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EFFECT OF MORINGA OLEIFERA (LAM) LEAF EXTRACTS ON GROWTH OF CHICKEN EMBRYO INDUCED BY ALKOHOL

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ABSTRACT

Fetal alcohol syndrome (FAS) is a condition associated with drinking alcohol during pregnancy and affect embryo development. FAS is the most common congenital disabilities in the world. The effects of FAS are mental retardation, cranial abnormalities, and heart defects. Moringa oleifera is a highly valued plant. It has an impressive range of biological activities, including antioxidant, antiproliferation, and anti-inflammatory. This study aims to assess the effect of Moringa oleifera leaf extracts in inhibiting growth retardation of chicken embryo growth induced by alcohol. The study was a true experimental design with posttest controlled group design. There were five groups with 5 of chicken embryo each group. The treatments contained 15% alcohol and aquadest as positive control (P+), without alcohol as negative control (P-), $P_{0.5}$ (0,5g/dl), P_5 (5g/dl), P_{50} (50g/dl) by adding Moringa oleifera leaf extracts, respectively. Moringa oleifera leaf extracts showed the improvement of length and head diameter as a benchmark for chicken embryo development with P_5 concentration showed the best results to overcome growth retardation in alcohol-induced chicken embryos. This finding implies against possibility Moringa oleifera leaf extracts can be alternative medicine to prevent growth retardation.

Kata kunci:
antioksidan
embrio ayam
fetal alcohol syndrome
Moringa oleifera

ABSTRAK

Fetal alcohol syndrome (FAS) adalah kondisi yang berhubungan dengan kebiasaan meminum alkohol selama kehamilan dan mempengaruhi perkembangan embrio saat kehamilan. FAS adalah cacat bawaan umum. Efek FAS adalah keterbelakangan mental, kelainan tengkorak, dan kelainan jantung. *Moringa oleifera* adalah tanaman yang memiliki berbagai aktivitas biologis mengesankan yaitu antioksidan, antiproliferasi, dan anti-inflamasi. Penelitian ini bertujuan untuk menilai pengaruh ekstrak daun kelor dalam menghambat gangguan pertumbuhan embrio ayam yang disebabkan oleh alkohol. Penelitian ini menggunakan desain *true experimental* dengan *posttest controlled group design*. Ada lima kelompok dengan 5 embrio ayam masing-masing kelompok. Perlakuan mengandung 15% alkohol dan aquades sebagai kontrol positif (P+), tanpa alkohol sebagai kontrol negatif (P-), $P_{0.5}$ (0,5g/dl), P_5 (5g/dl), P_{50} (50g/dl) masing-masing ditambahkan dengan ekstrak daun Moringa oleifera. Ekstrak daun Moringa oleifera menunjukkan peningkatan panjang badan dan diameter kepala sebagai patokan untuk perkembangan embrio ayam dengan hasil menunjukan pada konsentrasi P_5 memberikan hasil terbaik untuk mengatasi gangguan pertumbuhan pada embrio ayam yang diinduksi alkohol. Temuan ini memungkinkan ekstrak daun kelor dapat menjadi obat alternatif untuk mencegah gangguan pertumbuhan.

BACKGROUND

In 2015, the primary reason for neonatal deaths were prematurity (35.5%), asphyxia and trauma (21.6 %), and congenital abnormalities (17.1 %). Women who drink alcohol heavily during pregnancy trigger the high risk of mental disorders development against baby birth. The spectrum of disorders includes alcohol-related birth defects (ARDB), alcohol-related neurological disorders (ARNB), and the most prominent being fetal alcohol syndrome (FAS). Moreover, FAS causes a variety of physical, growth retardation, and central nervous system damage (Jones and Smith, 1973; Clarren et al., 1978; Sampson et al., 1997; Kalter, 2003). The alcohol exposure enhances growth disorder through disrupting the proliferative activity of glial and neural precursors. Cell proliferation in the developing CNS trigger mitogenic growth and anti proliferated growth regulators. It has been reported that alcohol inhibits cell proliferation and disturb signal transduction directly (Lou and Miller, 1998). Thus, alcohol exposure also affects Sonic hedgehog (Shh) signalling. Shh is crucial to neural development due to essential protein for embryogenesis. Shh also affects head development, reduces death cell of neural crest after alcohol exposure, and neural crest migration (Ahigren, Thakur & Fraser, 2002). Recently, it has been reported that chicken embryos exposed to alcohol trigger slower growth in body length (Carly, 2015).

The effect of alcohol appears more obvious when it is consumed during organogenesis, from the third to eight weeks after the implantation in humans (Parnells and others, 2006). In the chicken model, the "critical window" of sensitivity occurs in short period from gastrulation to cranial neural crest migration, or from 18 to 36 hours into development (Smith, 1997). The chicken is a suitable model for studies on FAS, since it allows for the determination of the ethanol effect in early pregnancy, without the influences of maternal under nutrition, concurrent drug use, acetaldehyde formation or impaired placental function (Rao and Chaudhuri, 2007). Furthermore, studies have revealed that FAS effect in the chicken model is comparable to those in humans (Cartwright and Smith, 1995).

Moringa oleifera belongs to Moringacae family and has enormous functions such as medicine for malnutrition. It has enormous essential phytochemicals in its leaves, pods, and seeds. In addition, it has variety bioactive compound include 7 times more vitamin C than an orange, 10 times more vitamin A than a carrot, 17 times more calcium than milk, 9 times more protein than yoghurt, 15 times more potassium than bananas and 25 times more iron than spinach (Rockwood and others, 2013). Moringa oleifera leaves also contain fiber, fat proteins, vitamin A (Beta carotene), vitamin B-chaoline, vitamin B1-thiamine, riboflavin, nicotinic acid, and ascorbic acid. There are various amino acids such as Arg, His, Lys, Trp, Phe, Thr, Leu, Met, Ile, Val. Moreover, there are phytochemicals such as tannins, sterols, saponins, terpenoids, phenolics, alkaloids, and flavonoids (Tejas et al., 2012). Aqueous, methanolic (70%), ethanolic extract (80%) of leaves of Moringa oleifera exhibit strong antioxidant and radical scavenging activity. Vergara et al (2017) reported that this antioxidant activity of Moringa oleifera leaves due to the presence of Kaemferol.

Moringa oleifera plays a role in preventing alcohol-induced malformations. The previous mechanism for ethanol-induced dysmorphogenesis has not been yet clarified. However, several studies have suggested that the excessive number of reactive oxygen species (ROS) produced by ethanol metabolism. It is a major trigger against the abnormal development of the embryo (Aberle et al., 2013). Variety metabolic reactions produce ROS. It is regulated at physiological reactions through the defence of antioxidant mechanism in the body, as well as by antioxidant in the diet (Memon and Pratten, 2009). ROS participated in various physiological mechanisms such as tissue remodelling, hormone signalling and germ cell function in adults as well as in embryos (Hossein et al., 2007). ROS cause malformations in the developing embryo when the number of antioxidants is not enough to maintain their physiological mechanism (Jauniaux et al., 2004).

In this present study, the effect of different dose (0,5g/dl, 5g/dl, 50g/dl) of Moringa oleifera extract therapy on ethanol-exposed chick embryos was studied to investigate antioxidant protection in developing chicken embryo from ethanol-induced growth retardation.

METHODS

Research Design

This research was true experimental design with posttest only controlled group design. Data was obtained from December 2018 - January 2019 at the Biochemistry Laboratory, Faculty of Medicine, University of Jember. Super java chicken eggs were purchased at Technical Implementation Unit (UPT), Polytechnic of Jember. There were five groups with 5 of chicken embryo each group. The treatments

contained 15% alcohol and aquadest as positive control (P+), without alcohol as negative control (P-), $P_{0.5}$ (0,5g/dl), P_5 (5g/dl), P_{50} (50g/dl) by adding Moringa oleifera leaf extracts, respectively. Simple random sampling and seven-day-old embryonic chicken egg were used in this study. The minimum number of samples per group was determined according to Federer's formula. Body length and head diameter of chicken was obtained by a millimetre-scale ruler.

Extraction of M. oleifera

The leaves of M. oleifera were collected from Perhutani KPH Jember, Indonesia. The M. oleifera leaves were washed with water. Then, M. oleifera leaves were 24 hours air dried and pulverised using a blender. A kilogram powder of M. oleifera was extracted with 4 L of 96% ethanol at 24 hours. The extract separated through filtration using Whatman filter paper. The filtrate was evaporated using a water bath at 60oC. Then, the filtrate was placed in a flask and refrigerated until needed for evaluation.

Treatment of Chicken Eggs

A day old eggs were washed with distilled water and cleaned with 70% alcohol. The eggs were incubated until the 7 days at a temperature of 37.5oC. Eggs were selected according to inclusion and exclusion criteria on day 7. Egg candling method was performed to determine the egg fertility by passing light from the bottom of the egg. Eggs with dark spot were considered as fertile eggs while eggs without dark spot were considered as infertile eggs. The eggs were grouped based on the exclusion and inclusion criteria. Then, eggs were incubated again during 12 days. Each egg was given two holes using mini-drill above the air sac with a gap between 0.5 cm on 12 days. The hole was used for the injection site.
Each group of eggs was injected with 250µl ethanol into the air sac while the eggs in the negative control were injected with 250µl of saline. The injection hole in the egg was sealed with adhesive tape. The eggs were re-incubated for 24 hours. Then, the eggs were injected with Moringa leaf extract based on the group. The injection site was sealed with adhesive tape again and the eggs were incubated until day 15.

Measurement of Body Length and Head Diameter in Chicken Embryo

15-day old eggs were broken and rinsed using normal saline. The embryo was placed on a petri dish. The body length and head diameter were measured by a millimetre-scale ruler. Analysis of embryo body size was determined by measuring the body length of the cranium following the vertebral groove to the tip of the coccyx using a millimetre-scale ruler.

Statictical Analysis

The normality of data distribution was analyzed by Saphiro Wilk and the data homogeneity was performed with Levene. Data were analyzed using Oneway ANOVA ($p > 0.05$) if the data was normal and homogeneous. Duncan analysis was to see which specific pairs of groups are different. All values are presented as the mean of at least 2 independent experiments. Values of $p < 0,05$ were considered statistically significant and indicated by $p < 0,05$.

RESULTS

The initial experiment experiment was performed to investigating whether Moringa oleifera leaf extract has ability to inhibit the teratogenic effects of alcohol on chicken embryo development. At 0.5 g / dl, 5 g / dl, 50 g / dl of Moringa oleifera leaf extract has impact on inhibit the teratogenic effects. In this study, chicken embryos were treated using alcohol and Moringa oleifera leaf extract. Thus, after 15 days, the body length and head diameter as an indicator of chicken embryo development were determined.

Body Length of Chicken Embryo Induced by Alcohol

P- were uninduced by alcohol (negative control), P+ were induced by alcohol without treatment, $P_{0,5}$, P_5, P_{50} were induced by alcohol and treated with 0,5g/dl, 5g/dl, 50g/dl of Moringa oliefera leaf extracts. Anova and Duncan test were used to determine significance between every group treatment and negative control. *$p < 0,05$. Each group treatment consists of 5 samples and described as 1st - 5th Sample.

As shown in Figure 1, positive control had smaller body length than negative control. These data indicated that alcohol had affect on embryonal development. Thus, among $P_{0,5}$, P_5, and P_{50}, showed treatment with 5g/dl of Moringa oleifera leaf extract indicated the optimum concentration based on the result of body lenght.

Head Diameter of Chicken Embryo Induced by Alcohol

P- were uninduced by alcohol (negative control), P+ were induced by alcohol without treatment, $P_{0,5}$, P_5, P_{50} were induced by alcohol and treated with 0,5g/dl, 5g/dl, 50g/dl of Moringa oliefera leaf extracts. Anova and Duncan test were used to determine sig-

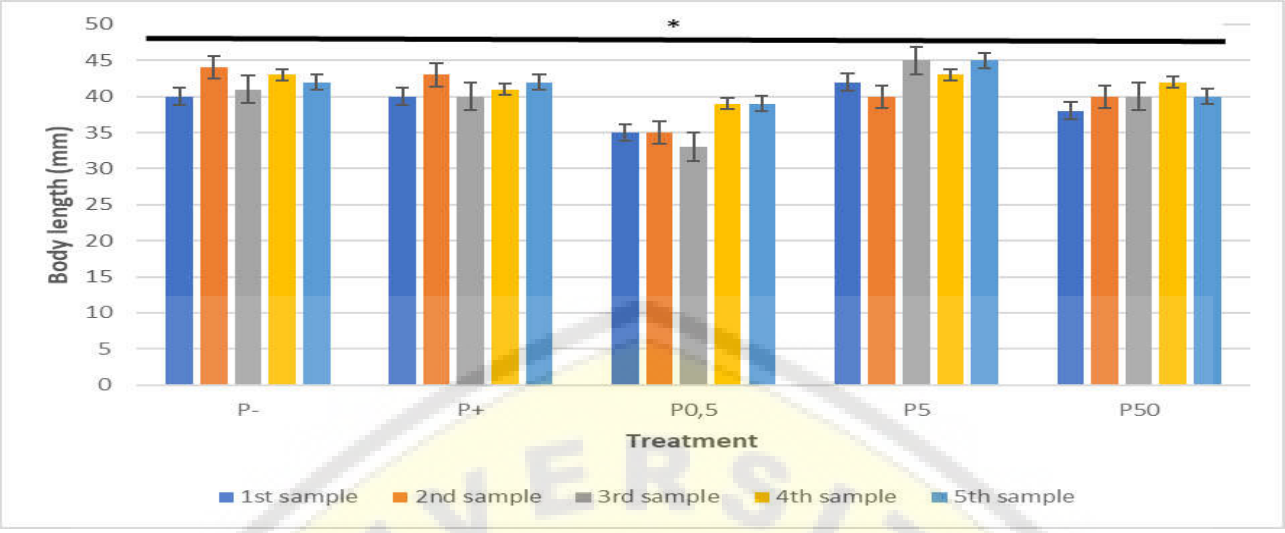

Figure 1. The Effect Of Moringa oleifera Leaf Extracts On Body Length Of Chicken Embryo Induced By Alcohol

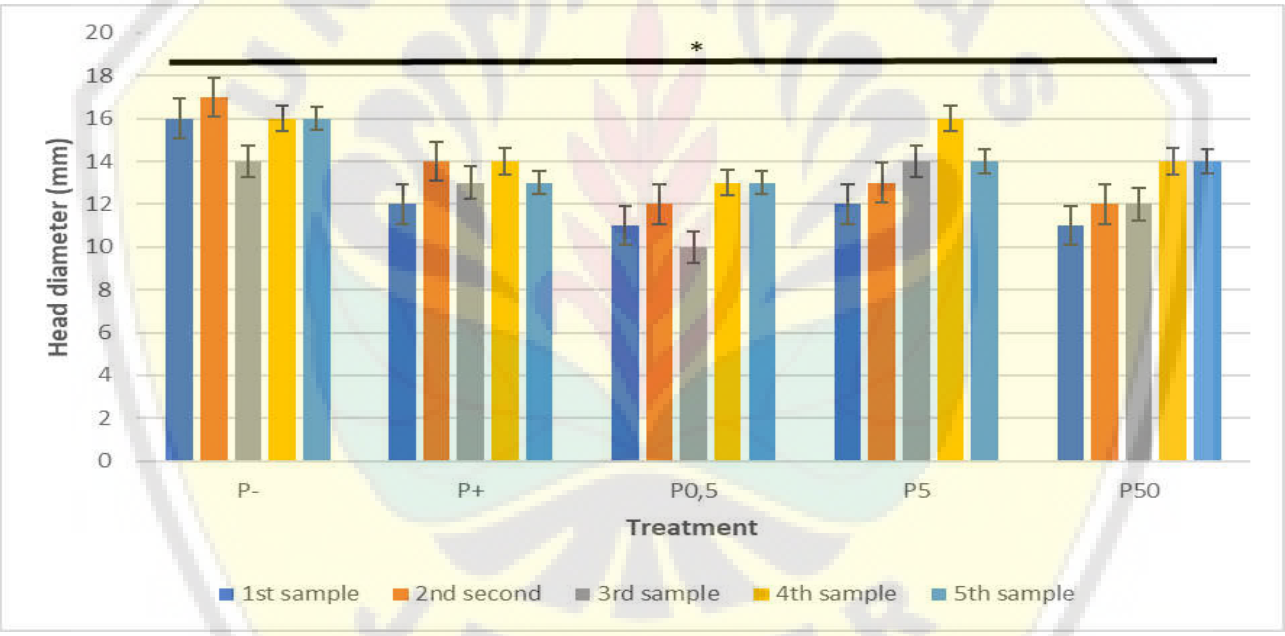

Figure 2. The Effect Of Moringa oleifera Leaf Extracts On Head Diameter Of Chicken Embryo Induced By Alcohol

nificance between every group treatment and negative control. *p<0,05. Each group treatment consists of 5 samples and described as 1st-5th Sample.

As shown in Figure 2, positive control had smaller head diameter than negative control. This finding indicated that alcohol had affect on head development and might be on cranio nerve system. Among $P_{0,5}$, P_5, and P_{50}, P_5 and P_{50} had similar effect in head development and much better than $P_{0,5}$.

DISCUSSION

Several studies have demonstrated the beneficial effects of Moringa oleifera in humans. The most used parts of the plant are the leaves, which are rich in flavonoids (Leone et al., 2015). Bajpai et al., 2005 reported that this antioxidant activity of moringa oleifera leaves due to presence of Kaemferol. Kaempferol (3,4', 5,7-tetrahydroxyflavone) is a natural flavonol, a type of flavonoid, found in various plant and plant foods (Wang et al., 2006). At the molecular level, kaempferol has been reported to modulate a number of key elements in the cellular signal transduction pathway associated with apoptosis, angiogenesis, inflammation, and metastasis. Significantly, kaempferol inhibits cancer cell growth, angiognesis, and induces cancer cell apoptosis, but on the other hand, kaempferol appears

to maintain normal cell viability, in some cases providing a protective effect (Chen and Chen, 2013).

As shown in figure 1 and 2, alcohol exposure in chicken embryo inhibited growth of body length and head diameter compared to the growth of chicken embryos unexposed with alcohol. These results were expected because alcohol exposure during development effects Shh signaling which is important to neural development. The impact of embryos exposure to alcohol associate with Shh signalling. It is an important period for the neural crest development. When exposed to alcohol, several cell signals are vital for neural development will disappear (Ahlgre, Thakur & Fraser, 2002).

At 5 g / dl of Moringa oleifera extract showed the good results for inhibiting the alcohol effect on the chicken embryos development. It is due to at high concentrations, polyol increases the activity or expression of antioxidant enzymes such as superoxide dismutase, catalase, and heme oxygenase-1 while at low concentrations, kaempferol acts as a superoxide remover, especially against highly reactive and peroxynitrite hydroxyl radicals (Montano et al., 2011). According to Kim et al (2015), Moringa leaf extract can inhibit the effects of alcohol in mechanism as an anti-oxidant by suppressing the release of Nitric Oxide (NO) and Prostaglandin E2 (PGE2), decreasing cellular adhesion of U937 cells to Fibronectin (FN), neutralizing radical generation, and reducing the level of mRNA expression from inflammatory genes that encode NO induction Synthase (iNOS), TNF-alfa, and Cyclooxygenase- (COX-) 2 in Lipopolysaccharide- (LPS-) and Sodium Nitroprusside- (SNP-) are mediated by RAW264.7 cells and peritoneal macrophages. Kaempferol reduces NF-?B levels (p65 and p50) and AP-1 (c-Jun and c-Fos) in the nucleus and their transcription activities.

These findings suggested that Moringa oleifera leaves extract implies an alternative medicine to inhibit growth disorders caused by exposure to alcohol.

CONCLUSION

Moringa oleifera leaves extract had affect on chicken embryo growth induced by alcohol. At 5 g / dl of Moringa oleifera leaf extract had the most inhibitory effect on the teratogenic effects compared to concentrations of 0.5 g / dl or 50 g / dl. The findings of this study implies that Moringa oleifera leaves extracts would be beneficial to be alternative medicine to prevent growth retardation. Further research should be examine the minimum and maximum doses that can cause therapeutic effects on the disruption of chicken embryo growth.

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