



Antioxidant effect of aqueous *Achyranthes aspera* L. extract on hydrogen peroxide (H₂O₂)-induced zebrafish Model

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ABSTRACT: Oxidative stress (OS) is caused by free radicals such as reactive oxygen species (ROS) in the human body. An excessive quantity of hydrogen peroxide (H₂O₂) would increase the levels of ROS, resulting in lipid peroxidation (LPO) and oxidative stress that can lead to further oxidative damage. Extract of *Achyranthes aspera* L. possesses well-documented antioxidant properties. The objective of the study was to the therapeutic value of *A. aspera* extract as a natural antioxidant in zebrafish. In this study, the H₂O₂ lethal dose (LD₅₀) and *A. aspera* extract lethal concentration (LC₅₀) on Zebrafish were determined. The results showed that the LD₅₀ of H₂O₂ was 0.639 mM and *A. aspera* extract LC₅₀ was 1110 µg/ml. Then, zebrafish adults were exposed to 0.639 mM H₂O₂ and treated with 1110 µg/ml *A. aspera* extract by co-induction of both on Zebrafish for 24 hours. Malondialdehyde (MDA) assay was performed to determine lipid peroxidation that occurred in the zebrafish. It was revealed that the *A. aspera* extract-treated group had substantially lower MDA levels than the negative control and normal groups, indicating a reduction in lipid peroxidation. The aqueous extract of *A. aspera* outperformed ascorbic acid as an antioxidant and also had beneficial therapeutic effects on the heart and brain of zebrafish.

Keywords: *Achyranthes aspera* L., antioxidant, hydrogen peroxide, MDA assay,



INTRODUCTION

Oxidative stress (OS) is generally an imbalance between the production of free radicals such as reactive oxygen species (ROS) and the capability of the body cells to neutralize or detoxify the damaging effects of these radicals (Pizzino *et al.*, 2017). Overproduction of these radicals can further cause oxidative damage, therefore leading to numerous pathological conditions, illnesses, and disorders (Pham-Huy *et al.* 2008). Superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), ascorbic acid, and tocopherol are the main antioxidant agents which play an important role in cell protection, due to their ability to eliminate free radicals (Zampelas & Micha, 2015). The most abundant free radicals related to human diseases are hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide (Lobo *et al.*, 2010). Hydrogen peroxide (H₂O₂) is a powerful oxidizing agent which can oxidize and destroy proteins, lipids, and DNA, thus resulting in oxidative damage (Phaniendra *et al.*, 2015). Besides that, H₂O₂ is widely utilized as a model chemical to examine *in vivo* and *in vitro* oxidative stress (Kalpana *et al.*, 2009; Liu *et al.*, 2001). Despite being used as a disinfectant to control bacteria and parasites in fish (Avendaño-Herrera *et al.*, 2006), excessive use of H₂O₂ in the aquatic environment may cause its molecules to pass easily through cell membranes, therefore causing oxidative stress (da Rosa *et al.*, 2008). Earlier *in-vivo* studies performed on different species of fish such as *Dicentrarchus labrax* (Roque *et al.*, 2010), *Paralichthys olivaceus* (Hwang *et al.*, 2016), and *Oreochromis niloticus* (Jia *et al.*, 2019) have reported numerous toxicological effects occurred upon exposure to H₂O₂. A previous study reported that phytochemicals can lower the damaging effect of H₂O₂ (Okoko *et al.*, 2012). Phytochemicals are abundant in plants and may possess strong antioxidant capabilities. The Amaranthaceae family plant *Achyranthes aspera* L. (*A. aspera*) has been widely used to cure a variety of diseases. Nations including Sri Lanka, South Korea, Indonesia, Philippines, Bangladesh, Pakistan, and Kenya utilized *A. aspera* as a traditional medicine (He *et al.*, 2017). *A. aspera* consists of various bioactive compounds including alkaloids, flavonoids, tannins, and saponins (Priya & Krishnakumari, 2007) have proven to act as antibacterial (Khan *et al.*, 2009), anti-inflammatory (Sukumaran *et al.*, 2009; Vetrichelvan & Jegadeesan, 2003), antidepressant (Barua *et al.*, 2009, Barua *et al.*, 2010), anxiolytic (Barua *et al.*, 2012), and antioxidant (Edwin *et al.*, 2008). In addition, this plant can be utilized to treat renal dropsy, fistula, scrofula, skin rash, nasal infection, chronic malaria, infertility, fever, asthma, piles, and snake bites (Ghimire *et al.*, 2014). In the present study, H₂O₂ was used to induce oxidation in zebrafish. This study also identifies the toxicity of *A. aspera* extract as well as the therapeutic potential of *A. aspera* extract as a natural antioxidant in the zebrafish model.



MATERIALS & METHODS

Animals: Adult zebrafish were placed in containers with adequate circulation and oxygen. The light-to-night ratio was maintained at 14:10, and the temperature of the tank system was maintained between 26-28.5°C. Non-chlorinated water was used and pH between 6.8 and 7.5 for zebrafish water was kept constant for all zebrafish groups. The system and water conditions in the tank were frequently checked and maintained, and the zebrafish were fed twice a day (Aleström et al., 2020).

Determination of H₂O₂ Lethal Dose (LD₅₀) on zebrafish: This test was performed according to Xavier et al., (2020). A total of 60 adult zebrafish were divided into six groups (n = 10). The control group was not treated with H₂O₂ and five experimental groups were given H₂O₂ with various concentrations (25.58 mM, 19.18 mM, 12.79 mM, 3.19 mM, and 0.639 mM). Various H₂O₂ concentrations were given to the experimental group by dissolving H₂O₂ in the tank water for 24 hours. After 24 hours of induction, observations were conducted and records of the results were prepared. An LD₅₀ was established.

Determination of A. aspera extract Lethal Concentration (LC₅₀) on zebrafish: This test was performed according to Xavier et al., (2020). Five groups (n = 10) were treated with A. aspera extract in the concentration of 313 µg/ml, 625 µg/ml, 1250 µg/ml, 2500 µg/ml, and 5000 µg/ml. The A. aspera extract was added to the tank water and incubated with the appropriate concentrations for 24 hours. Observations were made 24 hours following induction, and the results were recorded.

Treatment of A. aspera extract on H₂O₂-induced zebrafish: Data on LD₅₀ and LC₅₀ obtained from previous steps were used for this test. An in vivo antioxidant study was performed according to Issac et al., (2021). Briefly, 10 adult zebrafish were placed into four groups which were: (i) Normal group, which only given normal conditions water and food; (ii) Negative group, which was exposed to 0.639 mM H₂O₂; (iii) Positive group, which was exposed to 0.639 mM H₂O₂ and treated with 1110 µg/ml Ascorbic acid; (iv) A. aspera group, which was exposed to 0.639 mM H₂O₂ and treated with 1110 µg/ml of A. aspera extract. The extract and H₂O₂ were dissolved in the tank water for 24 hours. After 24 hours of induction, observations were conducted and records of the results were prepared.

Euthanization and tissue homogenization: Zebrafish were sacrificed by putting them into water containing ice cubes for five minutes. The preparation of zebrafish was according to Yoon et al., (2022) with slight modification. The zebrafish were dissected to remove their fresh organs, including their hearts and brains. Organ sample homogenates were produced by crushing the organ with 2 ml of phosphate buffer solution (PBS) in the mortar. After that, the homogenized



organ sample was put into a Falcon tube. The homogenous sample was centrifuged for 15 minutes at 4000 rpm.

Lipid peroxidation: Thiobarbituric acid reactive substance (TBARS) test was done according to Yoon et al., (2022). Firstly, 900 μ L of TBARS reagent was transferred into a microcentrifuge tube. Then, 100 μ L of homogenized tissue was added to the microcentrifuge tube. The mixture was boiled at 95°C for 15 minutes. After cooling for 35 minutes, the tubes were centrifuged for 15 minutes at 4000 rpm. The supernatant was then pipetted into a 96-well plate. The absorbance was calculated at 540 nm. The experiment was performed in triplicate.

Bradford protein assay: Protein assay was performed using a method according to Bradford (1976) with minor adjustments. Bovine serum albumin (BSA) was diluted with distilled water to provide a 5 mg/ml stock solution for the standard preparation. Starting with a stock solution of 5 mg/ml, further serial dilutions were made. Then, 10 μ L of supernatant from homogenized tissue samples and standards were added to a 96-well plate. After that, 90 μ L of Bradford reagent was pipetted into a 96-well plate. Prior to the measurement procedure, the solution was mixed. The absorbance was measured at 540 nm. The standard was used for a standard curve. This experiment was carried out three times.

Data analysis: Data were statistically analyzed using ANOVA, SPSS version 21. p-value less than 0.05 was considered statistically significant.

RESULT & DISCUSSION

Determination of H₂O₂ lethal dose (LD₅₀) on zebrafish: The mortality percentage was increased after zebrafish were exposed to H₂O₂ ranging from 0.639 to 25.58 mM. The LD₅₀ for H₂O₂ is 0.639 mM. (Figure 1)

Determination of A. aspera extract Lethal Concentration (LC₅₀) on zebrafish: A dose-response curve was created using the percentage of dead zebrafish from each concentration. At the concentration of 1250 μ g/ml, the extract caused 55% mortality of zebrafish. At the lowest dose, 0.313 mg/ml, the graph showed low mortality (30%). *A. aspera*'s LC₅₀ was determined to be 1110 μ g/ml (Figure 2).

Co-induction of A. aspera extract and H₂O₂ in zebrafish (MDA Assay): The MDA level in the negative control group was the highest compared to the other groups. While the treatment group displayed the lowest MDA level as compared to the other groups. The brain in the positive control group showed no significant MDA level than the normal group (Figure 3) The negative control group's hearts had the highest MDA levels. The treatment group had a lower heart MDA level

than the negative group. This was followed by the positive control group, which had a higher heart MDA level than the normal group (Figure 4). MDA levels were lower in the brain organ compared to the heart (Figure 5).

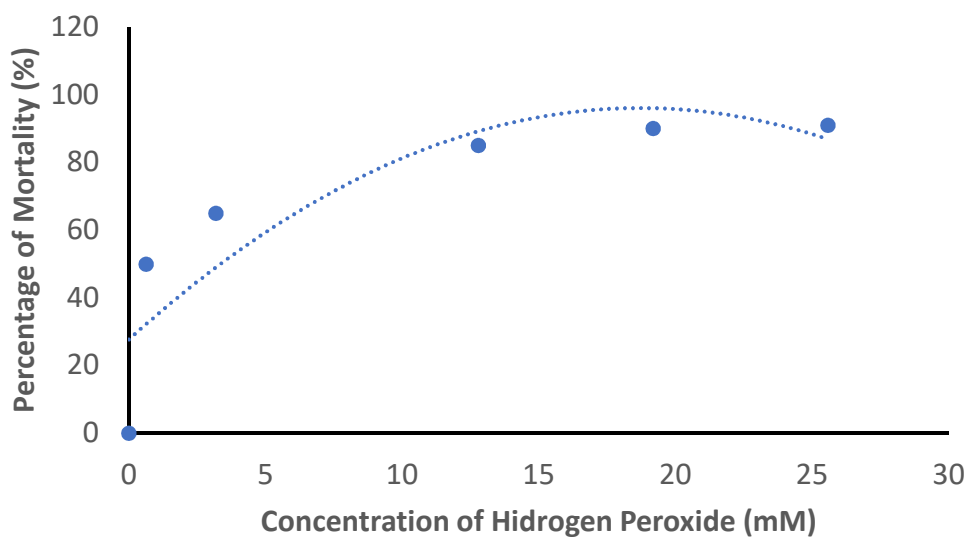


Figure 1: The effect of H₂O₂ on the mortality percentage of zebrafish.

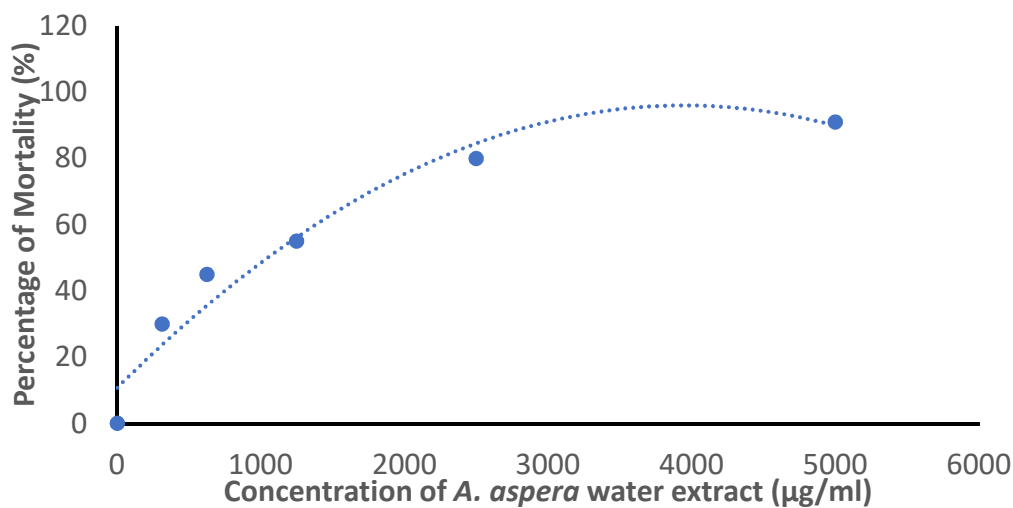


Figure 2: The effect of *A. aspera* extract on zebrafish mortality percentage.

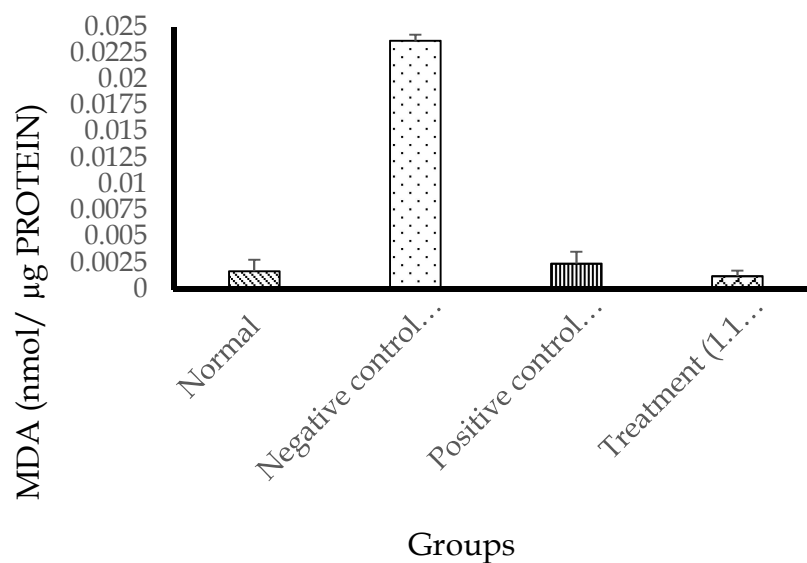


Figure 3: MDA levels in the brain for four different groups (normal, negative control, positive control, and treatment)

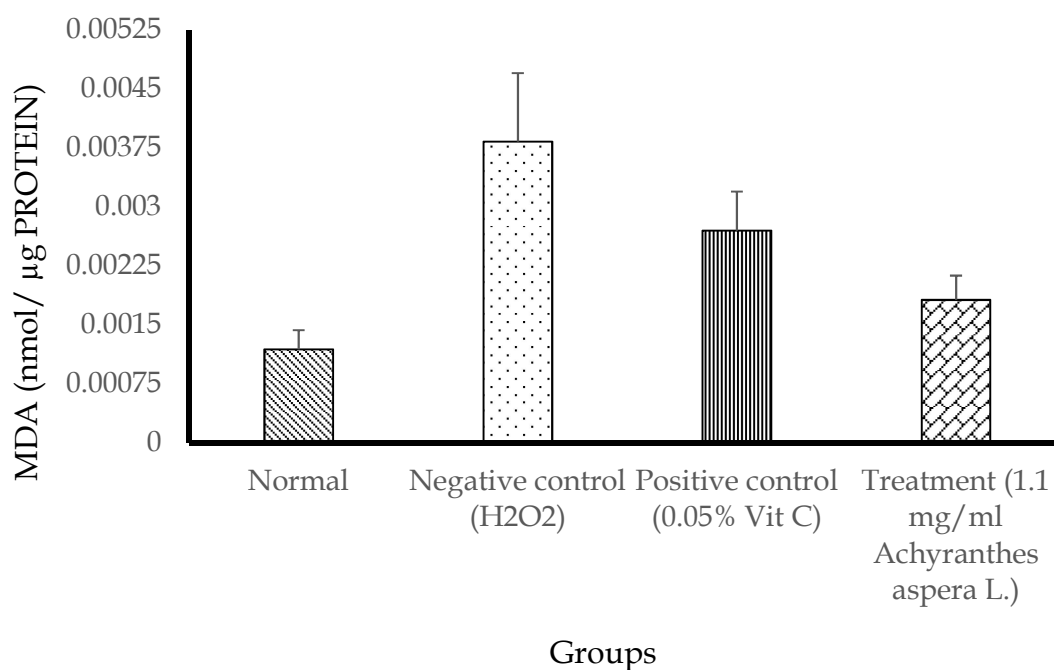


Figure 4: MDA level in the hearts of zebrafish for four different groups (normal, negative control, positive control, and treatment)

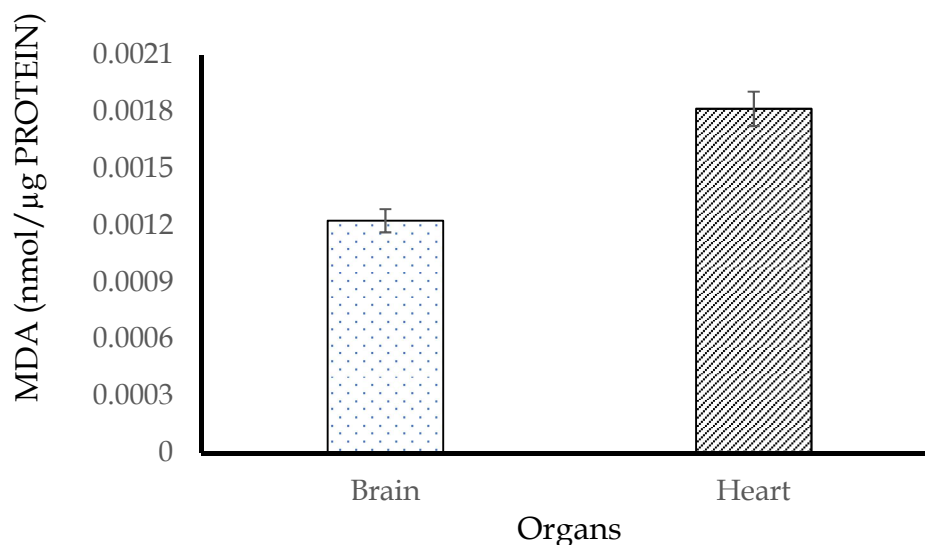


Figure 5: MDA levels in the two different organ samples (the heart and brain) after co-induction of *A. aspera* with H₂O₂. Data represent the mean ± SD.

DISCUSSION

The test for H₂O₂ toxicity in zebrafish showed an LD₅₀ value of 0.639 mM. The results of this test are consistent with a number of previous studies using zebrafish and H₂O₂ (Kim *et al.*, 2021; Ma *et al.*, 2018). Zebrafish may suffer oxidative damage when exposed to H₂O₂. Meanwhile, the LC₅₀ value of *A. aspera* is 1110 µg/ml which is considered low toxicity. The best method to evaluate toxicity is to determine the LC₅₀ value (Pacheco and Rebelo, 2013). If the LC₅₀ value is less than 1000 µg/l, the toxicity of a substance is considered very high (Kowan *et al.*, 2016). In a previous work on the toxicological study of *A. aspera* leaf extract associated with Nile tilapia fish seedlings demonstrated a low toxic effect with an LC₅₀ value of 1310.74 mg/L (Mandefro *et al.*, 2021). The current study result approved earlier study conclusions that *A. aspera* is not toxic and safe for freshwater species like Nile tilapia and zebrafish. Besides that, the MDA test showed varying MDA levels in four different groups. The results showed *A. aspera*-treated group had lower MDA levels. Compared to the brain of the normal group (0.0017 nmol/g protein), the brain of the negative control group (0.0237 nmol/g protein) exhibited a high MDA level. Increased MDA levels might be due to the effect of H₂O₂ molecules on the brain of zebrafish. The H₂O₂ molecules can traverse cell membranes and convert into highly reactive hydroxyl radicals that promote lipid peroxidation via oxidative signaling (Kim *et al.*, 2021). In addition, the central nervous system including the brain is particularly vulnerable to reactive oxygen species (ROS) due to its high metabolic rate, high concentration of polyunsaturated lipids, and relatively low activity of its antioxidant enzyme system (Hallwell & Gutteridge, 1985; Pratic, 2002). In addition, the LPO process also occurs in



the heart after H₂O₂ exposure. MDA levels in the hearts of the negative control group were marginally higher (0.0038 nmol/g protein) than those in the normal group (0.0012 nmol/g protein). In some circumstances, administering H₂O₂ can cause the release of nascent oxygen that directly damages heart cells, impairing enzymatic activity and the integrity of cell membranes or causing protein denaturation in the heart (Sellers *et al.*, 1962). Besides that, the positive control group showed lower (0.0024 nmol/g protein) MDA levels compared to the negative control group's (0.0237 nmol/g protein) for the brain. Similar to the heart, the positive control group (0.0027 nmol/g protein) had lower MDA than the negative control group (0.0038 nmol/g protein). These findings confirmed that ascorbic acid has the capacity to act as an antioxidant. In a prior investigation, it was discovered that harmful co-administration with ascorbic acid reduced LPO, which reduces oxidative stress in zebrafish (Paduraru *et al.*, 2021). Moreover, H₂O₂-induced LPO was considerably decreased by *A. aspera* extract treatment. In the *A. aspera* extract treatment group, the brain's MDA concentration was lower (0.0012 nmol/g protein) compared to the negative control group (0.0237 nmol/g protein). MDA levels in zebrafish hearts treated with *A. aspera* extract were also lower (0.0018 nmol/g protein) than those in the negative control group (0.0038 nmol/g protein). According to Paramanik *et al.*, (2019), zebrafish treated with an ethanol extract of *A. aspera* displayed improved behavior, a relative decrease in SOD, enhanced mitochondrial activity, and increased cell viability. According to Khan *et al.*, (2013), the flavonoids quercetin and polyphenols, which are abundant in *A. aspera*, are known to be potent antioxidants. Due to its antioxidant activity and removal of free radicals, *A. aspera* has been shown in other experiments to be capable of dissolving H₂O₂ (Khan *et al.*, 2013). In addition, treatment of *A. aspera* extract produced low MDA levels with the brain having slightly lower MDA levels than the heart. This demonstrates that *A. aspera* is marginally more sensitive to neuroactivity and might be possessing neurotherapeutic. *A. aspera* extract contains phenolic compounds, tannins, saponins, and flavonoids as its primary phytoconstituents (Verma *et al.*, 2020). All of these substances have been shown to be able to cross the blood-brain barrier (BBB) in order to reduce oxidative stress in the brain (Chen & Chen, 2013; Hu *et al.*, 2020; Stepnik, 2021; Teles *et al.*, 2018). Several studies have linked the antioxidant and anti-inflammatory properties of *A. aspera* extract to neuroprotective and cerebroprotective properties against oxidative stress (Paramanik *et al.*, 2019; Viswanatha *et al.*, 2019). These investigations corroborate the findings of this study, which indicate that *A. aspera* extract has superior neurological effects than cardiovascular effects. However, *A. aspera* extract does contain a variety of phytoconstituents that may help to reduce oxidative stress, a factor in both cardiovascular and neurological diseases (Sinan *et al.*, 2020).

CONCLUSION

This study showed that *A. aspera* extract may have a therapeutic effect in lowering lipid peroxidation induced by H₂O₂ in both the brain and the heart. Additionally, the extract has



insignificant toxicological effects on zebrafish. As a conclusion, *A. aspera* extract has a significant potential for antioxidant application as an alternative treatment for cardiovascular and neurological conditions in zebrafish. The extract of *A. aspera* might be useful to be used as a supplement for zebrafish to avoid the oxidation process.

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

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