EFFECTS OF ALPHA-MANGOSTIN ON HEMATOPOIESIS
IN ZEBRAFISH *Danio rerio* (Hamilton, 1822) EMBRYOS

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ผลของแอลฟาแมงโกสทินต่อการกำเนิดเม็ดเลือด
ในเอ็มบริโอของปลาม้าลาย *Danio rerio* (Hamilton, 1822)

น.ส.วรรณกานต์ กิตติภาสน์พัลลภ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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วรรณกานต์ กิตติภาสน์พัลลภ : ผลของแอลฟาแมงโกสทินต่อการกำเนิดเม็ดเลือด ใน เอ็มบริโอของปลาม้าลาย *Danio rerio* (Hamilton, 1822). (EFFECTS OF ALPHA-MANGOSTIN ON HEMATOPOIESIS

IN ZEBRAFISH *Danio rerio* (Hamilton, 1822) EMBRYOS) อ.ที่ปรึกษาหลัก : ศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาร่วม : ดร.วิทยา พิมทอง

แอลฟาแมงโกสทินเป็นสารในกลุ่มของแซนโทนซึ่งเป็นองค์ประกอบหลักที่ได้จากการ สกัดมังคุด *Garcinia mangostana* Linn. พบว่า แอลฟาแมงโกสทินมีประสิทธิภาพในการต้าน อนุมูลอิสระ ต้านการเติบโตของแบคทีเรีย และต้านการเพิ่มจำนวนของเซลล์มะเร็ง อย่างไรก็ตาม ข้อมูลเกี่ยวกับผลข้างเคียงที่มีต่อเซลล์ปกติ โดยเฉพาะผลที่มีต่อการกำเนิดเม็ดเลือดยังไม่สมบูรณ์ งานวิจัยนี้จึงมีจุดประสงค์เพื่อศึกษาผลกระทบของแอลฟาแมงโกสทินที่มีต่อการกำเนิดเม็ดเลือด โดยใช้เอ็มบริโอปลาม้าลาย *Danio rerio* เป็นโมเดล พบว่า แอลฟาแมงโกสทินทำให้เอ็มบริโอ ปลาม้าลายเกิดการตายเพิ่มตามระดับความเข้มข้นที่สูงขึ้น โดยมีค่าความเข้มข้นของสารที่ส่งผลให้ เอ็มบริโอปลาม้าลายตาย 50% (median lethal concentration, LC_{50}) ที่ 72 ชั่วโมง เท่ากับ 5.75 ± 0.26 μM นอกจากนี้ยังส่งผลให้เอ็มบริโอปลาม้าลายมีความผิดปกติ เช่น เยื่อหุ้มหัวใจบวม โต หางและลำตัวงอผิดรูป และการอุดตันของเลือด ยิ่งไปกว่านั้น คือ ทำให้ระดับฮีโมโกลบินใน หัวใจของเอ็มบริโอปลาม้าลายลดลงอย่างมีนัยสำคัญทางสถิติ นอกจากนี้แอลฟาแมงโกสทินยัง ส่งผลให้การแสดงออกของยีน *spi1b, mpx* และ *hbae1.1* ซึ่งเกี่ยวข้องกับการกำเนิดเม็ดเลือดใน ระดับของการถอดรหัสทางพันธุกรรมเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ แต่ในทางตรงกันข้ามกลับไม่ ส่งผลต่อการแสดงออกของยีนกลุ่มดังกล่าวในระดับของการแปลรหัสทางพันธุกรรม ถึงแม้ว่าก่อน หน้านี้ได้มีการรายงานฤทธิ์ทางชีวภาพของแอลฟาแมงโกสทินอย่างแพร่หลาย แต่ในงานวิจัยนี้แสดง ให้เห็นว่า การใช้สารดังกล่าวในระดับความเข้มข้นที่มากกว่า 3 μM ทำให้เกิดผลข้างเคียงต่อการ เจริญของเอ็มบริโอปลาม้าลายในระยะแรก และนำไปสู่ความเป็นพิษต่อระบบการกำเนิดเม็ดเลือด ได้

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Wannakarn   Kitipaspallop   :   EFFECTS OF ALPHA-MANGOSTIN ON HEMATOPOIESIS

IN ZEBRAFISH *Danio rerio* (Hamilton, 1822) EMBRYOS.   Advisor: Prof. Chanpen Chanchao, Ph.D. Co-advisor: Wittaya Pimtong, Ph.D.

Alpha-mangostin (AM) is in the xanthone group and a main compound extracted from mangosteen (*Garcinia mangostana* Linn.). It is effective in antioxidant, antimicrobe and anticancer activities. However, the underlying side effects on normal cells, especially in hematopoiesis have not been investigated completely. In this research, it was aimed to elucidate the possible effects of AM on hematopoiesis using zebrafish (*Danio rerio*) embryos as a representative model. The results showed that AM could cause the zebrafish embryonic mortality with the concentration dependent manner. The median lethal concentration (LC_{50}) of AM at 72 h was 5.75 ± 0.26 µM. In addition, AM induced a zebrafish embryonic malformation such as pericardial edema, tail malformation, body malformation and blood clotting. Furthermore, the hemoglobin level in a zebrafish embryo's cardiac region was decreased significantly by AM. In addition, at the transcriptional level, AM increased the gene expression of *spi1b*, *mpx* and *hbae1.1* which were representatives of hematopoietic related genes significantly. On the other hand, AM had no effect on the expression of those genes at the translational level. Although it has been widely reported that AM has many bioactivities, in this research, it revealed the side effects of AM at the concentration higher than 3 µM on the early stage of zebrafish embryonic development and the toxicity on the hematopoietic system.

Field of Study: Zoology	Student's Signature ....
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LIST OF ABBREVIATIONS

Abs	Absorbance
AM	Alpha-mangostin
ANOVA	Analysis of Variance
ALM	Anterior lateral mesoderm
°C	degree Celsius
DMSO	Dimethylsulfoxide
DI water	Distilled water
dpf	Days post fertilization
Gata1a	GATA binding protein 1a
g	Gram
IC_{50}	Half maximal inhibitory concentration
Hbae1.1	Hemoglobin alpha embryonic 1.1
h	Hour
HSCs	Hematopoietic stem cells
hpf	Hours post fertilization
IOD	Integrated optical density
ICM	Intermediate cell mass
IL-8	Interleukin 8
IEC	Intestinal epithelial cells
kg	Kilogram
LPS	Lipopolysaccharide
LD_{50}	Median lethal dose
ME	Mangosteen extraction compound
mg	Milligram
Mpx	Myeloid-specific peroxidase

min	Minutes
mm	Millimeter
OECD	Organisation Economic Co-operation and Development
µl	Microliter
µm	Micrometer
PLM	Posterior lateral mesoderm
PTU	Phenylthiourea
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcriptase real time polymerase chain reaction
REC	Retinal endothelial cells
ROS	Reactive oxygen species
VEGF	Vascular endothelium growth factor
SDS	Sodium dodecyl sulphate
Spi1b	Spi-1 proto-oncogene b
Tal1	T-cell acute lymphocytic leukemia 1
TBST	Tris buffered saline with tween
TNF-α	Tumor necrosis factor alpha

CHAPTER I

INTRODUCTION

1.1. Background and rationale

Over the past decades, natural plant products have been widely used as a traditional medicine for relieving human diseases. Due to their incredible potentials, more than 80 percent of the world's human populations use them as an alternative remedy [1]. Natural plant products have drawn the interest from researchers due to a great value of their pharmacological properties; thereby, they become the special issue. Mangosteen (*Garcinia mangostana* Linn.) is one such well-known plant which has been studied since a wide array of their pharmacological potentials [2].

There have had long stories about mangosteen in Southeast Asia used as traditional medicine. By scientific approach, mangosteen contains major secondary metabolite called alpha-mangostin (AM), one of the xanthone derivatives. AM exhibited stronger bioactivities than other derivatives such as decreasing inflammatory cytokines [3], high activity of free radical scavenging [4] and also suppressing the viability of cancer cell lines [5]. In recent year, the popularity and the consumption of mangosteen-based products have been increasing as dietary supplements, cosmetics, food additives and medicinal appliances [6]. Consumers use botanical supplements as an alternative way for health maintenance since they believe that natural products are safer than conventional drugs. This perception can lead to improper dose intake resulting in toxic effects [7, 8]. Supported by previous research, some health problems can be associated with dietary supplement consumption like hepatotoxicity, liver, kidney and heart toxicities [7-10]. In case of AM, although this compound has various benefits and is widely used in industry, only a few researches have studied about its toxicity profiles. Thereby, the information about side effects even the toxicities should be evaluated, especially on hematopoietic system.

The overall process of blood cell formation during embryonic development is called hematopoiesis. This process begins during the first week of gastrulation in animal development which is important for providing the oxygen and homeostasis. Hematopoiesis can adversely affect health and crucial for survival; therefore, this process has been intensively studied for understanding blood disorder, cytokine network as well as oncogenesis [11, 12]. Subsequently, this complex process can be influenced by various factors such as environment, chemical, food additive and drug therapy [13]. A defect in hematopoiesis can lead to unbalance in blood cell production. Decreasing the red and white blood cell concentration can cause oxygen supply as well as lead to insufficiency and inefficiency of the immune system [14]. Taken together, chemical effects on hematopoiesis is an essential part that should be considered.

To study toxic effects of hematopoiesis, using mammals are very difficult. Non-transparency of their body is not allowed to detect any defect in real time. Interestingly, zebrafish (*Danio rerio*) embryos are transparent resulting in the easiness to monitor and manipulate under a microscope [15]. Furthermore, at least one of 70% approximately of protein-coding genes in human have orthologs in zebrafish, and 84% of disease-related genes also have been identified in zebrafish [16]. Additionally, the molecular mechanisms in hematopoietic development between human and zebrafish are conserved. The mechanism for generating of all blood cell types in zebrafish has been shown to be similar to that of human, including erythroid, myeloid and lymphoid lineages. Moreover, the essential transcription factor genes which associated in hematopoiesis in zebrafish have orthologs to human [17, 18]. For mutagenesis screening, zebrafish can be used as the reference model for representing the defect in human hematopoiesis since the key genes have already been studied. Thus, zebrafish emerged as a powerful model to represent the effects on hematopoiesis such as leukemia, anemia, inflammation and innate immune response that contribute to clearly understand the core regulation of these diseases [19-21].

As described above, several researches reported the benefits of AM but only a few research concerned about the side effects despite evaluating the possible

toxicity is an essential step. Hence, the objective of this work is to investigate the effects of AM on hematopoiesis in zebrafish *Danio rerio* (Hamilton, 1822) embryos. Embryos were exposed to AM with different concentrations for screening the toxicity and selecting the working doses. Then, the effects on hematopoiesis by measuring hemoglobin and gene expression levels were focused. The expression levels of five genes related to hematopoiesis: *T-cell acute lymphocytic leukemia 1* (*tal1*), *GATA binding protein 1a* (*gata1a*), *hemoglobin alpha embryonic 1.1* (*hbae1.1*), *Spi-1 proto-oncogene b* (*spi1b*) and *myeloid-specific peroxidase* (*mpx*) were determined through quantitative reverse transcriptase real time PCR (qRT-PCR) and western blot assay, respectively. Finally, the obtained data will provide the safe dose and possible mechanism on hematopoietic system of AM *in vivo*.

1.2. Research Objective

To investigate the effects of alpha-mangostin on the early stage of embryonic development and hematopoiesis in zebrafish embryos.

1.3. Research Hypothesis

AM, especially in unsuitable dose, has side effects on hematopoietic system in zebrafish embryos.

1.4. Places of study

National Science and Technology Development Agency, National Nanotechnology Center, Phatum Thani, Thailand.

CHAPTER II

LITTERATURE REVIEW

2.1. Alpha-mangostin (AM)

Herbal medicines have more than thousands years of history in the world. Nowadays even though chemically synthesized medicines have been developed, many people still rely on herbal treatment [22]. Mangosteen is an outstanding example of natural product.

Because of many health benefits, mangosteen has been used as a traditional folk medicine for centuries. Every part of mangosteen, peel, rind, hull or ripe, has strongly potential for reliving human diseases. The rind of mangosteen can be used for treating diarrhea, fever and skin ailment. For mangosteen leave, some people use as food additive for dysentery, fever and thrush. The pericarps of this fruit are also used for defending skin infection, abdominal pain and trauma [23]. Additionally, mangosteen also has a great flavor and delightful smell, therefore, it is generally known as "the queen of fruits" [24]. Accordingly, mangosteen is used in many industries as an important ingredient for developing the products such as herbal supplements, beverages, and medical appliances. Hence, mangosteen-based products easily become the top selling in the United States [25].

For phytochemical studies, mangosteen contains several of secondary metabolizes, with great chemoprotective properties, including xanthones. More than 68 types of xanthones can be derived from the whole fruit of mangosteen, mostly in pericarp such as alpha-mangostin, beta-mangostin, gamma-mangostin and garcinones (Figure 1). For chemical structures, xanthones consist of a tricyclic aromatic system with a C_6-C_3-C skeleton, A and B benzyl ring are located by isoprene, methoxyl and hydroxyl groups in different location. Xanthones exhibited the remarkable biological activities; thereby, these compounds have been the most studied in several researches. Antioxidant, anticancer, anti-inflammation and antimicrobial properties

are some of main pharmacological properties of xanthones that have been reported [26-28].

Among xanthone derivatives, the first compound that could be extracted was alpha-mangostin (AM) (Figure 1). AM was found in 1855 by Schmid et al. and the structure was revealed by Dragendorff (1930) and Murakami (1932), respectively [29-31]. The molecular formula of this compound is $C_{14}H_{22}O_6$ with 410.46 of molecular weight. AM is the most abundant xanthone, presenting in a dried pericarp at the concentration 5.5 mg/g [32]. Among xanthone derivatives, AM gains the greatest attention from researchers because of the notable of their medicinal properties.
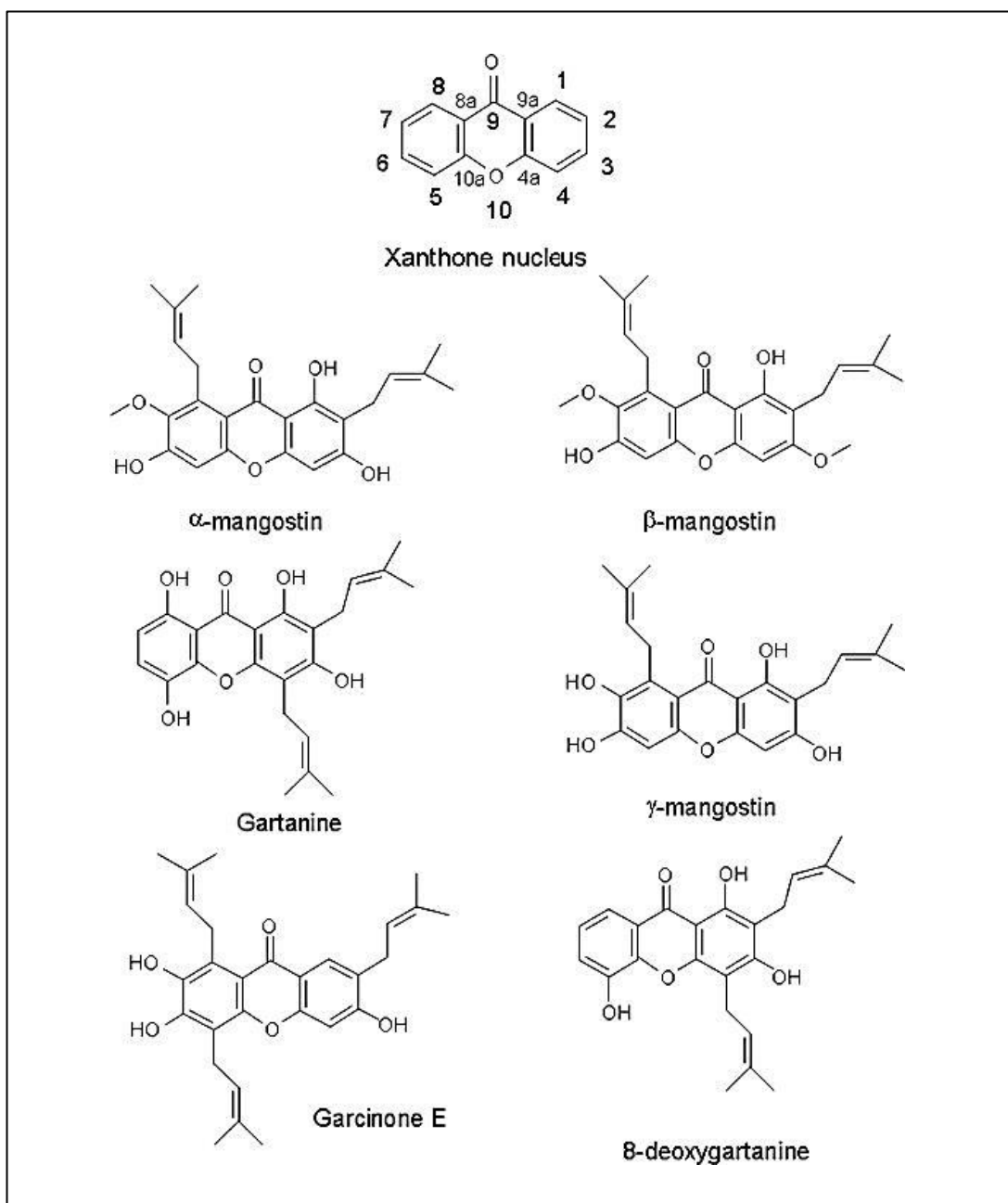
Figure 1 Chemical structures of xanthone derivatives [24]

2.1.1. Biological and pharmacological properties of AM

2.1.1.1. Anticancer properties

Cancer is a deadly disease caused by multifactor. More than million people died from cancer and the new cases of patients extensively increase every year. Nowadays, the best way for treating cancer is still incomplete; therefore, combination therapies are a good choice for patients. A plant-based diets, phytochemicals, also have been used for decreasing the risk of cancer and AM is an interesting phytochemical possessing a potential anticarcinogenic effect [33, 34]. One of anticarcinogenic activities of AM is suppressing cancer promotion and progression by initiation of cell cycle arrest as well as induction of apoptosis. Johnson et al. found that AM decreasing cell viability of human prostate cancer cells (LNCaP) with median maximal inhibitory concentration (IC_{50}) of 5.9 µM at 48 h. For mechanism of action, AM directly inhibited cyclins/cyclin dependent kinases which responsible for cell cycle promoting; therefore, cancer cell was loss its ability to proliferate [35]. Additionally, Matsumoto et al. also found the antiproliferative activities of AM in human colon cancer cells (DLD-1). This compound inhibited DLD-1 cell growth at 20.0 µM after 24 h of exposure and up-regulated the expression levels of p27 protein which playing a role in cell cycle machinery. Interestingly, AM could eliminate the hyperproliferation of neoplastic cells by inducing cell apoptosis. AM caused the loss of mitochondrial membrane potential in hepatocellular carcinoma cells (SK-Hep-1) resulted from mitochondrial caspase apoptotic pathway triggered with a half number of cell viability at the concentration of 19.6 µM at 48 h, approximately [36]. Subsequently, AM also acts as suppressing agent to suppress abnormal angiogenesis that resulted in inhibiting tumor cell growth and metastasis. Jittiporn et al. conducted the experiment through hypoxia-treated bovine retinal endothelial cells (REC) and reported that AM significantly decreased cell viability at the concentration greater than 8 µM after 24 h of exposure. In hypoxia-induced condition, AM reduced ROS formation that also resulting in suppressing vascular endothelial growth factor (VGEF) activities. This research also revealed the effects of AM on anti-angiogenesis that AM could inhibit VEGF migration, proliferation as well as hyperpermeability. From these

results, AM is useful for protecting abnormal neovascularization in retinal which is an important cause of vision loss [37]. Another interesting research was conducted by Matsumoto et al. The researchers examined the effects of six xanthones isolated from pericarp of mangosteen to human leukemia cell HL60. AM displayed the highest growth inhibitory effects compared to other derivatives with IC_{50} of 10 µM at 72 h. This effect was induced by cell apoptosis which activated through caspase-3 pathway [38]. Moreover, AM was also investigated for the anti-tumor activities performing in animal models, mostly in nude mice. For instance, Lee et al. reported the antitumor effect of AM on tongue mucoepidermoid carcinoma cells (Y-D 15) in BALB/c nude mice. YD-15 cells were subcutaneously injected to five weeks old mice and started treating with AM at the concentration 10 and 20 mg/kg BW by intraperitoneal injection when the tumors were palpable. The results showed AM treatment could significantly reduce tumor volume in apoptosis pathway, as confirmed by TUNEL assay [39]. Nabandith et al. studied, four weeks old male F344 rats were fed with a diet containing 0.02% and 0.05% of AM for a week and then subcutaneously injected with 1, 2-dimethyhydrazine, cancer inducing agent, 40 mg/kg BW for two weeks. After terminating at the fifth week, the result showed that AM in all concentrations inhibited the development of aberrant crypt foci, dysplastic foci and beta-catenin accumulated crypts which are a biomarker for short-term colon carcinogenesis. Therefore, exposure AM in long-term might suppress tumor development. This research pointed out the evidence of anti-cancer properties of AM as a chemoprotective substance by preventing preneoplastic cell proliferation in rat colon carcinoma cells [40]. For clinical trial, AM could use combining with modern chemotherapy drugs for increasing their efficiency as well as decreasing their side effects. Acute renal injury is the side effect from cisplatin (CDDP) which widely used as chemotherapeutic agent for treating the tumors in ovary, testis, bladder and endometrium. Sanchez-Perez et al. co-incubated proximal tubule renal epithelial cells (LLC-PK1) with CDDP (100 µM) and AM (5 µM) for 24 h. The researcher found that (1) AM protected CDDP-induced cells from apoptosis and increased cell viability, (2) AM reduced ROS generating from CPPD-induced cells and (3) this compound also suppressed the expression of apoptotic mediator protein, p53. Based on these

results, AM had a potential for protecting the cells from side effects which caused from chemotherapy drugs [41]. Accordingly, AM is one of promising phytochemicals that should be developed for cancer treatment and prevention.

2.1.1.2. Antioxidant properties

ROS is associated with human health, resulting in several health problems such as neurodegenerative, cancer and skin aging. To reduce the amount of ROS, many natural substances have a high antioxidant capacity and got a lot of attention from researchers [42]. AM is one of natural products that showed protective activity against the free radical. Devi Sampath et al. evaluated antioxidant properties of AM using isoproterenol (150 mg/kg BW) to induce myocardial infarction in Wistar rat model. This induction led to overexpress of ROS generation through elevating lipid peroxidase and decreasing the activities of endogenous antioxidants, superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and reduced glutathione. After orally pre-treated with AM at the concentration 200 mg/kg of body weight per day for 6 days and followed by isoproterenol for 2 days leading to significant reduce the levels of lipid peroxidase and restore the activities of antioxidant enzymes. Moreover, they examined the histology of heart in pretreated group and had not detected any signs of adverse pathology. This finding revealed the defensing capacities of AM against oxidative stress [43]. Additionally, Buelna-Chontal et al. also discovered the protective effect of AM on the reperfusion injury in cardiac area. The heart tissue was obtained from male Wistar rat and rapidly fixed onto a Langendorff heart perfusion system. The heart was subjected to ischemia for 30 min and followed by reperfusing for 60 min, respectively. During reperfused process, AM was treated to the heart at the concentration 2.5 μM. Finally, AM showed a role in the cardiac mechanic including reduced the infarction area, prevented the fall off cardiac ATP and phosphocreatine levels in the refused myocardium. This research also reported other protective properties of AM such as an ability for reducing oxidative stress, lipid peroxidation and glutathione content [44]. Moreover, AM had the protective effect against age-related macular degeneration by suppressing

oxidative stress performing. Fang et al. examined these effects on retinal heath both in *in vivo* and *in vitro* models. For *in vivo* model, female balb/c mice, 8 weeks old, were orally administered with 10 mg/kg BW and 30 mg/kg BW of AM for 7 days and exposed to white light for inducing retinal dysfunction. After 24 h of light exposure mice were scarified and conducted the retinal section. At 30 mg/kg BW, AM showed the abilities to maintain photoreceptor function and protect cell apoptosis in light-induced retina. For *in vitro* model, human retinal pigment epithelial cells (ARPE-19) were pretreated with AM (4, 8, 10, 12, 14, 16, 18, 20, 30 and 40 µM) for 24 h and then induced cell stressed by incubating in hydrogen peroxide (H_2O_2) (200 µM) for 24 h. AM in range of 4-12 µM could increase cell viability and reduce apoptosis rate in H_2O_2-induced cells. Furthermore, ROS and lipid peroxidase production were decreased in AM-pretreated cells. In summary, AM not only exert antioxidant capacities but also protect the possible effects on retinal health [45]. In clinical trial, Xie et al. found that consuming a mangosteen-based beverage (245 mL) everyday leading to increase antioxidant biomarkers in plasma. 60 healthy adults both men and women received mangosteen-based drink for 30 days. Every day in the morning, participants were drawn their blood for collecting the plasma after fasting for 8 h and recorded physical details, body weight, BMI, blood pressure and heart rate. For these physical measurements, the results were not showed significantly different between treated and non-treated group. For antioxidant status, AM had potentially increased antioxidant activities in the bloodstream, measured by oxygen radical absorbance capacity assay. Interestingly, the researches recommended mangosteen-rich drink because of its ability and did not alter the hepatic and renal functions [46]. From all information above, it should be noted that AM has a various role of antioxidation properties.

2.1.1.3. Anti-inflammatory properties

Inflammation is a pathological condition that characterized by vasodilatation, fluid exudation and neutrophil infiltration [47]. This condition can promote several diseases including cardiovascular diseases, cancer and chronic inflammation diseases.

To reduce the risk of inflammation, medicinal plant with anti-inflammation effects have been used as an alternative remedies [48]. AM has been prescribed to modulate an inflammatory response in human body. In the research conducted by Guierrez et al. showed that AM inhibited the production of nitric oxide, pro-inflammatory mediator, on lipopolysaccharide (LPS)-induced in murine macrophage cell line (RAW 264.7) at the concentration in range of 1-10 µM. Additionally, the researchers measured anti-inflammatory activities using biomarker of inflammation, interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α), and found that AM significantly attenuated the secretion of both inflammatory markers [49]. The potential effects of AM in anti-inflammatory property also revealed by Zou et al. via LPS-induced inflammatory model of rat intestinal epithelial cells (IEC-6). The results showed the ability of AM to decrease LPS-induced IEC-6 cells apoptosis as well as inflammatory cytokines production [50]. Thus, AM exerted a high anti-inflammatory activity for considering as a substitute substance to treat the diseases causing by inflammation.

2.1.2. Pharmacokinetic

Pharmacokinetics is the essential information for generating and approving the new drugs. It provides the mechanism and biological properties which are necessary for human clinical trials as well as adjusting the proper doses [51]. To follow pharmacokinetic process, absorption, distribution, metabolism and excretion have been quantified [52]. In case of AM, Li et al. investigated its pharmacokinetics in male Sprague Dawley rat plasma comparing between intravenous and oral application. In each rat was firstly exposed to AM (2 mg/kg BW) by intravenous injection. After washout period for a week, the same rat was received AM (20 mg/kg) by oral administration. Blood samples (500 µl) at 2, 5, 10, 20, 30 min, 1, 2, 4 and 6 h were used for analyzing pharmacokinetic factors. For intravenous application, the bioavailability was fast distribution and slow elimination with 3 min of half-life in distribution phase along with 3.5 h of terminal elimination phase, respectively. This result suggested that AM has a high tissue binding for intravenous administration. In

the contrary, oral administration was low bioavailability; therefore it was impossible to receive a complete concentration-time profile. Confirmed by Li et al., AM has low oral bioavailability owing to extensive first-pass metabolism as well as poor absorption [53, 54]. Interestingly about pharmacokinetics between AM pure active compound and AM in mangosteen extraction compound (ME), Choi et al. monitored their efficiency via tissue absorption, distribution, metabolism and safety using mouse model. After intraperitoneal administration, AM pure compound had a lower median lethal dose (LD_{50}) than AM in mangosteen extract, 150.0 mg/kg and 231.5 mg/kg, respectively, whereas all mice survived even at the maximum concentration (1.0 g/kg) in oral administration. For tissue distributions, the amount of AM in each organ tissues are variable depending on multifactor including administration routes, sampling time and chemical constitutes [55]. In conclusion, all findings about pharmacokinetics of AM are helpful to design the proper doses for human clinical trial and increase the awareness for safety.

2.1.3. Toxicity

Although AM has a great chemoprotective effects and gains a lot of attention from researchers, the information about its toxicity and safety are still limited. Ibrahim et al. demonstrated the toxic effects of AM from *Cratoxylum arborescens* both in *in vivo* and *in vitro* studies. In general observation, AM did not cause mortality and has no effects on behavior as well as on physical appearance in ICR mice even in the highest dose (1.0 g/kg). After autopsy, histopathology of mice in treated and non-treated groups did not showed any defects, necrosis, inflammation and cirrhosis. In an *in vitro* study, AM also had no toxic to normal liver cells (WRL-68) [56]. More interestingly, Lui et al. investigated the effects of AM on rat platelet. The results revealed that AM could cause non-aggregation of platelet cells in the range of 1-10 µM and directly induce platelets shape change as well as stimulate cytolysis of the platelet cells [57]. Importantly, platelet toxicant can lead to hemostasis failure and platelet dysfunction, resulting in cardiovascular toxicants [58].

Taken together, dietary supplement base on mangosteen extract is on a fast growing market in the USA. Many products had mangosteen extract as a main ingredient, for instance, antibacterial face mark, acne patch and wound-aid gauze pad from Germ Guard**®** Technology. These products were warranted for inhibiting bacterial growth both of gram negative and positive. Wound-aid gauze pad was used in clinical trial in patients with curing burn wound having 70% of dead tissue. After using this pad, the wound was healed completely within 2 weeks. Moreover, people are bombarded by many advertisements that usually refer to their health promoting properties while the safety dose-limiting may indeed fulfill. For AM, almost researches have focused on its protective effects but the information specifically on toxicities only a few researches have been reported. In fact, to develop traditional medicine for medicinal use, it is necessary to consider about safety and risk assessment for adjusting the suitable doses for clinical trial. As describe above, AM has side effects on hematopoietic system, it is one of the critical point that should be studied. Normally, the experimental about toxicity testing always comes from cell culture and rodent models, whereas in the hematopoietic system it is difficult to detect the possible side effects in real time since their bodies are non-transparently. One of the effective models that represent the transparent body with an impressive ability for toxicities is zebrafish (*Danio rerio*).

2.2. Zebrafish

2.2.1. Background of zebrafish

The zebrafish, *Danio rerio* (Hamilton, 1822), is a tropical freshwater fish that become a well-known vertebrate model organism using in many fields in scientific research. The name *Danio* is mean "of the rice flied" which is derived from the Bengali name "*dhani*" [59]. Zebrafish was originally from Himalayan region and nowadays they are available worldwide. They are omnivorous, consuming zooplankton, phytoplankton, insect as well as algae [60]. For taxonomy status, zebrafish belong to minnow family and the classification of this species is shown as below.

Kingdom   Animalia

Phylum   Chordata

Class   Actinopterygii

Order   Cypriniformes

Family   Cyprinidae

Subfamily   Danioninae

Genus   *Danio*

Species   *Danio rerio*

The standard lengths from the tip of nose to the posterior end of vertebra of zebrafish are around 3.0 cm in lenght, rarely exceeding 4.0 cm. Zebrafish have three types of pigment cell including dark blue melanophores, gold xanthophores and iridescent iridophores. The pigment performs after 24 hours post fertilized (hpf) and can be inhibited by phenylthiourea (PTU) within 24 h leading to visual the internal organs obviously [61]. The main color of their body is silvery white with blue strips line on the side of their body which similarly to zebra's stripes. Zebrafish have sex differentiation that can be used to identify male and female. Male zebrafish are tend to pinkish and yellowish coloration with lager anal fins and slender shape while female zebrafish tend to have white and blue coloring and rounded shape with lager belly (Figure 2) [62].

Figure 2 Adult Male and female zebrafish [63]

In reproduction behavior, all gonads start developing as ovaries and completely differentiate to normal testis at the third month after hatching. Zebrafish become sexual maturity around 3-4 months and no seasonal breeding cycle in domesticated zebrafish. Females are capable to spawn twice a week containing hundreds of eggs in each clutch [64]. Zebrafish eggs are transparent and non-adhesive with 0.7 mm. of diameter, approximately. Heart beating of embryos starts around 24 h and appears all precursors of major organs within 36 h and subsequently following by hatching between 48-72 h. Zebrafish embryos develop rapidly comparing with other vertebrates [65]. The reproduction and development potentials are the reasons making zebrafish become popular as the vertebrate model in several flied researches, especially in toxicology.

2.2.2. The advantages of zebrafish

Zebrafish firstly have been used as alternative vertebrate model organism in the late 1960s by George Streisinger. They have numerous advantages that providing the new insights in Biology; therefore, the number of scientific researches using zebrafish was increasing rapidly [66].

One of the beneficial properties of zebrafish is the ease to maintain and care than other animal models because they are robust and small size so the large numbers of zebrafish can be raised in a laboratory. Additionally, they require only the small space for housing and the maintenance cost also cheaper comparing to other model organism such as rodent [67].

Another main advantage of zebrafish is the high potential of reproduction and rapid development. A single female can generate hundreds of offspring in each week that ready for supporting the large experimental sample in researches. Furthermore, adult zebrafish have an external fertilization that can control their ovulation by light and the eggs are optically clear which easy to display the embryo insight. Because of the complete transparent of embryos, it is easy to monitor and manipulate the internal organs as well as the development stage in real time under simple microscopy without scarification the fish. Specifically, transparent embryos are useful

for investigating the effects in toxicology testing with non-invasive and powerful techniques that increasing an accurate outcomes. Moreover, the rapid development of zebrafish helps researchers screening their experimentation endpoint easily and also saving time for producing the transgenic lines. Hence, zebrafish have been used as a model for studying the basic development such as cell-fate determination, organ and neuronal development [67-69].

The highly genetic pathways conservation between zebrafish and human is also beneficial. Zebrafish share a high degree of their sequence and functional gene 70%, approximately, similarly to human. Additionally, 84% of human diseases genes match in zebrafish genome therefore zebrafish widely used for getting in deep the mechanism of diseases specifically in hematopoiesis [16, 70]. The hematopoietic system between zebrafish and human are highly conserved. The important steps and signaling molecules as well as the blood cell types in the underlying system of zebrafish are similarly to human [71]. Together, the advantages allow researchers to create transgenic development, gene mutation and specific gene knockdown which can be revealed the gene function. In overall, zebrafish posse a high potential laboratory model for deeper understanding as well as providing the new knowledge in genetically researches.

Finally, using zebrafish as a vertebrate laboratory model extensively provides many beneficial properties over mammalian model. This species is invaluable not only providing the new information but also applying in various researches.

2.2.3. Zebrafish model for toxicity testing

Toxicity testing for safety and risk assessment of substances is an essential process that should be established for developing the new drugs. In preclinical toxicity testing, it is recommended the information including the toxicity mechanism, toxicodynamic, toxicity endpoint and the proper dose response. For screening the compound impact, numerous researches studied via animal models and zebrafish is one of a great representative organisms. In brief, zebrafish are advantageous more than other vertebrate models because zebrafish embryos can prescreen the toxicities

of several chemicals at a time. They are small size so many replicates can be performed together at the same time and the embryos able to absorb the testing chemicals that have been added to medium easily [67]. Additionally, toxicity testing using this species decreases the waste disposal and reduces the chemical volume [72]. In case of heart malformed embryos, they can survive continuously more than 24 h because the embryos directly exchange the gas with environment whereas the gas exchange of rodent embryos depend on circulation system therefore rodent embryos with an abnormal heart always die *in utero*. This advantage allows the researcher exposing the chemical to abnormal embryos while they still alive [73]. Another evident that supports the effective of zebrafish as an alternative model for chemical testing is a case of thalidomide defect. Thalidomide is a sedative drug released in the late 1950's that had been widely used for treating morning sickness in pregnant women. This drug distributed around the world and easily became the top selling drug in the market with the advertisement that it is completely safe. Soon after, thalidomide was banned in 1961 because this drug can cause peripheral neuropathy and severe birth defects. Oddly in drug screening, mice were not susceptible to thalidomide damage meaning different species showed different reactions [74]. On the other hand, zebrafish model revealed the defects that caused by thalidomide such as lack of pectoral fin, loss of embryonic development-related genes and stopped blood vessels development through inhibiting actin skeleton dynamics [75-77]. This evident suggests that zebrafish model has a high sensitivity and a great representative for chemical testing that can prevent the possible effects and improve drug safety. Overall, toxicity testing strategies using zebrafish provide the meaningful impacts in safety chemical which could be related to human health specifically in hematopoietic system.

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2.3. Hematopoiesis

Hematopoiesis is an essential system which amenable for all steps of blood cell formation responsible for carrying the oxygen, promoting organ development as well as protecting organs from pathogens [78]. This process maintains the blood

production throughout organism's lifetime [79]. Hematopoiesis comprises of multi-potent stem cells firstly deriving to progenitor cells that have no the self-renewal property. Next, all progenitor cells differentiate to precursor cells in specific linage and lastly turn into the mature cells. The process initiates in early stage of embryos depending on the species which influenced by the action of growth factors and cytokines [80]. Hematopoietic system is a paradigm for studying general stem cell biology leading to understand cellular interaction, the mechanism of aging and diseases [81, 82]. Importantly, hematopoietic system has an intensive activity of cells proliferation therefore this system is very sensitive to chemical substances. Additionally, radiation, virus and infective agents also act as extrinsic damaging factors to this system which leading to a cause of death or hematopoietic failure [83]. Together, the effects of each compound to hematopoietic system are a critical point that should be analyzed. To overcome this problem, zebrafish is a suitable model for evaluating the effects on hematopoietic system, particularly.

2.3.1. Zebrafish and mammalian hematopoiesis

As mentioned above, zebrafish become an attractive vertebrate model to study in many field researches. The developmental biology of blood is one of systems that zebrafish could be a great representative as vertebrate model. Unlike mammals, zebrafish emerge outstanding benefits such as optical clarity of their body and external fertilization which are easily accessible the development of phenotypes during embryogenesis, while mammals are non-transparent body and intrauterine growth. In case of absence the circulatory system, early stage zebrafish can survive by exchanging the gas via diffusion directly, in contrast, mammals with abnormal circulation die *in utero* [84]. In addition, zebrafish can generate numerous mutants related in hematopoietic defects that leading to a deep understand in this system.

Generally, hematopoiesis of all vertebrates comprise of two waves known as the primitive and definitive waves, respectively. In mammals, the earlier wave establishes in an extraembryonic yolk sac responsible for producing erythrocytes and primitive macrophages. In zebrafish, the first wave occurs at intraembryonic area

where is located above the yolk tube; the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM). These sites become intermediate cell mass (ICM) which is equivalent to an extraembryonic yolk sac in mammals [85]. Like mammals, the primitive wave of zebrafish generates primitive erythroid cell and primitive macrophages at PLM and ALM, respectively. Primitive erythroid cells respond for supporting tissue oxygenation during the embryos growth, while primitive macrophages remodeling the tissue development via apoptosis pathway [81]. Then, progenitor cells enter into the circulation system at 24 hpf and persist until 4 days post fertilization, approximately (Figure 3).
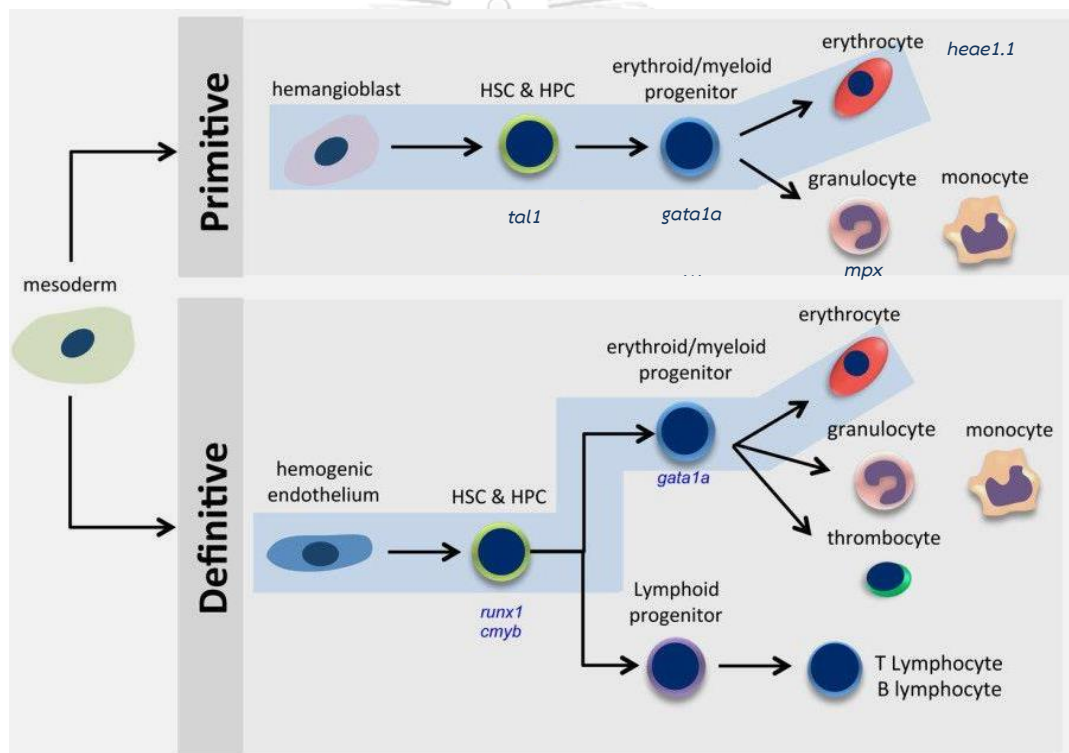

Figure 3 Overall of hematopoiesis of zebrafish in both primitive and definitive wave as well as gene regulation in each development step [21]

To begin the first wave of hematopoietic, several transcription factors play a vital role. The first transcription factor, T-cell acute lymphocytic leukemia (*tal1*) also known as stem cell leukemia (*scl*), is expressed during the 2 to 4 somite stages or 10 hpf both in ALM and PLM. This factor is a marker for a primitive hematopoietic stem cells (HSCs) formation. Knocking down the gene, the primitive and definitive wave cannot perform leading to completely loss of all blood linages. In addition, *tal1* knockdown embryos had severely effects in both HSCs formation and endothelial differentiation [86, 87].

Next step of the first wave is conversing stem cells to myeloid and erythroid linages. For erythroid linages, GATA binding protein 1a, *gata1a*, is expressed at 5-somite stage or 12 hpf at PLM. It is crucial for performing erythroid progenitor cells and activating the expression of erythroid-specific genes. The erythroid cells of zebrafish have a flattened elliptical shape and restrained nucleus. Considering the progenitor and mature erythrocyte of this species, proerythroid cells have less cytoplasm and larger nucleus while the mature erythrocytes are distinct from mammalian mature erythrocyte that retaining nucleus whereas mammalian erythrocytes are not nucleated [84]. From previous research, it was shown that mice with lacking *gata1a* failed to differentiate erythroid progenitor to mature erythroid cells and died during gastrulation phase [88]. The role of *gata1a* is not only promoting an erythroid, but also suppressing myeloid differentiation. Interestingly, the genes regulation between erythroid and myeloid cell is antagonistic action. In *gata1a* knockdown studies, it was found severe effects in erythropoiesis and the increasing of myeloid cells which resulting from the blood cells switching their fate and expressing myeloid genes instead. This transformation performs for balancing the amount of primitive erythroid and myeloid cells generation [89].

In the restriction area for myelopoiesis, ALM, the cells in this area generate the early myeloid cells including macrophages and granulocytes. The transcription factor calls spi-1 proto-oncogene b (*spi1b*) plays a key role in controlling myeloid cells development [90, 91]. As described above, primitive erythroid and myeloid regulated genes have a cross inhibitory control. In the absence of *spi1b,* myeloid

cells have a potential for differentiating to erythroid cells and maturing to hemoglobin alpha embryonic 1.1 (*hbae1.1*) instead than *mpx* [92].

After that, erythroid progenitor cell develops to mature erythrocyte and then circulates throughout the body for 4 days post fertilization (dpf). Mature erythrocyte expresses erythroid specific gene, *hbae1.1*, which responds for hemoglobin synthesis. Hemoglobin structure is a tetrameric molecules composed of globin subunits which regulated by intrinsic transcription factors and extrinsic signals. Embryo with *hbae1.1* mutation leads to red blood cell anomalies and deficiency of oxygen supply [21, 93].

Subsequently, myeloid progenitor cell commits to mature myelocyte and expresses the granulocyte-specific marker, *mpx* by 18 hpf [91]. Zebrafish with *mpx*-deficiency had no an ability to reduce the concentration of H_2O_2 after tissue damaging. Additionally, *mpx*-deficiency can alter the inflammatory response by impaired the host defense system [94, 95].

Owing to the results from hematopoietic disorder as describe above, the effects of every compound should be completely investigated for avoiding the feasible hematotoxicity. Even though zebrafish and mammals have a few different in hematopoietic system, they have common blood cell types and also strong conservation processes of hematopoiesis [96]. Therefore, zebrafish emerged as a representative model for evaluating a precise understanding in hematopoiesis.

Although AM offers effective properties and gains a lot of popularities among people, the awareness about its toxicity and safety are low leading to the possible hazards. In addition, the effects of this compound on hematopoietic system were not evaluated adequately. Here, this present study aims to examine the effects of AM on hematopoiesis using zebrafish embryos as a model for fulfilling the lacking knowledge and practicing of the safe use of AM.

CHAPTER III

MATERIALS AND METHODS

3.1. Methods

3.1.1. Zebrafish maintenance and embryos collecting

All animal procedures were approved by National Science and Technology Development Agency (NSTDA) International Animal Care and Use Committee and all zebrafish were raised in National Nanotechnology Center (Nanotec). Adult zebrafish were housed in controlling system at 28.5 °C, pH 6.0-8.0, conductivity in range of 300-1,500 µS, with a 14/10 h light/dark photoperiod. Fish were fed by live brine shrimp twice daily both in the morning at 8.00 a.m. and in the evening at 4.00 p.m. To get embryos, natural spawning was done by mating male and female zebrafish. In brief, male and female zebrafish were kept in a hatchery box and separated by a partition in a ratio of 2:2. The next day, a partition had been removed for one hour. Fertilized eggs were selected by using a stereomicroscope and maintained in 10 mL E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$•2H$_2$O and 0.33 mM MgSO$_4$) in petri dish at room temperature.

3.1.2. Zebrafish embryos acute toxicity assay

Stock solution (5 mM) of alpha-mangostin (AM) was prepared in 100% dimethylsulfoxide (DMSO). The E3 medium was used to dilute the AM stock solution to required final concentrations which were 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, and 12.0 µM with 0.48% DMSO. The concentrations were designed based on the preliminary experiment. According to the previous research, zebrafish embryos could expose to levels of up to 1% DMSO without any sign of toxicities. Therefore, the amount of DMSO in this experiment was safe and did not cause embryonic toxicities

[97]. To perform the acute toxicity assay, the protocol was adopted from Economic Co-operation and Development guideline 236 (OECD 236, 2013). Zebrafish embryos of 4-16 cells stage were selected and placed in a 12-well plate (30 zebrafish embryos per well). Zebrafish embryos had been exposed to AM at different concentrations for 72 h. Later, test solutions were refreshed at every 24 h. The plate was further incubated at 28.5 °C with a 14/10 h light/dark cycle. Dead zebrafish embryos were recorded and removed daily. Morphology and heart rate were observed directly under a stereomicroscope at 72 h incubation. The data were obtained from three independent experiments.

3.1.3. Hemoglobin staining by *o*-dianisidine solution

For visualizing hemoglobin in erythrocytes, *o*-dianisidine staining was performed as descripted previously in Duan et al. [98]. Briefly, zebrafish embryos were divided into treated and non-treated groups. In each group, zebrafish embryos of 4-16 cells stage were selected and placed in a 12-well plate (30 zebrafish embryos per well). The plate was further incubated at 28.5 °C with a 14/10 h light/dark cycle. Zebrafish embryos had been exposed to AM combined with 0.0015% (w/v) 1-phenyl2-thiourea for 24 h in order to inhibit pigment formation both in treated and non-treated groups. At 72 h exposure, zebrafish embryos were washed by distilled (DI) H_2O three times. Then, the embryos were incubated in 0.6 mg/mL *o*-dianisidine solution with 30% hydrogen peroxide in the dark for 15 min and fixed with 4% paraformaldehyde in phosphate-buffered saline. Then, the embryos were observed and photographed under a stereomicroscopy. To quantify hemoglobin levels, the ImageJ software was used. The rounded shape tool was selected to create the bounding cover hemoglobin positive staining in cardiac regions and then, the average integrated optical density (IOD) was calculated, subsequently.

3.1.4. Gene expression by quantitative reverse transcriptase real-time PCR (qRT-PCR)

To investigate the effect of AM on gene expression, transcriptional levels of five genes (*tal1, gata1a, spi1b, mpx* and *hbae1.1*) related to hematopoietic development were detected. According to acute toxicity testing, 0, 1/2 LC_{50} and LC_{50} of AM were chosen as designed concentrations. The concentration at 1/2 LC_{50} represented no observed adverse effects and LC_{50} represented severe effects. After 72 h exposure, total RNA was extracted from thirty zebrafish embryos per condition by using a RNeasy Plus Mini Kit. The absorbance (Abs) at 260 and 280 was measured by nanodrop 2000/2000c spectrophotometer. The concentration and purity of total RNA could be calculated according to the below formulas. The ratio of A_{260}/A_{280} should be around 2.0 indicating the purification of extracted RNA.

Concentration of total RNA (ng/µL) = (Abs_{260} nm) x 40 x dilution factor

Purity of total RNA = Abs_{260} / Abs_{280}

Next, 1 µg of RNA template were conversed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR according to manufacturer's instruction. Quantitative real-time PCR was performed as describe previously in Nugitrangson et al. (2016) on Biorad CFX96 Touch using SsoAdvanced Universal SYBR Green Supermix. Primers for five genes were commercially designed. A reaction mixture for 20 µl final volume consisted of 1x Ssoadvanced universal SYBR green supermix, 1x of each forward and reverse primer, 5 ng cDNA templates, and ultrapure DI H_2O. A reaction was performed under the prime PCR cycling condition: 1 cycle of 95 °C, 2 min for activation, followed by 40 cycles of 95 °C, 5 sec for denaturation, 60 °C, 30 sec for extension, and 65-95 °C, 5 min for performing melting curve (0.5 °C increments). The mRNA expression level of target genes was normalized to the mRNA expression level

of a reference gene, *gapdh*, using a comparative C_T method ($2^{-\Delta\Delta C_T}$). The data were presented in relative gene expression values comparing between control and treated group. All qRt-PCR reactions were performed with three independent experiments.

3.1.5. Protein expression by western blotting

In order to characterize the expression level of hematopoietic relating proteins (Tal1, Gata1a, Spi1b, Mpx and Hbae1.1), western blotting was performed. Zebrafish embryos treated with AM at the concentration of 0, 1/2 LC_{50} and LC_{50} were used. After 72 h exposure, the chorions of zebrafish embryos were removed and dechorionated zebrafish embryos were washed three times by ultrapure DI H_2O. Dechorionated zebrafish embryos were homogenized in 200 μl cold lysis buffer (Appendix B). The samples were centrifuged at 10,000x g, 4 °C for 5 min and the supernatants were then transferred to a new tube. The concentration of total protein was determined by a Pierce BCA protein assay kit with bovine serum albumin standard (Appendix C). Later, 12% sodium dodecyl sulphate polyacrylamide gel (SDS-polyacrylamide gel) was prepared (Appendix B). The amount of 30 μg lysate proteins combining with SDS sample buffer (Appendix B) were heated at 95 °C for 5 min and loaded into 12% SDS-polyacrylamide gel, respectively. Subsequently, the gel was electrophoresed at 90 volts for 2 h.

The gel was removed from the glass plates and soaked in transblotting buffer (Appendix A) for 5 min. For activating the membrane, immun-blot polyvinylidene difluoride (PVDF) membrane was soaked in methanol for 5 min and followed by transblotting buffer, respectively. Protein on the gel was transferred to PVDF membrane by electroblotting in semi-dry condition at 23 V for 0.30 h. For visualizing of protein in membrane, ponceau s solution was used. The membrane was washed in phosphate buffered saline with 0.1% tween (TBST) and followed by incubating with ponceau s solution on agitator for 10 min. The membrane was destained by washing extensively with 0.1% TBST until the protein bands were completely visible. Next, a non-specific protein in blotted PVDF membrane was blocked with 5% bovine serum albumin in 0.1% TBST at 4 °C for 2 h with gently agitation on a shaker. The

blotted membrane was incubated with primary antibody (1 µg/ml Tal1, 1 µg/ml Hbae1.1, 1 µg/ml Gata1a, 1 µg/ml spi1b, 2 µg/ml mpx, and 0.925 µg/ml gapdh) in blocking buffer at 4 ℃, overnight. Subsequently, the blotted membrane was washed using 0.1% TBST, 5 min for 3x. Next, the blotted membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (0.1 µg/ml mouse and 0.1 µg/ml rabbit) in blocking buffer at room temperature for an hour and washed with 0.1% TBST. After washing the membrane, ECL chemiluminescence reagent was used for visualizing proteins in membrane according to manufacturer's instruction. Briefly, detection solution A and detection solution B were mixed together in an equal volume. The final volume was 0.125 ml/cm^2 of membrane area. The membrane was incubated in mixed solution for 1 min and drained the excess solution, respectively. Amersham imager 600 was used for visualizing and capturing protein bands. The bands were defined as a selected region in a rectangle shape and measured the intensity using ImageJ software. Protein expression level of target proteins was normalized to Gapdh as an internal control protein. This process was generated in three independent experiments. The details of all used solutions were shown in appendix B.

3.1.6. Statistical analysis

Data were calculated by SPSS software 22.0 and were shown as mean ± S.D. for mortality rate, heart rate, and IOD of treated and control groups. For relative gene and protein expression, the data were shown as mean ± S.E.M. All information were distinguished by one-way analysis of variance (ANOVA) following by Tukey's multiple comparisons. The level of statistical significance was at $p < 0.05$.

CHAPTER IV

RESULT

4.1. The effects of alpha-mangostin (AM) treatment on zebrafish embryos

To investigate the acute toxicity of AM, zebrafish embryos of 4-16 cells stage were exposed with AM for 72 h in various concentrations. In control group (0.48% DMSO), we did not find a mortality or any defect. In AM-treated groups, AM caused embryonic mortality in dose dependent manner. The mortality was 14.4% at 4.5 μM and reach 100% of mortality at 7.5 μM (Figure 4A). The median lethal concentration (LC_{50}) of AM at 72 h was 5.76 ± 0.26 μM. Moreover, AM significantly decreased the heart rates of the embryos at 6 μM as compared to that of control group (Figure 4B). Additionally, AM treatment at 4.5 and 6.0 μM resulted in embryos abnormalities, such as tail malformation, pericardial edema, yolk edema and blood clotting (Figure 4C).
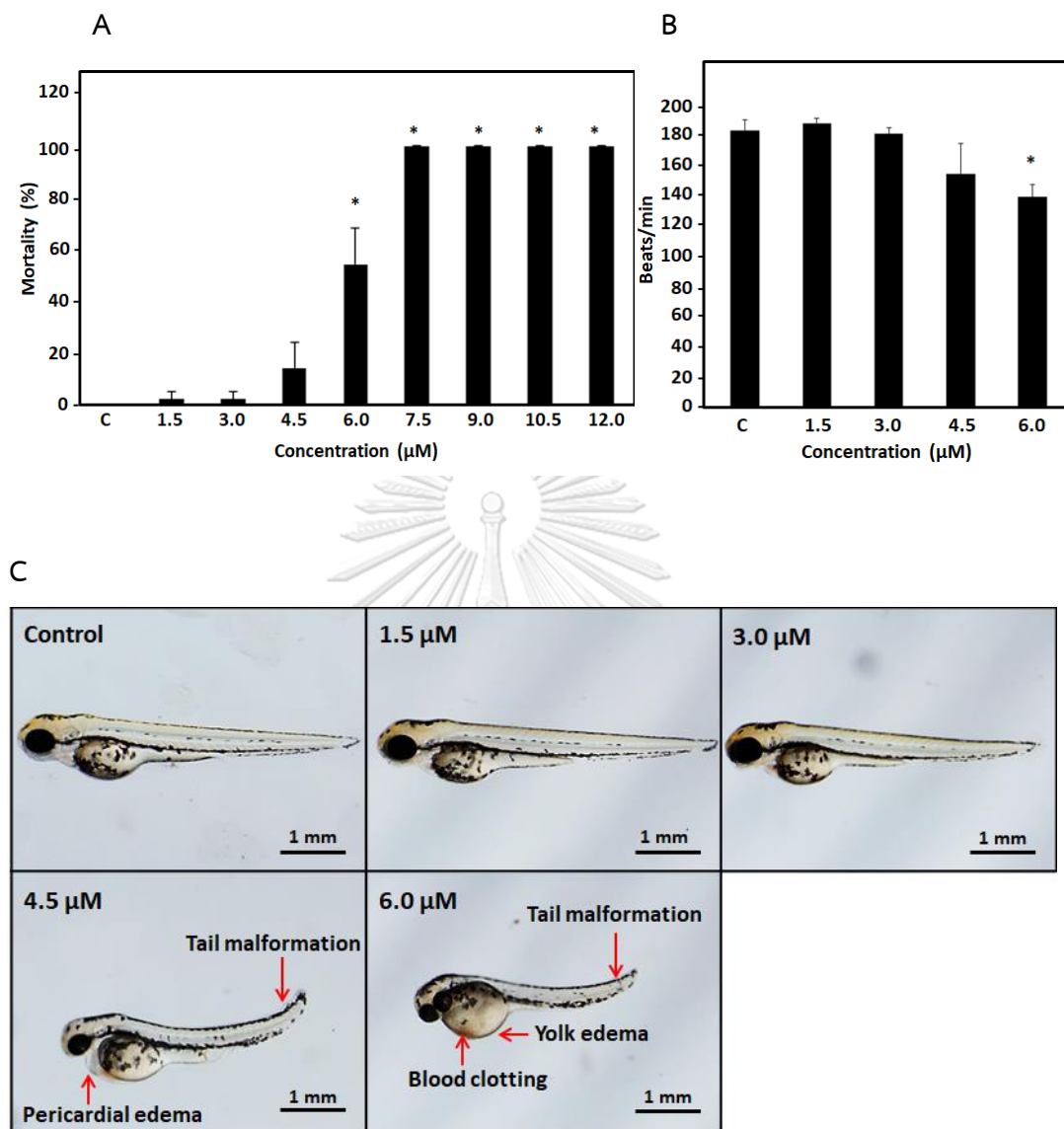
Figure 4 Acute toxicity testing of zebrafish embryos after treated with AM in different concentrations for 72 h. (A) The percentages of embryo mortality exposed to AM. (B) Heart rate of the embryos. (C) Images of representative zebrafish embryos in treated and untreated groups, the red arrows show malformation that can be visualized. The scale bars are 1 mm. The experiments of AM in all concentrations were obtained from 3 independent experiments and expressed as mean ± S.D (n=30). (*$p<0.05$).

4.2. The effects of AM on hemoglobin level

To verify whether AM affected hemoglobin in erythroid cells, mature hemoglobin staining was performed using *o*-dianisidian. Based on the information from acute toxicity testing, two treatment conditions were selected. AM at 3 µM showed no observed adverse effect but 6 µM elicited severe effects on morphology. The result showed that embryos in untreated group exhibited hemoglobin positive staining in cardiac region more than treated groups (Figure 5A). For quantification, integral optical density values (IOD) were used to represent hemoglobin positive staining in cardiac section. The result demonstrated that IOD values of control group significantly higher than those of treated group at 6 µM (Figure 5). Together, the results indicated that AM can decrease hemoglobin levels in the region of cardiac in zebrafish embryos.
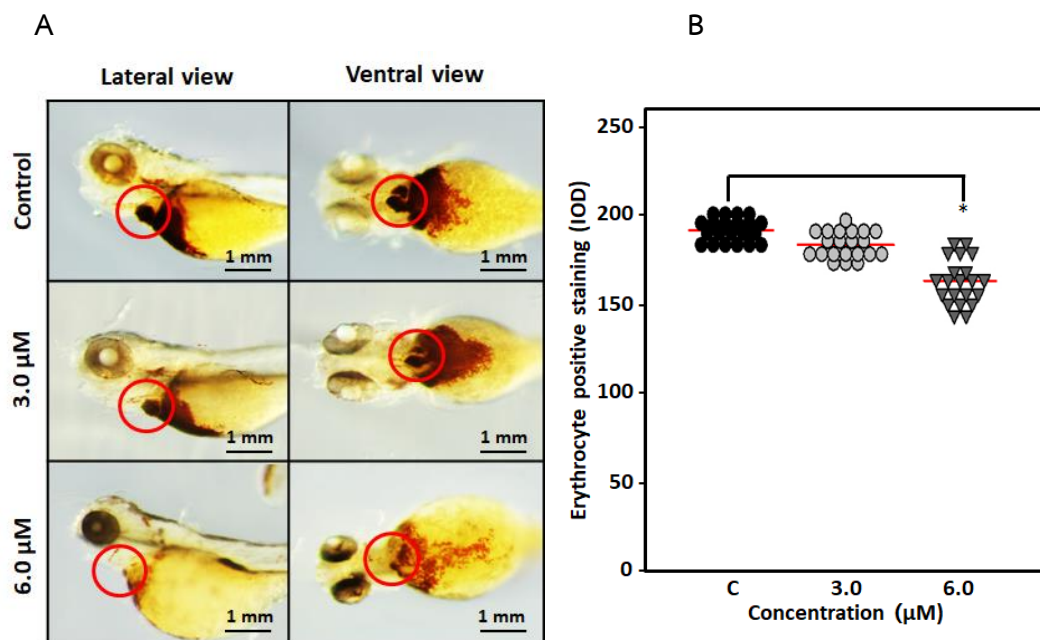
Figure 5 Hemoglobin detection in cardiac area of AM-treated in selected condition at 72 h. (A) Representative images of positive area in cardiac region (red circle). The scale bars are 1 mm. (B) Quantification of hemoglobin staining in the region of cardiac represented as integral optical density values (IOD) calculating by imageJ software. The values showed as mean ± S.D. of 3 independent experiments (n=20). (*p<0.05)

4.3. The effects of AM on the expression of hematopoietic genes

To get a closer insight into the mechanism of AM on hematopoiesis, mRNA expression levels that related to hematopoiesis pathway were measured in whole embryos both in treated and untreated groups of AM at 72 h by qRT-PCR. Compared to the control group, no induction effect and statistically different on mRNA expression levels of *tal1* and *gata1a* in treated groups, while the expression levels of *spi1b* were significantly up-regulated only at 3 μM of AM. The levels of *mpx* were up-regulated in dose dependent manner, whereas *hbae1.1* showed a significant up-regulation at 3 μM, but not at 6 μM (Figure 6).
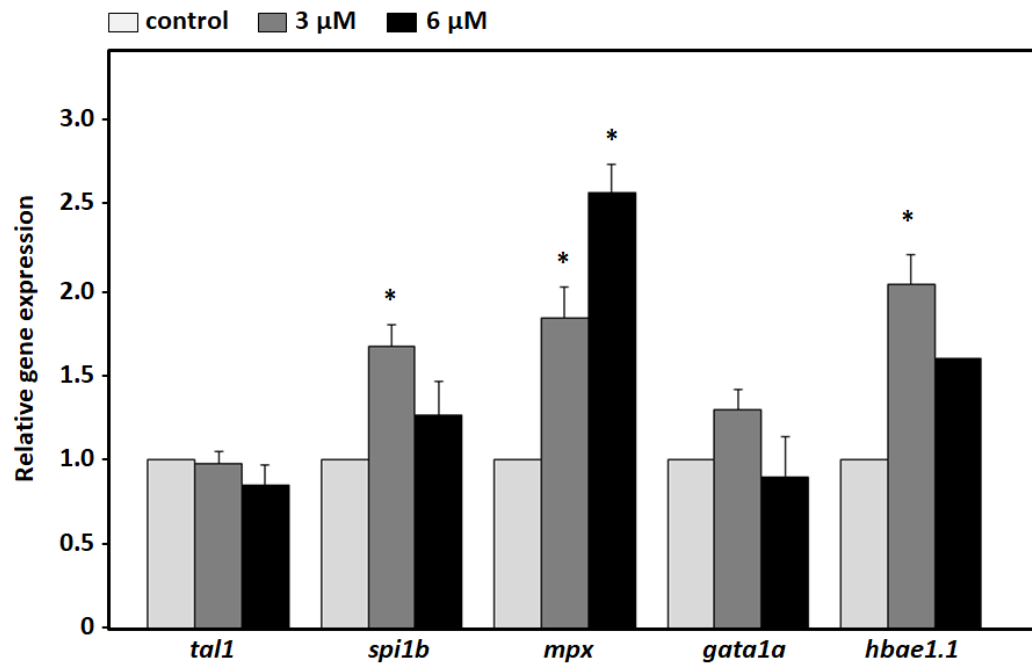
Figure 6 Relative gene expression levels of hematopoietic-related genes
of zebrafish embryos exposed to AM in several doses at 72 h. B-actin was used as a
housekeeping gene. Values are shown as ± S.E.M. of 3 independent experiments
(n=30). (*p<0.05)

4.4. The effects of AM on the expression of hematopoietic proteins

The expressions of proteins in hematopoiesis pathway were examined using western blot. At 72 h of exposure, the results demonstrated that the translation level of all genes in AM-treated groups have no statistically different from control group. Tal1 and Hbea1.1 proteins slightly dropped at the highest concentration of AM-treated. Subsequently, protein expression levels of Spi1b and Gata1a have the highest value at 3 μM while the lowest value generated at 6 μM. In the contrary, in Mpx protein at 6 μM exhibited the highest level while 3 μM showed the lowest level of protein expression, respectively, in Mpx protein (Figure 7).

Figure 7 Protein expression in zebrafish embryos exposed to AM in different concentrations at 72 h. (A) Relative protein expression levels of hematopoietic-related proteins. Gapdh was used as an internal control protein. (B) Representative images of hematopoietic-related proteins band. Values are shown as mean ± S.E.M of 3 independent experiments (n=30). (*$p<0.05$)
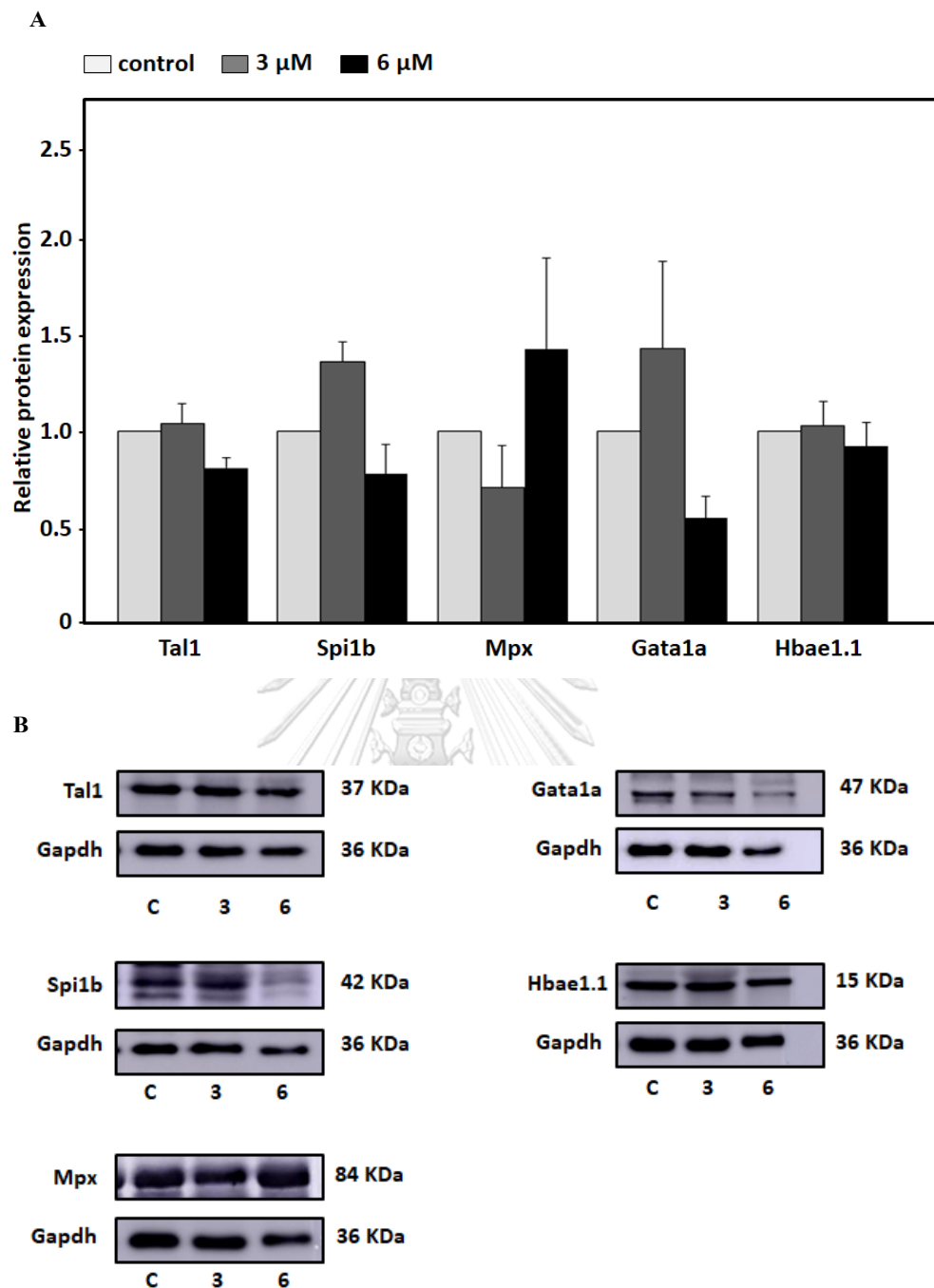
CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATION

Nowadays, natural products have been widely used among people worldwide. Some people still mainly rely on traditional medicines and use them as an alternative remedy. Unlike the modern medications, herbal substances and related products are easy to find, but some of them have no safety data especially the proper dose [99, 100]. The toxic and health effects of each natural compound must be evaluated to avoid risks. One of the outstanding natural compounds has been isolated from mangosteen, namely alpha-mangostin (AM). Many previous studies have been reported about the protective properties of AM, while the toxicity studies were available in a few reports. Furthermore, the researches often study through the abnormal-induced model for revealing the protective potential. On the other hand, the normal model has been rarely used for studying, therefore, the safety data of AM still unclear.

In this study, the effects of AM on early embryonic development and hematopoiesis were evaluated using zebrafish embryos as a vertebrate model. Zebrafish presented extraordinary advantages to murine models and a suitable alternative. For example, zebrafish embryos grow and develop rapidly with almost completely body pattern and functional organ within 48 hours post fertilization (hpf). In the chemical screening process, zebrafish can produce many eggs that supporting the number of experimental samples; therefore, the experiments can repeat multiple times to prove the result [65]. Interestingly, zebrafish had DMSO limiting dosages higher than cell culture. Hence, the chemical with poor solubility which had to dissolve in the high percentage of DMSO can be treated in zebrafish without the side effects of this solvent [97]. Considering all of the beneficial properties, in this study, zebrafish embryos were used as a model for predicting the effects of the substance. First of all, acute toxicity was tested in zebrafish embryos at the concentrations in a range of 1.5 – 12.0 µM for screening the possible effects and determination the LC_{50} value. These concentrations were designed based on the

evaluation of LC_{50} value. The data showed that AM caused embryonic mortality and malformation, with the LC_{50} of 5.76 ± 0.26 µM at 72 hpf (Figure 4A). The embryonic abnormalities included body malformation, bent tail, blood clotting, pericardial edema and yolk edema (Figure 4B). This result agreed with a previous research from Nugitrangson et al., which also found the compound induced zebrafish embryo mortality with the LC_{50} of 9.4 µM at 72 h of exposure. The difference could be owing to the different system for maintaining the zebrafish. However, they did not report about the effects on the phenotype of the embryos [101]. In this study, the concentrations of AM were used in the same range of the concentrations in pharmaceutical properties. For instance, AM inhibited the cell growth of the leukemia cell lines (K562, NB4, and U937) at 5.0 – 10.0 µM, 5.0 µM of this compound protected proximal tubule renal epithelial cells (LLC-PK1) from cisplatin CDDP-induced apoptotic death and AM also induced cell death effects on PC12 rat pheochromocytoma cells with the EC_{50} of 4 µM [102-104]. Importantly, because of the lacking selectivity of some anticancer agents, a normal cell can be destroyed during chemical treated [105]. Therefore, the effects of each compound should be parallelly tested in both normal cells and cancer cell lines. The present study also investigated the heart rate of the zebrafish embryos for assessing the effects of the compound (Figure 4B). It was also found that heart rate was significantly decreasing in the treated embryos at 4.5 and 6.0 µM. These results indicated the correlation between heart rhythm malfunction and bodies malformation. Explanation of these phenomena was reported by Phungphong et al. The cardiovascular toxicities of AM were studied in rat and rabbit models [106]. The researchers reported the inhibitory potential of AM to cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) with the IC_{50} of 6.47 ± 0.7 µM. For inhibiting SERCA, resulted in myocardial relaxation dysfunction which causing the heart malfunction. Together, the results indicated that the compound induced cardiac toxicities on zebrafish embryos [107]. Even AM has many beneficial properties; the toxic effects should not be neglected due to its toxicities to zebrafish embryos. Here, this study is useful for dosage management and safety assessment specifically in food and drug development.

Hematopoiesis is a vital process which regulating blood cells formation in every lineage. Lacking this process leads to a hematological disorder such as anemia and leukemia [78]. To study the effects on this process, zebrafish also emerged as an effective model. Zebrafish offer various features to predict the possible defects in human blood disorder especially the conserving of molecular mechanisms in hematopoiesis to human [78]. Therefore, this study took the benefit of the genetic feature of zebrafish model to demonstrate the chemical effects on hematopoiesis. Since AM induced blood clotting and decreased heart rate, it was highly possible that AM affected to blood cells in the circulatory system. To investigate the effect on hematopoiesis, hemoglobin was detected. AM decreased hemoglobin levels significantly in cardiac region at 6 μM (Figure 5) indicating that AM had the toxic effect on hematopoiesis. Hemoglobin mainly responds for transporting the gaseous molecules and maintains cellular homeostasis, therefore, decreasing hemoglobin levels resulted in inefficiency of their abilities [108]. Even though it has no previous research reporting about the effect of AM particularly in this system, Lui et al. (2015) found that AM-treated altered rat's platelets activities such as changing their shape, inhibiting aggregation and inducing cytotoxic [57]. Together, it was possible either AM decreased hemoglobin levels in erythrocytes or reduced the number of erythrocytes in zebrafish embryos. Accordingly, further studies are necessary to confirm and get in deep the underlying mechanism for keeping away from hematopoiesis toxicant. However, our results firstly pointed out the toxicity of AM to hemoglobin levels in the cardiac area of zebrafish embryos.

To get a deep insight into the mechanisms of AM on hematopoiesis, the effects of AM on the expression of hematopoietic-related genes, *tal1*, *spi1b*, *gata1a*, *mpx* and *hbae1.1*, in both transcription and translation level. It was shown that AM affected mRNA expressions (Figure 6). The first step of primitive hematopoiesis, *tal1* is a master regulatory gene [87]. The result indicated that AM did not affect HSCs, HSCs formation can be generated normally. The second step of primitive wave is conversing stem cells into the unique linage between erythroid and myeloid cell fates. At 3 μM, AM slightly increased mRNA expression of *gata1a* and also up-regulated the expression of *spi1b*, significantly. By contrast, AM decreased mRNA

expression in both *gata1a* and *spi1b* at 6 μM. This finding indicated that AM at sub-lethal concentration induced mRNA expression whereas reduced the expression of mRNA at the lethal concentration. Beside progenitor regulated genes, mature marker genes, *mpx* and *hbae1.1*, also the possible target genes of AM. Our result demonstrated that AM at 3 μM also increased the expression of *hbae1.1* and *mpx*, significantly. At 6 μM, AM markedly increased *mpx* expression but not *heae1.1*. S*pi1b* and *mpx* control primitive myelocyte generation which regarded as pathological protection. The upregulation of these myelocyte related genes might be caused by the tissue-damaging and/or inflammation.

After the study of the effects of AM on hematopoietic protein expression, AM showed no statistical difference in protein expression levels in all samples. For progenitor-related proteins, AM also slightly increased protein expression levels at 3 μM but dropped the expression of these proteins at 6 μM. The direction between transcription and translation level were likely to fluctuate in each gene. Previous studies explained the low correlation between mRNA and protein expression that can be affected by various factors such as translational regulating process and protein half-lives [109]. Another factor that might a reason for this phenomenon was the instability of Gapdh protein level. From the result of western blot assay, Gapdh expression levels were reduced in dose-dependent manner (Figure 7). Interestingly, Gapdh played a role in hematopoiesis-related processes such as hemoprotein maturation, heme regulation mechanism and participation in iron homeostasis [110]. From these evident, it was highly possible that Gapdh protein was also AM target; therefore, this protein might not be proper as an internal reference gene. However, gene expression is still a well-known marker for diseases. Transcription level is useful for screening disease-associated genes whereas translation level serves as the mechanisms determination as well as phenotype prediction [111]. Interestingly, Hbae1.1 is a mature erythrocyte marker protein that had no significantly different of its expression after treated with AM, conversely, hemoglobin molecules in the cardiac region were found to decrease their amount. It is possible that AM decreasing only the amounts of hemoglobin without the concomitant decreasing the number of erythrocytes. These consequences could be caused by erythroid heme biosynthesis

disorder. For the disorder was generally described by heme synthesis and degradation. Previous researches reported the factors that have relevance for heme syntheses such as aminolevulinate synthase, ferrochelatase and coproporphyrinogen oxidase [112]. Interestingly, Hanaoka et al. found that knock-down coproporphyrinogen oxidase (CPO) leads to inhibit the production of hemoglobin without reduction of blood cells [113]. Importantly, heme synthesis is important for globin synthesis during erythropoiesis. Disorder in this process could imply erythropoietic interruption. Although AM had no effect on Hbea1.1 protein, there are other mechanisms that associated in erythropoietic regulation. Not only the intrinsic factors, transcription factors, but extrinsic factors like erythropoietin also a key regulation in erythropoiesis. Erythropoietin is a glycoprotein playing a role as an extrinsic factor in erythropoiesis. Knockdown erythropoietin could impair erythropoiesis leading to sever anemia, mortality and tissue hypoxia [114]. However, the effects of AM on hemoglobin regulation have not been reported. Herein, the present study had firstly demonstrated the effects of AM on hemoglobin levels. In summary, it is indicated that AM affected hematopoietic-related genes only in transcription level, by contrast, the translation level can retain its function. For further study, the roles of extrinsic factors and heme biosynthesis pathway are interesting in order to get the mechanism in deeper.

In conclusion, AM affected to hematopoiesis in zebrafish embryos and induced acute toxicities which possible triggered the inflammatory response and led to up-regulation of myeloid regulated genes. In addition, AM reduced hemoglobin levels in cardiac areas without affecting erythroid marker protein. Finally, this study firstly reported the effects of AM on embryonic hematopoiesis in the animal model. All together, we should be aware of the possible risks using AM. The toxicity profiling of AM must be studied in other models before using the compound for clinical trials [80].

For recommendations, in order to develop AM using in clinical trials, dosage management is really important. The outcomes of this research showed the concentration greater than 3 µM of AM inducing the mortalities, malformations as

well as hematopoiesis malfunction. Therefore, the people who have a problem with the hematopoietic system should carefully use AM.

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APPENDICES

จุฬาลงกรณ์มหาวิทยาลัย
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APPENDIX A

1. Materials

1.1 Chemicals

2-Mercaptoethanol (C_2H_6OS, M.W. 78.13), Sigma-Aldrich, USA

3-Amino benzoic acid ethyl ester ($C_9H_{11}NO_2$, M.W. 165.19), Sigma-Aldrich, USA

30% Acrylamide solution (C_3H_5NO, M.W. 71.08), PanReac Applichem, Germany

30% Hydrogen peroxide (H_2O_2, M.W. 34.01), Merck, USA

Alpha-mangostin ($C_{24}H_{26}O_6$, M.W. 410.46), catalog # 6147-11-1, Sigma-Aldrich, USA

Ammonium persulphate (($NH_4)_2S_2O_8$, M.W. 228.20), Sigma-Aldrich, USA

Antibody, Abcam, UK

      Anti-gapdh antibody [EPR16891], catalog # ab181602

      Anti-gata1 antibody, catalog # ab85466

      Anti-hemoglobin subunit alpha antibody [EPR3608], catalog # ab92492

      Anti-myeloperoxidase antibody, catalog # ab210563

      Anti-pu.1/spi1 antibody, catalog # ab209983

      Anti-tal1 antibody, catalog # ab172144

      Goat anti-mouse IgG H&L (HRP), catalog # ab6789

      Goat anti-rabbit IgG H&L (HRP), catalog # ab6721

Ammonium persulfate (($NH_4)_2S_2O_8$, M.W. 228.18), Sigma-Aldrich, USA

Bovine serum albumin, Sigma-Aldrich, USA

Bromophenol blue ($C_{19}H_{10}Br_4O_5S$, M.W. 669.96), Sigma-Aldrich, USA

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, M.W. 147.01), Sigma-Aldrich, USA

Dimethyl sulfoxide (C_2H_6OS, M.W. 78.13), VER Life Science, USA

Ethanol (C_2H_6O, M.W. 46.07), Merck, USA

ECL chemiluminescence reagent, catalog # RPN2209, GE Healthcare, USA

Ethylene diamine tetraacetic acid, ($C_{10}H_{16}N_2O_8$, M.W. 292.24), Sigma-Aldrich, USA

Glycerol ($C_3H_8O_3$, M.W. 92.09), Sigma-Aldrich, USA

Glycine ($C_2H_5NO_2$, M.W. 75.07), Biorad, USA

Hydrochloric acid (HCl, M.W. 36.46), Merck, USA

IScript reverse transcription supermix for RT-qPCR, catalog # 1708841, Biorad, USA

Magnesium sulfate (MgSO$_4$, M.W. 120.37), PanReac Applichem, Germany

Methanol (CH$_3$OH, M.W. 32.04), Fisher Chemical, USA

Methyl cellulose, catalog # 9004-67-5, Sigma-Aldrich, USA

N,N,N',N'-tetramethylethylenediamine (C$_6$H$_{16}$N$_2$, M.W. 116.24), PanReac Applichem, Germany

N-Phenylthiourea (C$_7$H$_8$N$_2$S, M.W. 152.22), Sigma-Aldrich, USA

Nonidet P-40, catalog # 9016-45-9, Sigma-Aldrich, USA

o-Dianisidine (C$_{14}$H$_{16}$N$_2$O$_2$, M.W. 244.29), Sigma-Aldrich, USA

Paraformaldehyde (CH$_4$O$_2$, M.W. 48.0), Sigma-Aldrich, USA

Pierce BCA protein assay kit, catalog # 23227, Thermo Scientific, USA

Ponceau S solution (C$_{22}$H$_{12}$N$_4$Na$_4$O$_{13}$S$_4$, M.W. 760.57), catalog# 6226-7905, Sigma-Aldrich, USA

Potassium chloride (KCl, M.W. 74.55), Merck, USA

Precious plus protein dual color standard, catalog # 1610374, Biorad, USA

PrimePCR SYBR green assay for zebrafish, Biorad, USA

> *gapdh*, assay id: qDreCED0021000
>
> *gata1*, assay id: qDreCID0013676
>
> *mpx*, assay id: qDreCID0017849
>
> *alpha-globin*, assay id: qDreCID0018899
>
> *pu.1*, assay id: qDreCID0004109
>
> *tal1*, assay id: qDreCID004320

Protease inhibitors cocktails, catalog # P8340, Sigma-Aldrich, USA

Sodium acetate (C$_2$H$_3$NaO$_2$, M.W. 82.03), Sigma-Aldrich, USA

Sodium chloride (NaCl, M.W. 58.44), Merck, USA

Sodium deoxycholate (C$_{24}$H$_{40}$O$_4$, M.W. 392.57), Sigma-Aldrich, USA

Sodium dodecyl sulphate (NaC$_{12}$H$_{25}$SO$_4$, M.W. 288.37), Biorad, USA

Ssoadvanced universal SYBR green supermix, catalog # 172-5271, Biorad, USA

Tris (C$_4$H$_{11}$NO$_3$, M.W. 121.14), Biorad, USA

Triton X-100, catalog # 9002-93-1, Sigma-Aldrich, USA

Tween-20, catalog # 1706531, Biorad, USA

RNeasy plus mini kit, catalog # 74134, Qiagen, Germany

Ultra-pure distilled (DI) H_2O, catalog # 10977015, Invitrogen, USA

1.2 Instruments

Auto pipettes (0.1–2.5, 0.5–10.0, 2.0–20.0, 10.0–100.0, 20.0–200.0, and 100.0–1,000.0 µL), Eppendorf, Germany

Centrifuge tube (15 and 50 mL), Corning Incorporated, USA

Electrophoresis transfer cell, Mini –PROTEAN Tetra cell 2-Gel system, catalog # 1658005, Biorad, USA

Extra thick blot paper, catalog # 1703966, Biorad, USA

Fish hatchery box, AG global sourcing solutions, Philippines

Gel documentation, Amersham imager 600, GE Healthcare, USA

Glassware (Beaker, volumetric cylinder, and glass bottle), Schott Duran, Germany

Hard shell PCR plate, catalog # hss9601, Biorad, USA

High speed homogenizer, PRO-200, Sonics and Materials, USA

High speed refrigerated micro centrifuge, MX-305, Tomy Digital Biology, Japan

Immun-blot polyvinylidene difluoride, catalog # 1620177, Biorad, USA

Incubator, Metrology Technical, Thailand

Microplate reader, Spextramax M5, Molecular Devices, USA

Micro tube (0.2, 0.6, 1.5, and 2.0 mL), Axygen Scientific, USA

Mini centrifuge, MC-6, A and E Lab, UK

Mini shaker, PSU-2T, Biosan Laboratory, USA

Nanodrop, 2000/2000c spectrophotometer, Thermo Scientific, USA

PCR plate sealing film, catalog # MSB1001, Biorad, USA

Pipette tips (0.5–10.0, 1.0–100.0, 1.0–200.0, and 1.0-1,000.0 µL), Axygen Scientific, USA

Petri dishes, Corning Incorporated, USA

Power supply, Powerpac HC, Biorad, USA

Real time PCR detection system, CFX96 Touch, Biorad, USA

Rocker shaker, MR-12, Biosan Laboratory, USA

Semidry transfer cell, catalog # 170-3940, Biorad, USA

Stereo microscopes, S7X7 and SZX16, Olympus, Japan

Tweezers, Electron Microscopy Sciences, USA

Transfer pipette, Biologix, USA

Thermal cycler, T100, Biorad, USA

Thermo mixer, Thermomixer R Mixer, Eppendorf, Germany

Vortex, SI-0236, Scientific Industries, USA

Well plate, Corning Incorporated, USA

Western blot equipment (casting frames, combs, 0.75 mm. shot plates and spacer plates), Biorad, USA

Zebrafish housing system AB-074, Yakos65, Taiwan

APPENDIX B

Reagents for preparing western blot assay

1. Lysis buffer (100 mL)

5 M Sodium chloride	3.0 mL
0.5 M Ethylene diamine tetraacetic acid, pH 8.0	1.0 mL
1 M Tris, pH 8.0	5.0 mL
Nonidet P-40	1.0 mL
10% Sodium deoxycholate	5.0 mL
10% Sodium dodecyl sulphate	1.0 mL
Deionized water	84.0 mL

After mixing all ingredients, 1% protease inhibitor cocktail was freshly added before using.

2. 2X SDS sample buffer (10 mL)

1 M Tris-HCl, pH 6.8	1.0 mL
10% Sodium dodecyl sulphate	4.0 mL
Glycerol	2.0 mL
2-Merceptoethanol	2.5 mL
1% Bromophenol blue	0.5 mL

3. SDS-polyacrylamide gel electrophoresis

3.1 12% Separating gel (15 mL)

30% Acrylamide solution	6.0 mL
1.5 M Tris, pH 8.8	3.9 mL
10% Sodium dodecyl sulphate	0.15 mL
Deionized water	4.8 mL
10% Ammonium persulphate (APS)	0.15 mL
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.015 ml

TEMED and APS were lately added before pouring the gel because the gel solution will be polymerized quickly after adding.

3.2 3% Stacking gel

30% Acrylamide solution	0.804 mL
0.5 M Tris, pH 6.6	3.9 mL
10% Sodium dodecyl sulphate	0.60 mL
Deionized water	3.57 mL
10% Ammonium persulphate (APS)	0.60 mL
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.006 mL

4. 10x Electrophoresis buffer (1,000 mL)

Tris	24.4 g
Glycine	114.7 g
Sodium dodecyl sulphate	8.0 g
Deionized water to 1,000 mL	

5. 10x Tris buffer saline with 0.1% tween (1,000 mL)

Tris	30.0 g
Sodium chloride	88.0 g
Potassium chloride	0.20 g
Tween-20	1.0 mL
Deionized water to 1,000 mL	

6. Transblotting buffer (1,000 mL)

Tris	3.03 g
Glycine	14.4 g
10% Sodium dodecyl sulphate	10.0 mL
Methanol	200.0 mL
Deionized water to 1,000 mL	

APPENDIX C

Bovine serum albumin standard curve was performed to determine protein concentration.

$$y = 0.9791 + 0.0379$$
$$R^2 = 0.9964$$
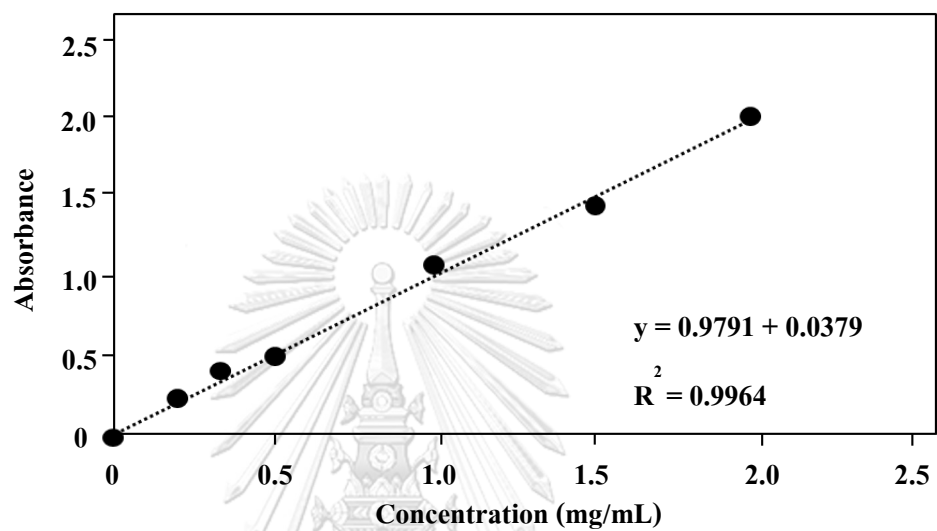
APPENDIX D

Malformation rate of zebrafish embryos after treated with AM for 72 h.

Toxic effects	control	1.5 µM	3.0 µM	4.5 µM	6.0 µM
Tail malformation	0	1.19	1.19	77.51	94.87
Yolk edema	0	2.38	0	53.41	93.73
Blood clotting	0	0	0	7.99	38.12
Pericardial edema	0	0	0	7.11	23.12

จุฬาลงกรณ์มหาวิทยาลัย

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AWARD RECEIVED   -

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