



Toxicity of *Anacardium occidentale* shoots (Pucuk Gajus) Extract on Zebrafish Embryos

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Abstract

The use of various medicinal plant to remedy specific disease has become more popular and grown intensively due to the preference for using medicinal plant instead of synthetic drugs. However, it is vital to know the safe dose limit of natural products consumption. In this present study, the medicinal plant such as *Anacardium occidentale* (cashew) shoots which is commonly consumed as *ulam* or as a salad in some Asian countries such as Malaysia and Indonesia was used. This study aims to evaluate the antioxidant capacity of the cashew shoots by using 70% methanolic:water and to determine its toxicity effect on the *Danio rerio* (zebrafish) embryos. The antioxidant capacity was calculated in IC₅₀ value where the *A. occidentale* methanolic extract was found to have higher antioxidant activity (IC₅₀ = 4.02 ± 0.02 µg/mL) as compared to ascorbic acid (7.44 ± 0.02 µg/mL) (p<0.05). At higher concentration of *A. occidentale* (9.802 µg/mL), it showed the toxicity effects on the mortality, hatchability, and several deformations (spinal curve, heart complication, and the presence of oedema). Therefore, it is recommended to use lower concentration of extract to prevent toxicity.

Keywords: *A. occidentale*, *Danio rerio* (zebrafish) embryos, antioxidants

Abstrak

Penggunaan pelbagai tumbuhan berasaskan perubatan untuk merawat penyakit tertentu telah menjadi popular dan berkembang secara intensif disebabkan meningkatnya pilihan untuk menggunakan tumbuhan perubatan berbanding ubat sintetik. Walau bagaimanapun, adalah penting untuk mengetahui had dos selamat penggunaan produk semulajadi tersebut. Dalam kajian ini, tumbuhan perubatan seperti pucuk *Anacardium occidentale* (jambu gajus) yang biasanya dimakan sebagai ulam atau salad di beberapa negara Asia seperti Malaysia dan Indonesia telah digunakan. Kajian ini bertujuan untuk menilai kapasiti antioksidan pucuk jambu gajus dengan menggunakan campuran metanol 70%:air dan untuk menentukan kesan toksiknya terhadap embrio *Danio rerio* (zebrafish). Kapasiti antioksidan dihitung dalam nilai IC₅₀ di mana ekstrak metanol *A. occidentale* didapati mempunyai aktiviti antioksidan yang lebih tinggi (IC₅₀ = 4.02 ± 0.02 µg/mL) berbanding asid askorbik (7.44 ± 0.02 µg/mL) (p<0.05). Pada kepekatan tinggi *A. occidentale* (9.802 µg/mL), ia menunjukkan kesan toksik terhadap kematian, kebolehan menetas, dan beberapa deformasi (lekukan tulang belakang, komplikasi jantung, dan kehadiran edema). Oleh itu, disyorkan untuk menggunakan kepekatan ekstrak yang lebih rendah untuk mencegah toksisiti terhadap sel.

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Kata kunci: *A. occidentale*, *Danio rerio* (zebrafish) embrio, antioksidan

Introduction

Anacardium occidentale (*A. occidentale*) leaves or cashew shoot is one of the herbal plants that are popular among Southeast Asians. People consume *A. occidentale* shoots as a remedy for certain diseases. For instance, in southeast Asian countries, particularly in Malaysia, *A. occidentale* leaves are consumed raw (ulam) since this herb is known to have many benefits to our health. These leaves are known to have antioxidant and anti-inflammatory properties (Souza et al 2017). Indonesian have use its shoots as an antiseptic due to the presence of the ethanolic compound found in the leaves (Jingga et al., 2019).

Zebrafish or *Danio rerio* (**Figure 1**) is a freshwater fish that is natively found in Asia such as India, Pakistan, Bangladesh, and Nepal. The male zebrafish is shaped like a torpedo with a golden- blue stripe across the

body from the head to the tail. Meanwhile, the female zebrafish appears to have a giant abdomen and silver-blue stripes (Zeng, 2017). The female has a big abdomen and is fatter than the male because they carry eggs inside. The lifespan for zebrafish is around 2-3 years, and they can grow up to 6.4 cm (2.5 in) in length. The zebrafish among other fishes, that are integrated in the internationally accepted OECD Guidelines to assess systemic toxicity in fish, i.e., *The Testing of Chemicals with the Fish Acute Toxicity Test* (OECD 203) and *The Fish Embryo Acute Toxicity Test* (OECD 236). Currently the European Commission Directive 2010/63/EU, permits experimentation in fish embryos at earliest life stages without being regulated as animal experiments.



Figure 1: An adult female (A) and male (B) zebrafish (*Danio rerio*) at 3-month-old

Based on the statistical evaluation by National Pharmaceutical Control Bureau and the Health Ministry's Malaysian Adverse Drug Reaction Advisory Committee in 2013, approximately 11,437 cases were caused by the drug reactions, and 0.2% of the cases were due to the over consumption of the medicinal or herbal plants (Chadardehi et al. 2020). Study done by Anaziah et al. (2023) have shown that no toxic effect exerted on the kidney, liver and small intestine of the wistar rat. In the present study, the embryo toxicity test was done to determine the safe dose to be consumed by human. *Danio rerio* or zebrafish embryo was used as an animal model to test the toxicity of *A. occidentale*. However, the toxicity studies of the cashew shoots (*A. occidentale*) on the zebrafish embryos are still lacking. Hence, thorough toxicity studies need to be done to evaluate and determine the toxicity of *A. occidentale* using zebrafish embryos.

Materials and Methods

Collection of sample and plant extract preparation

The fresh shoots of *A. occidentale* were purchased at the local market in Temerloh, Pahang. The healthy, fresh, brownish green colour shoots were selected as samples. The selected shoots were washed thoroughly under running tap water to remove the dirt. Then, it was allowed to dry at room temperature before being weighed to obtain the initial weight of the shoots. Then, the shoots were packed into the zipper bag and ready to be freeze dried.

Extraction

The powdered leaves were extracted by using maceration method, where 150g of the powder shoots were added into the 70% (v/v, methanol/water). The extract was macerated for 24 hours in the shaker (100rpm, 25°C). After 24 hours, the extract was filtered by using a cheesecloth and centrifuged at

5000rpm for 5 minutes at 25°C. The methanol in the extract was removed by using the rotary evaporator, and the remaining solution was freeze-dried again to remove excess water since 70% methanol was used in this experiment. After three to four days, the freeze-dried process was completed, the sample was weighed to obtain the yield of the extraction. The yield of extraction was calculated by using the following equation:

$$\text{Yield of extraction (\%, w/w)} = \frac{Wt1}{Wt2} \times 100\%$$

Wt1 and Wt2 represented the final weight of the dried extract and the primary weight of the leaf powder, respectively (Murugesu et al., 2019).

DPPH Radical scavenging Assay

The DPPH scavenging activity assay was conducted following the method mentioned in Fadhli et al., (2019) with a slight modification, where, 11 concentration of standards were used instead of six standards. The ascorbic acid was used as a positive control in this experiment. Ninety-six well microplates with a flat bottom were used in this analysis. The serial dilution was done where 50µl of 1000 µg/mL sample was pipetted into the microplates. Approximately, 80µl of DPPH was added into all the well containing sample and covered with aluminium foil. After 30mins of incubating, the microplate reader was measured at the absorbance of 517nm. The following equation was used to calculate the

DPPH scavenging activity (%):

$$\text{DPPH Scavenging Activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

A_{sample} represent absorbance of the sample or the positive control and A_{control} represent the absorbance of the negative control or blank (Burlingham & Widlanski, 2003).

Toxicity assessments using zebrafish embryos

The toxicity test followed the guideline by the Organization for Economic Co-Operation and Development (OECD) to observe the toxicity effect of *A. occidentale* towards the zebrafish embryos (OECD, 2013). The breeding process was done in the Centre of Research Equipment and Management (CREAM), IIUM Kuantan prior to the collection of the embryo. According to the Fish Embryo Acute Toxicity guideline by OECD 2013, the 24-well plate was used as a medium to place the embryos up until 96 hours of post fertilization. After the breeding process, the embryos was transferred into the mini aquarium before being observed under the microscope for further selection. Embryos with the following criteria were selected and transferred into the well (healthy and have the same development stage of embryos). One embryo was allocated for one well.

Microscopic evaluation

The lethality of the embryo was observed every 24 hours for 4 days (24hpf, 48hpf,

72hpf and 96hpf). The lethality observed were based on the OECD guidelines where it stated that four parameters indicate the lethality which were lack of somite formation, coagulation, non-detachment of the tail and lack of heartbeat. All of these parameters were observed using the Danioscope software (Noldus Information Technology, Wageningen, The Netherlands). After 96 hours, the median lethal concentration (LC_{50}) were calculated based on the number of lethality by using the Probit analysis (Pamanji et al., 2015). The SPSS software was used to calculate the Probit value. The following equation was used to calculate the rates of mortality:

Rate of mortality (%) =

$$\frac{\text{Number of dead embryos}}{\text{Initial number of embryos}} \times 100$$

Results and discussion

Antioxidant activity assay

The antioxidant scavenging capacities for the methanolic extract of *A. occidentale* are shown in **Figure 2** below. Methanolic extract of *A. occidentale* leaves exhibited high potential antioxidant activity where the extract showed a lower IC_{50} value which was 7.21 $\mu\text{g/mL}$ as compared to ascorbic acid (7.44 $\mu\text{g/mL}$) as shown in **Table 1**.

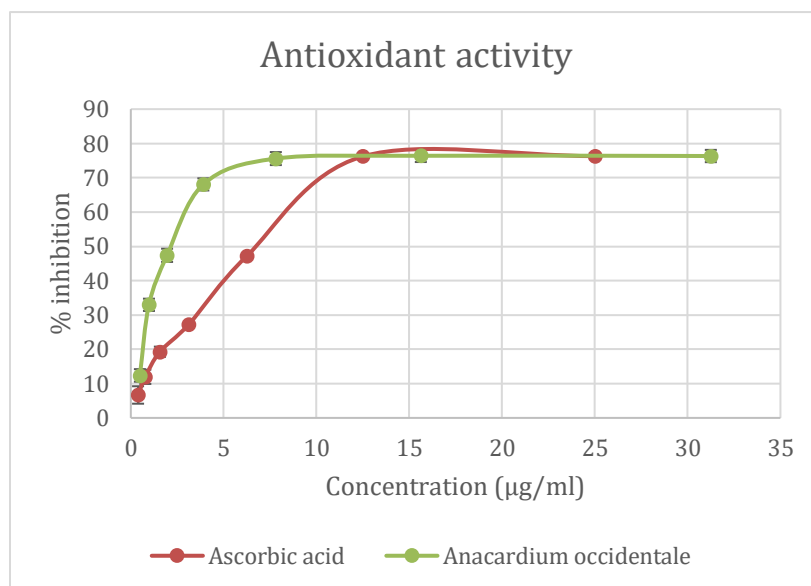


Figure 2: Percentage of inhibition for *A. occidentale* methanolic extract in comparison to ascorbic acid standard

Based on **Figure 2**, the concentration of *A. occidentale* was directly proportional to the percentage of inhibition until all of the free radicals were taken up and caused the graph to be constant at percentage inhibition of 70%. *A. occidentale* methanolic extract showed lower IC_{50} value ($4.02 \pm 0.02 \mu\text{g/mL}$) which was about two times lower than the IC_{50} value of ascorbic acid ($7.44 \pm 0.02 \mu\text{g/mL}$) ($p < 0.05$). This result could be due to the presence of large amount of flavonoid and phenolic compound that can be found in the

A. occidentale leaf extract (Sassi et al. 2022). In the previous study conducted by Souza et al., (2017), they mentioned that, *A. occidentale* leaf contains a high vitamin C level, about five times that of oranges, as well as a high mineral value. This is due to the potent antioxidant compound found in the leaf such as tannin, saponin and alkaloids. The IC_{50} values for both methanolic extract of *A. occidentale* and ascorbic acid were shown in **Table 1** below.

Table 1: The half maximal inhibitory concentration (IC₅₀) of *A. occidentale* shoots and ascorbic acid

Sample	DPPH IC ₅₀ (µg/mL)
<i>A. occidentale</i> methanolic extract	4.02 ± 0.02*
Ascorbic acid standard	7.44 ± 0.02

p<0.05 (significant difference)

Toxicity assessment of *Anacardium occidentale* using Zebrafish embryos

Lethal concentration dose (LC₅₀)

Lethal concentration dose (LC₅₀) was calculated using probit analysis by quantifying the quantity of toxicant (mg) per body weight (kg) which necessary to kill 50%

of the population of the animal examined (**Figure 3**). The LC₅₀ value obtained for the *A. occidentale* extract was 9.802 µg/mL which considered the extract to be very toxic at that concentration. According to Nguta et al. (2012), any compound that have less than 1000mg/L of LC₅₀ value was considered toxic.

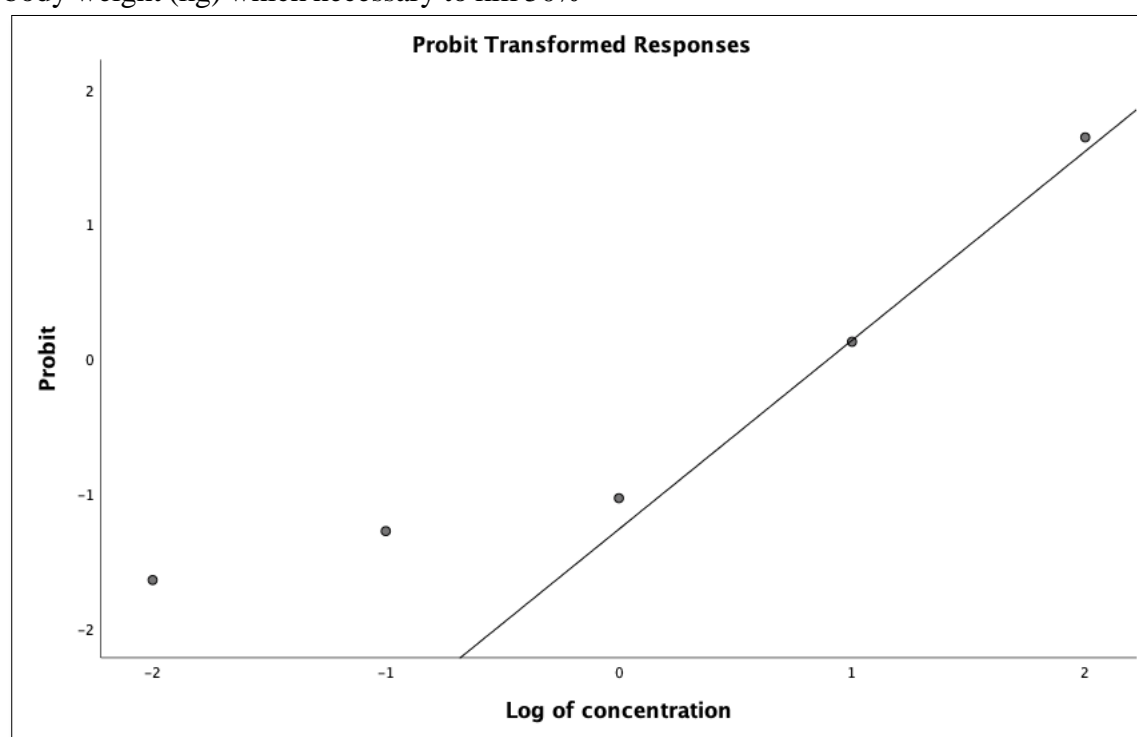


Figure 3: Lethal concentration doses (LC₅₀) value of *A. occidentale* based on probit analysis calculation

The morphological defect on Zebrafish embryos

A control group using the E3 medium, positive control using 3,4-dichloroaniline and six different doses of *Anacardium*

occidentale extract were observed for the toxicity effects by using Danioscope evaluation for its morphological defects as shown in **Figure 4**.

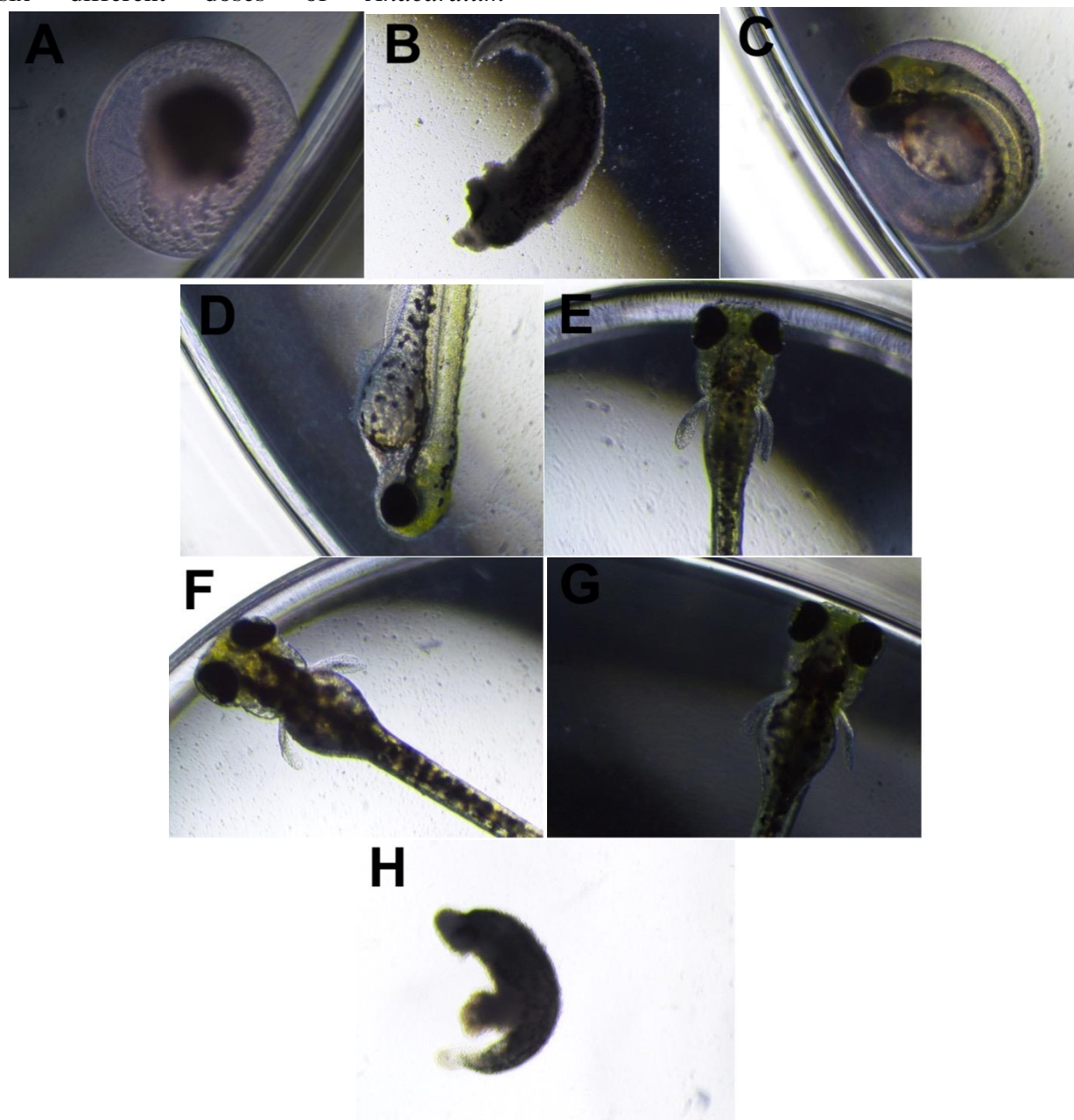


Figure 4: Morphological observation of the zebrafish embryos treated with different concentration of *A. occidentale*; A: 1000 µg/mL, B: 100 µg/mL, C: 10 µg/mL, D: 1 µg/mL, E: 0.1 µg/mL, F: 0.01 µg/mL, G: negative control (E3 medium) and H: positive control (3,4-dichloroaniline) at 72 hpf

The teratogenic defects and deformations of varying concentrations of *A. occidentale* leaves at 72 hpf in *D. rerio* larvae

Deformation of the zebrafish embryos was a common phenomena when treated with high doses of extract. Deformities such as

malformation, crocked backbone, heart complication and oedema of the embryos can be seen in **Figure 5**. All of these deformities may be cause by the high doses and the positive control administrated into the embryos.

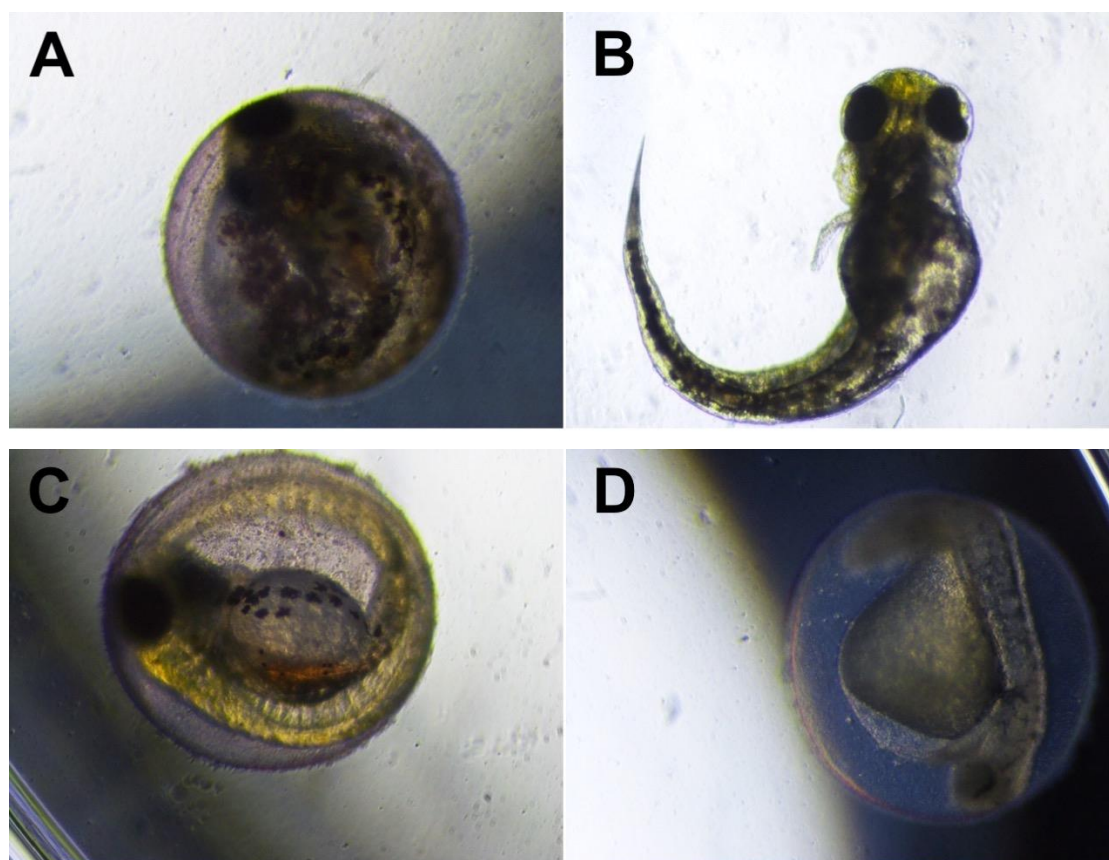


Figure 5: Deformation of *D. rerio* embryos towards *A. occidentale* extract (A: malformation, B: crocked backbone, C: heart complication, D: oedema)

The teratogenicity characteristics reported for normal, and embryos treated with different doses of *A. occidentale* extract at 72 hpf are shown in **Table 2** below. Growth retardation is an essential teratogenicity test parameter. Embryos with the lowest dosages (0.01 $\mu\text{g/mL}$) were absence of all mentioned

criteria. A large percentage of embryos in the 100 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$ concentrations were discovered dead. Embryo tested with 10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ portrayed some embryos having twisted backbones (scoliosis), heart complication and oedema.

Table 2: Observation of delayed hatch, scoliosis, oedema, and heart complication of *D. rerio* towards *A. occidentale* extract

Concentration (µg/mL)	Delayed hatch	Scoliosis	Oedema	Heart complication
0.01	-	-	-	-
0.1	-	-	-	-
1	-	-	-	-
10	/	/	/	/
100	/	-	-	-
1000	-	-	-	-
Positive control	-	-	/	-
Negative control	-	-	-	-

Mortality of zebrafish embryo

Coagulation and the absence of a heartbeat in zebrafish embryos were indicators of death (Romagosa et al., 2016). **Table 3** shows the mortality rate of *D. rerio* that treated with different concentration of *A. occidentale* extracts. At 96 hpf, the treatment with the lowest dosage of the plant extract saw 5% mortality. In contrast, the embryos treated with the maximum dosage of 1000 µg/mL

had 100% mortality rate at 24 hpf and on the following days of treatment (48 hpf , 72 and 96 hpf). This concluded that the concentration of 1000 µg/mL of sample was considered as toxic to the embryos. Plant concentration of 10 µg/mL has slightly over 50% of mortality which indicated that the LC₅₀ estimation must be between 1 µg/mL to 10 µg/mL.

Table 3: Mortality rate (%) at 0hpf to 96hpf of the *D. rerio* embryos treated with different concentration of *A. occidentale*

CONCENTRATION (µg/mL)	0hpf	24hpf	48hpf	72hpf	96hpf
1000	0	100	100	100	100
100	0	15	15	75	95
10	0	10	10	55	55
1	0	5	5	5	15
0.1	0	5	5	10	10
0.01	0	0	0	5	5
Negative control	0	0	5	5	5
Positive control	0	85	100	100	100

Hatchability of zebrafish embryo

Hatching denotes the effective development of the embryo into larvae, which happens between 48 and 72 hours after fertilisation. However, as the concentration of the *A. occidentale* extract increased, the hatchability

of the embryos was lower than that of the control. **Table 4** below shows the hatchability in % for normal embryos and embryos that treated with various doses at 0hpf, 24hpf, 48hpf, 72 hpf and 96hpf. The hatchability of the embryos was found to be normal and

complete in both the negative control and the groups treated with the two lowest plant concentration of 0.1 µg/mL and 0.01 µg/mL . The hatching of zebrafish embryos after 48

hours of development indicated successful embryonic development (Parichy et al., 2011).

Table 4: Hatchability rate (%) at 0hpf to 96hpf of the *D. rerio* embryos treated with different concentration of *A. occidentale*

CONCENTRATION (µg/mL)	0hpf	24hpf	48hpf	72hpf	96hpf
1000	0	0	0	0	0
100	0	0	0	0	0
10	0	0	0	65 (13/20)	65
1	0	0	0	90 (19/20)	90
0.1	0	0	0	100 (20/20)	100
0.01	0	0	5	100 (20/20)	100
Negative control	0	0	0	95 (19/20)	100
Positive control	0	0	0	0	0

Lange and colleagues (1995) previously examined the teratogenicity of substances by utilising the zebra fish embryo versus cell line (RTG-2). In most situations, the zebrafish embryo outperformed adult zebrafish. They observed that the embryos were more sensitive and properly indicated acute toxicity than the RTG-2 cytotoxicity test.

The low LC₅₀ value of the plant extract indicated that this plant fraction was harmful to zebrafish embryos. Some morphological alterations were detected in zebrafish treated with plant concentrations close to the LC₅₀ value of the listed in the results section. In this work, embryos exposed to various concentrations of plant extract and had numerous morphological defects during development in a concentration and time dependent way. Some deformation such as the deformation of the embryo spine known as scoliosis, heart complication and the oedema can be observed during 72 hpf. Oedema can be categorized as a deformation yolk sac with buildup of bodily fluid that disrupts the usual structure of the

yolk mass in the hatched larvae of this group (Murugesu, 2018).

The varied concentrations of the plant extract influenced embryo hatchability, whereas increasing the extract concentration induced decreased hatchability. The reduced of hatchability and delayed hatching process indicated the development embryos was slowed. The delayed hatching might be related to developmental anomalies in the growth of zebrafish embryos, resulting in the inability to break off the chorion, and could also be explained by morphological abnormalities detected in embryos that limit hatching. The rate of hatching in zebrafish embryos is irregular at higher doses of Hfr, which might be due to a weaker chorionic membrane or an increase in chorionase enzyme activity caused by the treatment (Murugesu, 2018).

The increase of plant extract concentration causes some deformities which are the increasing size of oedema in embryos and spinal curvature. The presence of oedema may be due to the failure of osmoregulatory system that associated with toxicant in the

plant extract. Meanwhile, the spinal curvature may be due to the lack of neuromuscular coordination. All of these defects could be occur due to the decreasing amounts of collagen in the spine cause by higher concentration of plant extract.

Although (Tédong et al., 2007) in their study found that *A. occidentale* hexane extract is non-toxic when administered orally up to a maximum dosage of 14 g/kg to the mice, however, the *A. occidentale* showed toxicity effect to the zebrafish embryos. This may be due to the early juvenile life stage of the zebrafish embryos that makes them fragile and extremely sensitive to the extract (Mu et al., 2015). The LC₅₀ of the *A. occidentale* may be different when a larger animal model such as mice was used.

Apparently, the varied concentrations of the *A. occidentale* extract influenced embryo hatchability, whereas increasing the extract concentration induced decreased hatchability. The delayed hatching might be related to developmental anomalies in the growth of zebrafish embryos, resulting in the inability to break off the chorion, and could also be explained by morphological abnormalities detected in embryos that limit the hatching process.

The secondary metabolites compound of *A. occidentale* is also one of the possibilities that make it toxic to the zebrafish embryos. Secondary metabolites such as phenols poses high chance of giving toxicity effect to the embryos. According to Downs & Wills (2019), even though phenol was important for its antimicrobial and therapeutic properties, phenol was also found to be a protoplasmic toxin with a wide range of toxicity effects. Its combined hydrophilic

and lipophilic qualities allow it to easily penetrate cellular membranes, denaturing proteins along the way and eventually leading to cell death and necrosis. A caustic effect can also occur, resulting in coagulation necrosis. Phenol spreads widely and, due to its high toxicity, it has pathologic consequences in nearly all organ systems. This can be seen in several observation of the embryo where the embryo was coagulated when high dose of *A. occidentale* was administrated.

Conclusion

It can be concluded that, eventhough the *A. occidentale* shoots extract showed a high antioxidant capacity, however, this extract was toxic to the zebrafish embryo at 9.802 µg/mL concentration. The most significant toxicity effect was seen at concentration higher than 10 µg/mL where some major deformities were observed such as the coagulation of the embryo, spinal curvature, heart complication, hatchability of the embryos and the oedema formation. Some improvement can be done in further research studies such as the identification of bioactive compound of *A. occidentale* may be further discuss to understanding the underlying reason of the toxicity effect. The concentration range of the *A. occidentale* for toxicity test can be reduced. Thus, the IC₅₀ calculation can be more reliable.

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