus with open eyelids are not found in the higher dose groups (doses 50 and 500 mg/kg bw).

Besides fetal open eyelids, the research also found fetal hemorrhage. Statistical analysis showed no significant differences on the number of fetal hemorrhage between treatment groups. Thus, exposure ethanol extract of *S. polyanthum* during

the period of organogenesis did not have a significant influence on fetal hemorrhage. Thus, hemorrhage were found in the research was not influenced by ethanol extract of *S. polyanthum*. Hemorrhage were found allegedly occurred spontaneously, due to small numbers (each 1 fetus at doses 5, 50, and 500 mg/kg bw).

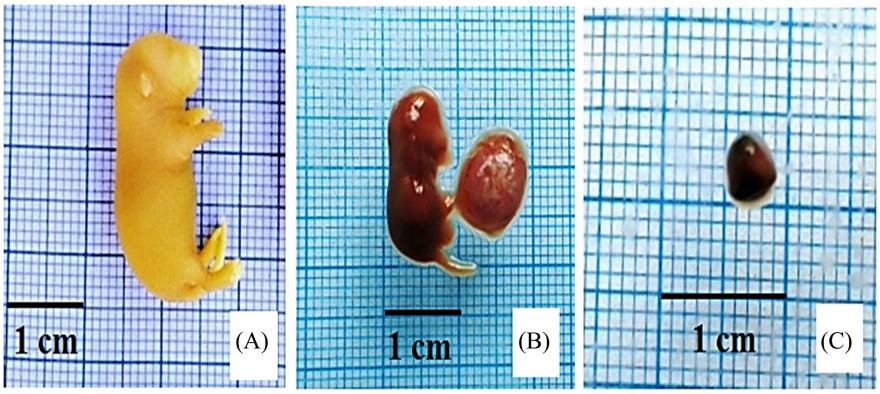
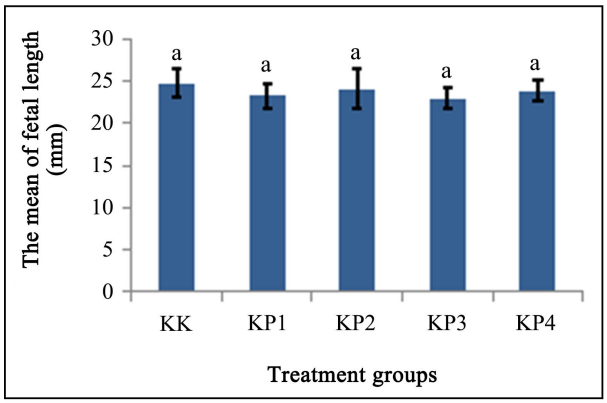
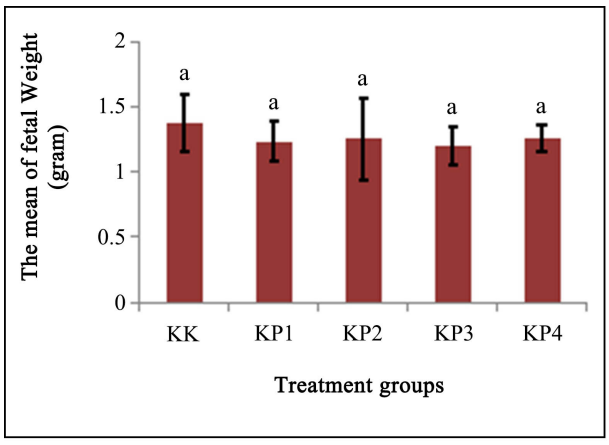


Figure 2. (A-C) Differences in normal fetuses (A), late resorption (B), and early resorption (C). [Source: Private Documentation].



(A)



(B)

Figure 3. (A-B) Diagram mean of fetal body length (A) and fetal weight (B). Line (bar) shows the standard deviation. Letter a indicates the level of significance ($P > 0.05$). *KK, KP1, KP2, KP3, KP4: 0, 0.5, 5, 50, and 500 mg/kg bw.

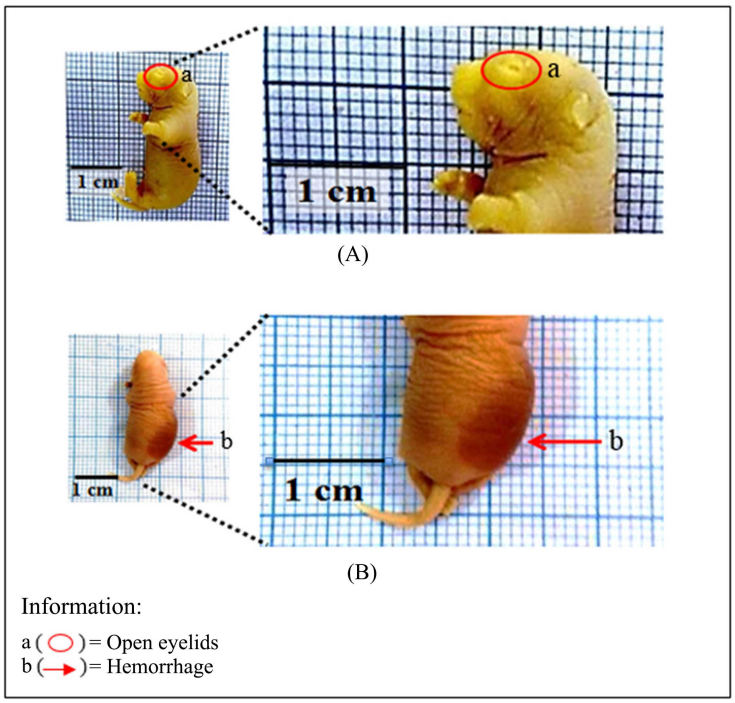


Figure 4. Fetal external malformation [Source: Private Documentation].

Table 1. The number of fetal external malformation.

Treatment	The number of fetuses (n)	Open eyelids (n)	Hemorrhage n (%)
KK	64	0	0
KP1	71	0	0
KP2	61	1 (1.63%)	1 (1.63%)
KP3	60	0	1 (1.67%)
KP4	68	0	1 (1.47%)

Note: KK, KP1, KP2, KP3, KP4: 0, 0.5, 5, 50, and 500 mg/kg bw.

3.4. Sex Ratio of Fetus

Observations parameters of sex ratio in the research aim to determine the tendency of sexes in mice with external malformations. Statistical test results showed the average number of male and female fetuses homogeneous and there are no significant differences between treatment groups. Thus, exposure ethanol extract of *S. polyanthum* not affect the formation of the fetal sex. The whole sex on research perfectly shaped and can be distinguished clearly between male and female fetuses (Table 2). The sex determination based on distance between anus and genitalia hole can be seen in Figure 5. In addition, the results showed that there are two forms of external malformation, open eyelids and hemorrhage. One female fetus was found with open eyelids. Meanwhile, 2 female fetuses and 1 male fetus were found with hemorrhage.

Table 2. Average the number of male and female fetuses.

Treatment	The number of fetuses		Male ($\bar{x} \pm SD$)	Female ($\bar{x} \pm SD$)	Percentage of male and female fetus (%)
	Male	Female			
KK	35	29	5.80 ± 2.16	5.80 ± 2.16	55:45
KP1	43	28	5.60 ± 2.07	5.60 ± 2.07	60:40
KP2	36	25	5.00 ± 2.44	5.00 ± 2.44	59:41
KP3	31	29	5.80 ± 1.92	5.80 ± 1.92	52:48
KP4	29	39	7.80 ± 1.78	7.80 ± 1.78	43:57

Note: KK, KP1, KP2, KP3, KP4: 0, 0.5, 5, 50, and 500 mg/kg bw.

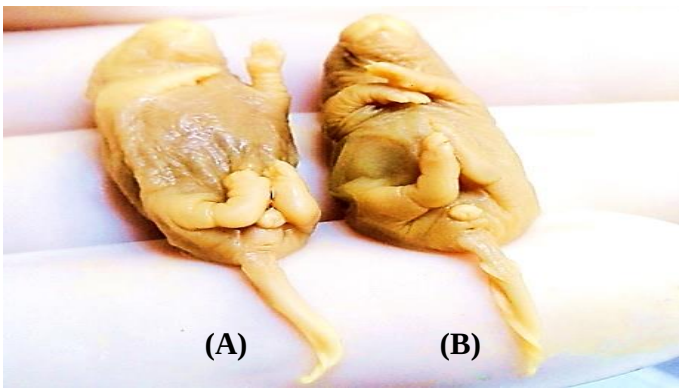


Figure 5. The difference of male (A) and female fetuses (B). [Source: Private Documentation].

4. Discussion

Calculation the number of corpus luteum aims to determine the number of implantation and intrauterine death. The number of implantation was found in the research consisted of a number of fetal life and resorption. The number of implantation is in accordance with the number of corpus luteum per individual. Thus, the number of corpus luteum of each group can be compared. These results are supported by observations that showed the average number of the corpus luteum is still in normal range. The average number of normal corpus luteum in mice is approximately 9 - 16 corpus luteum each cycle [13]. In addition, morphology of the corpus luteum is still normal. The normal corpus luteum saw as red or pink and have many blood vessels. Meanwhile, abnormal corpus luteum will look white or pale, no blood vessels, and its smaller size compared to normal corpus luteum [13].

The implantation consists of fetal life and resorption. The results showed the entire embryo successfully implanted and the average number of fetal life still in normal range. The average number of normal fetal life in mice is about 6 - 15 fetus [8]. The number of resorption in the treatment group and control group percentage less than 10%, thus showing that ethanol extract of *S. polyanthum* not embryotoxic and embrioletal [14]. Total resorption in each individual is still

in the normal range, which is about 1 - 3 resorption [15]. Therefore, resorption is not affected by ethanol extract of *S. polyanthum.*

Compounds contained in extracts of S. polyanthum allegedly did not include the requirement characteristics of compounds that can pass through placental barrier. The requirement of a compound that can pass through placental barrier is the weight of compound should be less than 500 Dalton, nonpolar, not bound protein, and easily ionized [16]. *S. polyanthum* ethanol extract contains flavonoids and tannins. Flavonoids and tannins are polar compounds with a molecular weight of about 500 - 3000 Dalton, and can form complex compounds with proteins [17]. Thus, extracts of *S. polyanthum* not teratogen. Resorption found in the research is believed to occur spontaneously. One of the factors that can cause spontaneous resorption is immunological factors [18].

Based on immunological factors, the success of pregnancy depends on balance between mother and fetus immune. Macrophages are the main immune cells in the uterus that can engulf pathogens and abnormal cells by means of secreting a wide range of cytokines. Macrophages can keep the embryo from infection, but if the excessive activation, macrophage will secrete tumor necrosis factor (TNF) -α which can cause spontaneous resorption [19]. Tumor necrosis factor (TNF) -α can inhibit proliferation trophoblasts and activate natural killer cells (NK) become lympokine-activated killer (LAK) which can destroy trophoblasts up to necrosis [20]. Research also reported an increase (TNF) -α in placental tissue of fetal mice undergo resorption [21].

The average weight and length of fetuses in the treatment group were still in the normal range. Based on literature, body length (crown-rump) normal mice is about 19 - 23 mm and the average weight of normal fetal mice around 0.5 to 1.5 g [8] [22]. Flavonoids have the ability as chelator ferrous metals that cause iron deficiency. Iron deficiency during pregnancy can lead to fetal weight loss. Nevertheless, the results of research, shows that fetus exposed flavonoids (quercetin) had a mean weight was not significantly different from control group [23]. In addition, tannin is thought to bind to proteins, so that it can interfere with the absorption of protein. It is thought to be able to lose weight fetus. The results, shows calliandra leaf contains tannin with high levels do not lose weight fetal mice. Thus, flavonoids and tannins is not expected to be a teratogen [24].

There are two forms of external malformations in the results of this study, fetal open eyelids and hemorrhage. Exposure ethanol extract of *S. polyanthum* during period of organogenesis did not have a significant influence on fetal open eyelids. Fetal open eyelids were found allegedly occurred spontaneously. That is because only 1 fetus (1.63%) and only in the group at dose 5 mg/kg bw. Fetus with open eyelids are not found in the higher dose groups (doses 50 and 500 mg/kg bw). The literature states that the possibility of birth defects in mice experienced spontaneous open eyelids, cleft palate, cleft lip, and polydactyly is quite high [25]. In addition, other literature states that the open eyelids defect occurs due to a spontaneous mutation autosomal recessive, so that occurs randomly and is not known for certain mechanisms. The incidence rate of disability is also

very small [26]. Therefore, the suspected cause of fetal open eyelids on research is caused by spontaneous defects.

Hemorrhage is any profuse internal or external bleeding from blood vessels and accumulating in tissues or under the skin area [14]. Hemorrhage can occur due to an imbalance between osmotic pressure of liquid intraembryonic with extraembryonic fluid, due to the presence of a particular compound [27]. *S. polyanthum* ethanol extract contains flavonoids and tannins. Flavonoids and tannins are polar compounds with a molecular weight of about 500 - 3000 Dalton, and can form complex compounds with proteins [17]. Compounds that can cross the placental barrier have a weight of less than 500 Dalton, nonpolar, not bound protein, and easily ionized [16]. Exposure ethanol extract of *S. polyanthum* during the period of organogenesis did not have a significant influence on fetal hemorrhage.

Flavonoids and tannins that contained in the ethanol extract of *S. polyanthum* not teratogen and can not pass through placental barrier. Flavonoids and tannins are polar compounds with a molecular weight of about 500 - 3000 Dalton, and can form complex compounds with proteins, so it does not include the characteristics of a compound that can pass through placental barrier. Therefore, the compound does not affect the osmotic pressure between intraembryonic and extraembryonic fluid [17]. Thus, hemorrhage were found in the research was not influenced by ethanol extract of *S. polyanthum*. Hemorrhage were found allegedly occurred spontaneously, due to small numbers (each 1 fetus at doses 5, 50, and 500 mg/kg bw). The literature states, hemorrhage is spontaneous congenital malformation that common in mice [28].

Observations parameters of sex ratio in the research aim to determine the tendency of sexes in mice with external malformations. The sex determination based on distance between anus and genitalia hole. The distance between anus and genitalia hole in the male fetus is more than 1 mm, while in the female fetus is less than 1 mm [29]. Determining the sex of mice is determined by chromosomes in the process of fertilization and rarely influenced by environmental factors. The sex differentiation process begins on the 12th day of pregnancy characterized by the formation of the testes in the male fetus [30]. Based on these results, mice experienced external malformation occur randomly on male and female. Thus, this research found no effect of ethanol extract of *S. polyanthum* to the formation of fetal sex and found no defects in the tendency of a specific gender.

5. Conclusion

The treatment of *Syzygium polyanthum* ethanol extract at doses of 0.5; 5; 50 and 500 mg/bw during the period of organogenesis did not have a significant influence on morphology of fetal mice (*Mus musculus* L.) strain DDY.

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Authors' Contribution

MA is the main contributor in this project, design methodology, data processing, writing the submission, and laboratory working; AO, IDD Laboratory working and data collected; AM, LS Writing and revised the submission.

Conflicts of Interest

The authors declare no conflicts of interest.

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